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Transfer of T Cell Surface Molecules to Dendritic Cells upon CD4$^+$ T Cell Priming Involves Two Distinct Mechanisms[1]

Annette Busch,[2]* Thomas Quast,† Sascha Keller,‡ Waldemar Kolanus,† Percy Knolle,* Peter Altevogt,‡ and Andreas Limmer[2]*

Activation of CD4$^+$ T cells by APCs occurs by multiple Ag recognition events including the exchange of costimulatory signals and cytokines. Additionally, the T cells acquire APC-derived surface molecules. Herein, we describe for the first time the transfer of human and murine T cell surface receptors to APCs after Ag-specific interaction. This transfer occurs in two qualitatively different phases. The first group of molecules (e.g., CD2) derived from the T cell surface was transferred rapidly after 2 h of interaction, was strongly bound on the DC surface (acid wash-resistant), was strictly dependent on dendritic cell-T cell contact, and transferred independently of T cell activation. The second group, including the CD3/TCR complex, CD27, and OX40, was of intracellular origin, transferred later after 10–16 h in a cell-cell contact-independent fashion, was noncovalently bound, and was strictly dependent on Ag-specific T cell activation. Functionally, murine dendritic cells that received TCR molecules from OVA-specific CD4$^+$ T cells after Ag-specific interaction were less efficient in priming naive CD4$^+$ T cells of the same specificity without losing their ability for CD8$^+$ T cell stimulation, indicating that the transferred TCR molecules mask the Ag-bearing MHC II molecules, thereby reducing their accessibility to following Ag-specific CD4$^+$ T cells. While the first group of transferred T cell surface molecules might facilitate the detachment of the CD4$^+$ T cell from the dendritic cell during the early scanning phases, the second group could play an important immunomodulatory role in intraclonal competition of T cells for APC access, making the physical presence of CD4$^+$ T cells unnecessary. *The Journal of Immunology,* **2008, 181: 3965–3973.**

The adaptive immune response is initiated in secondary lymphoid organs by cognate physical interaction of APCs and Ag-specific CD4$^+$ T cells. This Ag-specific activation of CD4$^+$ T cells by APCs is a dynamic process including distinct phases of interactions (1) and it leads to conditioning ("licensing") of APCs. During this cognate interaction the cells not only deliver signals via receptor-ligand interactions and cytokine production, they also exchange cell surface molecules (2, 3).

Surface molecule transfer has been observed for various cell types but has been predominantly reported for the transfer of dendritic cell (DC)[3] surface molecules to T cells in a unidirectional way, mostly in the form of exosomes that contain MHC I/II-peptide molecules (4–8) and costimulatory molecules such as CD80/86 (8–12), ICAM-1 (9, 13), and OX40L (14). The documented molecule transfer after cell-cell interaction is dependent on cognate interaction and physical contact as well as the presence of the ligand of the observed transferred molecule, for example, the appropriate TCR for transferred MHC-peptide molecules (5, 9, 11).

The transfer kinetics and the fate of the exchanged molecules are variable and dependent on the cell type and activation status of the cells. APCs transfer MHC, ICAM-1, and CD80/86 to T cells in less than 1 h (5). However, in case of the MHC I-peptide transfer, the complex is lost on the cell surface to 50% after 4 h and is undetectable after 16 h (5). Presumably TCR internalization accounts for this rapid loss of the acquired molecules. In the case of CD80 transfer, the molecules disappear even faster from the T cell surface and are shuttled to endocytic compartments (9). APCs are known to direct absorbed ligands to endosomes to allow Ag processing and subsequent loading of MHC molecules. Using this internalization process they have been shown to acquire Ag from other cells by direct contact (15, 16) or in the form of tumor- or APC-derived exosomes (17–19). On the other hand, follicular DCs do not internalize exosomes but carry them on their surface for long time periods (20). To date, only one report describes the transfer of rat T cell molecules to B cells and DCs by exosomes (21). The transferred MHC II-peptide complexes from activated CD4$^+$ T cells were recognized by allogenic responder T lymphocytes and caused them to proliferate. However, mouse CD4$^+$ T cells do not up-regulate MHC II upon activation, and the influence

*Institute of Molecular Medicine and Experimental Immunology (IMMEI) and †Life and Medical Sciences (LIMES) Institute, University of Bonn, Bonn, Germany; and ‡Division of Cellular Immunology, German Cancer Research Center, Heidelberg, Germany

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[2] Address correspondence and reprint requests Dr. Andreas Limmer, or Dr. Annette Busch, Institute of Molecular Medicine and Experimental Immunology (IMMEI), Sigmund-Freud-Strasse 25, University of Bonn, Bonn 53105, Germany. E-mail addresses: annette.busch@ukb.uni-bonn.de and andreas.limmer@uni-bonn.de

[3] Abbreviations used in this paper: DC, dendritic cell; SEB, staphylococcal enterotoxin B superantigen.

of other T cell molecules on DC function involving their ability to prime following $CD4^+$ T cells or $CD8^+$ T cells has not been investigated. We now show that, after Ag-specific interaction, human and murine DCs can acquire T cell surface molecules, including the CD3/TCR complex. Furthermore, we demonstrate that the transfer from DC surface molecules to naive T cells during the priming process is conducted in two phases. The first group of molecules originates from the T cell surface and is transferred rapidly in a cell-cell contact-dependent manner, whereas the second group is transferred through the release of intracellularly formed surface molecules during the later priming phase. Finally, we investigated the accessibility of these licensed, T cell surface molecule-carrying DCs to naive $CD4^+$ T cells. Our observations showed a reduced capacity of DCs that had received the TCR complex by cognate $CD4^+$ T cell-DC interaction to stimulate naive $CD4^+$ T cells without losing their ability for $CD8^+$ T cell stimulation, indicating a role of the transferred $CD4^+$ T cell molecules in the specific feedback regulation of the adaptive $CD4^+$ T cell immune response.

Materials and Methods

Mice and reagents

Mice were maintained under specific pathogen-free conditions at the animal facilities of the House of Experimental Therapy and the Institute for Molecular Medicine in Bonn. Experiments were performed according to animal experimental ethics committee guidelines. C57BL/6 and BALB/c mice were purchased from Charles River Laboratories. $OVA_{323–339}$ TCR transgenic OT-II (22) and DO11.10 (23) mice and OT-I $OVA_{257–264}$ TCR transgenic mice (24) have been described previously and were bred in our animal facility.

Staphylococcal enterotoxin B superantigen (SEB) and OVA (grade II) were purchased from Sigma-Aldrich. The following human Abs were used: CD2-FITC, CD3-FITC, CD27-FITC, and CD28-FITC (ImmunoTools); OX40-FITC and CD69-FITC (eBioscience); HLA-DR-Alexa Fluor 647, CD94-FITC, CD62L-FITC, and CD158b-FITC (BioLegend); HLA-DR blocking (clone L243, kindly provided by Gerhard Moldenhauer, Heidelberg, Germany); CD2 blocking (clone MEM-63, AbD Serotec); annexin V (apoptosis detection kit, Roche); and annexin I and CD3ζ (BD Biosciences). Murine cells were stained with CD2-FITC (Serotec); CD11c-Alexa Fluor 647 (eBioscience); and CD3-PE, CD62L-PE, TCRβ_5-PE, and $TCR_{KJ1–26}$-PE (BD Biosciences). For secondary staining of biotinytated proteins, streptavidin-Alexa Fluor 647 (Invitrogen) was used.

Generation of human monocyte-derived DCs, human $CD4^+$ T cells, and NK cells

Human PBMC were isolated from buffy coats obtained from healthy donors using LSM 1077 lymphocyte separation medium (PAA Laboratories). Monocytes were purified from PBMC using a human CD14 EasySep selection kit (StemCell Technologies), and DCs were generated by culturing monocytes in serum-free CellGro DC medium (CellGenix) containing antibiotics, GM-CSF (800 U/ml; ImmunoTools), and IL-4 (1000 U/ml; ImmunoTools) for 6 days. $CD4^+$ T cells and NK cells were purified from buffy coats by positive selection using CD4 or CD56-biotin plus streptavidin-coupled magnetic beads (Miltenyi Biotec), respectively.

Purification of murine $CD11c^+$ DCs and $CD4^+$ T cells from spleen

For murine DC preparation, collagenase-digested spleen cells (collagenase A, Roche) were pressed through a sterile fine wire mesh, suspended in MACS buffer (PBS, 1% FCS, 2 mM EDTA), and separated using CD11c magnetic beads (Miltenyi Biotec). The resulting DCs were routinely ≥85–90% positive for CD11c as determined by flow cytometry. $CD4^+$ T cells were purified by positive selection using CD4 magnetic beads (Miltenyi Biotec).

Transfer experiments

For transfer experiments, DCs and $CD4^+$ T cells were incubated in RPMI 1640 medium (Invitrogen) containing GM-CSF (mouse) or GM-CSF plus IL-4 (human). T cell activation was achieved by adding SEB (1 μg/ml), OVA protein (10 μM), or $OVA_{323–339}$ peptide (1 μM). For cell-cell contact-dependent transfer, 3.3×10^5 DCs were cocultured with 2.3×10^6 T cells (ratio of 1:7) in the upper compartment of 24-well transwell plates (membrane with 0.4-μm pore size; Nunc); for cell-cell contact-independent transfer, 5×10^5 DCs were cultured in the lower compartment. To obtain sufficient material for biochemical analysis of the transferred molecules by Western blotting, bulk cultures of 1×10^7 DCs were cocultured with 7×10^7 T cell blasts (ratio of 1:7) supplemented with 1 μg/ml SEB plus 20 ng/ml IL-12. T cell blasts were generated from freshly buffy coat-isolated $CD4^+$ T cells that were stimulated with 5 μg/ml PHA (Sigma-Aldrich) for 48 h. Cells were then restimulated with 200 U/ml IL-2 (ImmunoTools) every 3 d for 14 d. Supernatants from DC/SEB-activated T cell blasts were collected after 24 h, purified from remaining cells and debris by centrifugation at 1500 rpm for 10 min, followed by another centrifugation step at 4000 rpm for 20 min, and subjected to ultracentrifugation followed by biochemical analysis via Western blotting.

NK cell-DC transfer experiments were conducted as described above, and NK cell activation was achieved by addition of 20 ng/ml IL-12 (ImmunoTools).

Generation of apoptotic and necrotic T cells

To induce apoptosis, $CD4^+$ T cells were UV irradiated at 100,000 $\mu J/cm^2$ (254 nm UV) using a Ultraviolet Products crosslinker. After overnight culture, the cells were 52% apoptotic and 20% dead as verified by annexin V/Hoechst 33258 staining. For induction of necrosis, T cells were subjected to five freeze-thaw cycles. Thereafter, all cells were dead as verified by trypan blue staining.

Biotinylation of cell surface proteins

Cells were washed thoroughly with PBS and incubated with 200 μM *N*-hydroxysuccinimido-biotin (NHS-x-Biotin, Interchim) in PBS for 15 min. The reaction was stopped by addition of an equal volume of FCS for 1 min and the cells were washed thoroughly thereafter.

Acid wash

Cells were washed twice in PBS and resuspended for 4 min at 20°C in citrate buffer (0.133 M citric acid and 0.066 M Na_2HPO_4 (pH 3.3)) at a density of 5×10^6 cells/ml. The treatment was stopped by the addition of an excess of 5% FCS in PBS. After being washed, the cells were stained for flow cytometry.

Confocal microscopy

For better identification, cells were labeled with CellTracker orange CMTMR ((5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethyl-rhodamine)) or green CMFDA (5-chloromethylfluorescein diacetate) (Invitrogen) as suggested by the manufacturer. Cells were washed with MACS buffer after coculture, stained with designated Abs, and fixed with 4% (w/v) paraformaldehyde in PBS for 20 min. After a final wash, cells were mounted onto slides with Gel-Mount mounting medium (BioMeda) containing 50 mg/ml DABCO (1,4 diazobicyclo-(2.2.2) octane; Sigma-Aldrich) and samples were examined by laser scanning confocal microscopy. Confocal data were acquired and processed with an Olympus FluoView1000 confocal microscope performing two-dimensional reconstructions of Z-stacks (mean width 0.9 μm/slice) and scanning with 40 μs/pixel using Kalman mode. Images were taken with a PLAPON60×O/TIRFM-SP (numerical aperture 1.45) oil immersion lens at room temperature. Reconstructions were made with the Olympus FluoView software. All images were collected in a sequential mode to minimize the crossover between channels, and microscope parameters for the collection of images were kept constant between samples.

Flow cytometry

Single-cell suspensions were stained with designated Abs in MACS buffer for 10 min at room temperature, washed once with MACS buffer, and immediately processed for measurement. Appropriate isotype controls were always included. Dead cells were excluded from the analysis by Hoechst 33258 incorporation. DC-T cell aggregates were electronically gated out by large forward scatter width exclusion (digital acquisition) and strong streptavidin staining. Cells were measured on a LSR II fluorocytometer (BD Biosciences) and data were analyzed using the FlowJo software (Tree Star).

Biochemical analysis

Before Western blotting, cell culture supernatants were fractionated by ultracentrifugation at 100,000 × *g* for 2.5 h. Thereafter, supernatants were TCA-precipitated, adding 10% volume of 100% TCA. Precipitated pellets were washed twice with ether, air dried, and redissolved in SDS sample

buffer. Samples were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to an Immobilon membrane using semidry blotting. After blocking for 30 min with 5% skim milk in TBS, the blots were developed with the respective primary Ab overnight followed by peroxidase-conjugated secondary Ab and ECL detection.

Ag-specific T cell activation

Murine DCs from C57BL/6 mice were incubated with $CD4^+$ T cells from OT-II mice at a ratio of DC-to-T cells of 1:4 for 24 h with 10 μM OVA protein. The cells were washed thereafter, stained with CD11c Ab, and separated from the coculture by cell sorting using a FACSVantage (BD Biosciences). Dead cells were excluded from the analysis by Hoechst 33258 incorporation. DC-T cell aggregates were electronically gated out by large forward scatter width exclusion (digital acquisition). DC purity was consistently >95% as confirmed by flow cytometry. OT-II, DO11.10, and OT-I cells were purified by magnetic bead separation and labeled with CFSE. Sorted DCs and isolated T cells were cultured at a 1:1 cell ratio in 96 wells at 5×10^4 cells/ml for 24 h (cytokine secretion) or 72 h (proliferation). Quantitative ELISA for IL-2 were performed using commercially available Abs (BD Biosciences) according to the manufacturer's recommendations. Proliferation was assessed by visualization of CFSE dye dilution upon cell division using flow cytometry.

Results

Human $CD4^+$ T cells transfer surface molecules to DCs during cognate interaction

The transfer of DC-derived surface molecules to T cells has been extensively investigated. Here we asked the question whether the same occurs in a reverse way, that is, from T cells to DCs. To show surface protein transfer and exclude endogenous up-regulation of surface molecules by the DCs, all T cell surface receptors were biotinylated. After coculture of these labeled $CD4^+$ T cells with DCs, biotinylated proteins were detected by streptavidin staining. Twenty-four hours after coculture of biotinylated T cells and DCs in the presence of SEB, these biotinylated surface molecules could be detected on the DC cell membrane (Fig. 1*A*). They colocalize with the T cell surface molecule CD2, a receptor generally not expressed by DCs (Fig. 1*B*).

Transfer of various T cell surface receptors to DCs

To obtain more detailed information on which other T cell surface molecules besides CD2 were transferred, the DCs were stained for various T cell surface Ags after coculture with biotinylated T cells in the presence of SEB. Results showed a great number of T cell surface Ags on the DC surface, including CD2, CD3, CD27, OX40, CD69, CD5, and PD-1, whereas others (CD28, CD45, CD11a, CD29, CD7, CD4) were not transferred (selected markers shown in Fig. 2*A*). The fact that only particular T cell surface proteins were transferred indicates the measured transfer is not due to culture artifacts (e.g., bound cell debris). Similar results were obtained with murine OVA-specific $CD4^+$ T cells after unspecific stimulation by superantigen-induced TCR crosslinking to MHC II on DCs or Ag-specific stimulation with OVA peptide-pulsed DCs (Fig. 2*B*). In addition to the surface molecules transferred from human T cells to DCs, the specific TCR of transgenic T cells was detected on the DC surface. The amount of transferred molecules achieved by superantigen stimulation was similar to that observed by Ag-specific activation. These findings strongly suggest that the transfer observed in the human system is not due to artifacts caused by SEB stimulation but are rather reflecting a process conducted by Ag-specific activation of $CD4^+$ T cells. Similar to the human system OX40, CD5 and CD27 were transferred by murine $CD4^+$ T cells whereas CD28 was absent from the DC surface (data not shown).

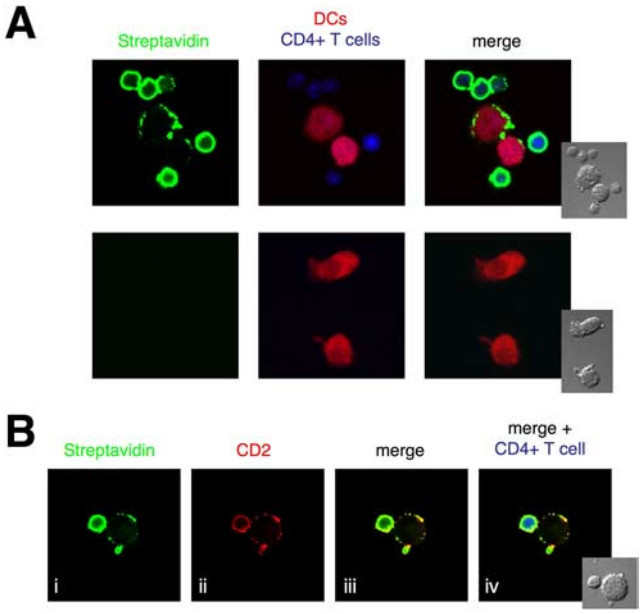

FIGURE 1. Transfer of biotinylated T cell surface molecules and CD2 to human DCs. *A*, DCs (CMFDA, red) were cultured with (*upper panels*) or without (*lower panels*) biotinylated human $CD4^+$ T cells (CMTMR, blue) for 24 h in the presence of SEB and examined by confocal microscopy as described in *Materials and Methods*. Fluorescence channels: Alexa Fluor 647 (*left*), Alexa Fluor 647 merged with CMFDA (*middle*), and all three fluorescences merged (*right*). *B*, Colocalization of CD2 (anti-CD2-FITC, red) and biotin (streptavidin-Alexa Fluor 647, green). Coculture of biotinylated $CD4^+$ T cells (CMTMR, blue) and DCs (unstained) in the presence of SEB. Fluorescence channels: Alexa Fluor 647 (*i*), FITC (*ii*), Alexa Fluor 647 merged with FITC (*iii*), and all three fluorescences merged (*iv*). Overlapping areas are displayed in yellow. Data shown are taken from one out of three experiments with similar results.

Transfer of $CD4^+$ surface proteins to DCs by two different mechanisms

To investigate whether the transferred molecules were only cell-cell contact-dependently or also independently transferred, we employed a transwell system containing an upper well with biotinylated $CD4^+$ T cells and DCs with SEB separated from DCs alone in the lower well by a cell-impermeable membrane (0.4 μm). T cell activation was mediated by SEB. Surprisingly, some of the analyzed molecules such as CD3, CD27, or OX40 did not require DC-T cell contact for transfer: They were transferred cell-cell contact-independently in the transwell system (Fig. 3*A*). In contrast, CD2 is transferred only through cell-cell contact. Again, CD28 was neither transferred cell-cell contact-dependently or contact-independently. The cell-cell contact-dependently transferred $CD4^+$ T cell surface molecules in the upper well originate from T cell molecules present on the cell surface since a great amount of biotin was transferred onto the DCs. Acquisition by trogocytosis or ectodomain shedding are possible transfer mechanisms. In contrast, the cell-cell contact-independently transferred $CD4^+$ T cell surface molecules in the lower well are not of cell surface origin since biotin was absent on the surface of DCs, which indicates the secretion of intracellular assembled molecules rather than shedding or nibbling of biotinylated surface molecules. Since is has been reported that activated $CD4^+$ T cells secrete exosomes bearing the CD3/TCR complex (25), we assumed that this transfer of T cell surface molecules might be mediated by T cell-secreted exosomes, that is, vesicles that are formed intracellularly and subsequently exocytosed. For the reverse transfer of DC surface

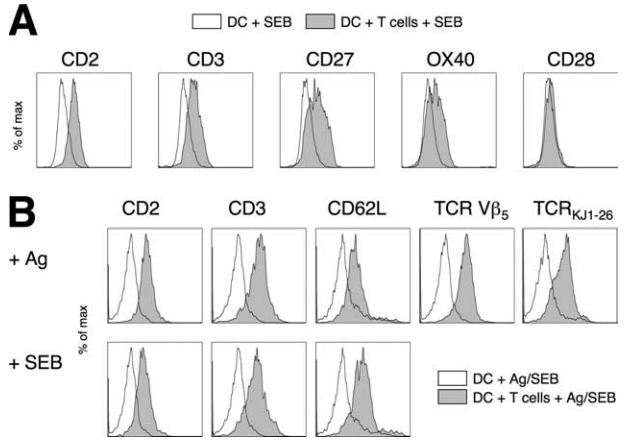

FIGURE 2. Transfer of various human and murine T cell molecules to DCs. *A*, Twenty-four-hour coculture of human DCs and biotinylated $CD4^+$ T cells with SEB. For FACS analysis, DC-T cell aggregates were electronically gated out by large forward scatter width exclusion (digital acquisition) and strong streptavidin staining. Cocultured DCs (filled histograms) were compared with DCs that were cultured alone (open histograms). *B*, Coculture of murine OT-II $CD4^+$ T cells and C57BL/6 DCs in the presence of $OVA_{323–339}$ peptide for 24 h. For SEB-mediated activation, $CD4^+$ T cells of C57BL/6 mice were cocultured with C57BL/6 DCs. For detection of transgenic $TCR_{KJ1–26}$, $CD4^+$ T cells from DO11.10 mice were cocultured with BALB/c DCs in the presence of $OVA_{323–339}$ peptide. Data shown are taken from one out of five experiments with similar results.

molecules to other cells, exosomes are known to be important vehicles (17, 26).

Intracellularly derived but not cell surface-derived T cell molecules are dissociated by low pH

Noncovalent protein-protein interactions like association of β_2-microglobulin with the MHC I H chain or Ab binding can be dissociated by acid wash treatment. Therefore, we tested the sensitivity of the transferred $CD4^+$ T cell surface molecules to this low pH treatment to obtain information about their binding nature. Interestingly, surface proteins that were intracellularly synthesized after the initial biotinylation and transferred in the transwell system such as CD3, CD27, and OX40 could be removed by lowering the pH to 3.3, whereas the T cell surface-derived, strictly cell-cell contact-dependently transferred $CD4^+$ T cell surface molecule CD2 remained bound to the DC surface (Fig. 3*B*). Thus, transferred T cell surface proteins that were cell surface-derived are considerably more stably bound than were intracellularly derived molecules.

Transfer of T cell molecules is receptor-mediated

To investigate whether the cell surface-derived $CD4^+$ T cell surface molecules such as CD2 and/or the intracellularly derived molecules such as CD3 or CD27 are transferred by specific receptor-ligand interaction or randomly by unspecific membrane transfer, we used blocking Abs to their receptors on the DC surface (Fig. 3*C*). Interaction of CD2 with CD58 was blocked by an anti-CD58 Ab, and interaction of the CD3/TCR complex with its MHC II counterpart was inhibited by an anti-HLA-DR Ab. To prevent inhibition of T cell activation by anti-HLA-DR before intracellularly derived T cell molecule transfer, T cells were activated by DCs and SEB in the absence of anti-HLA-DR for 24 h and fresh DCs were incubated subsequently with the resulting cell-free supernatants in the presence/absence of anti-HLA-DR. Both Abs inhibited the T cell surface molecule transfer to DCs in a dose-dependent manner

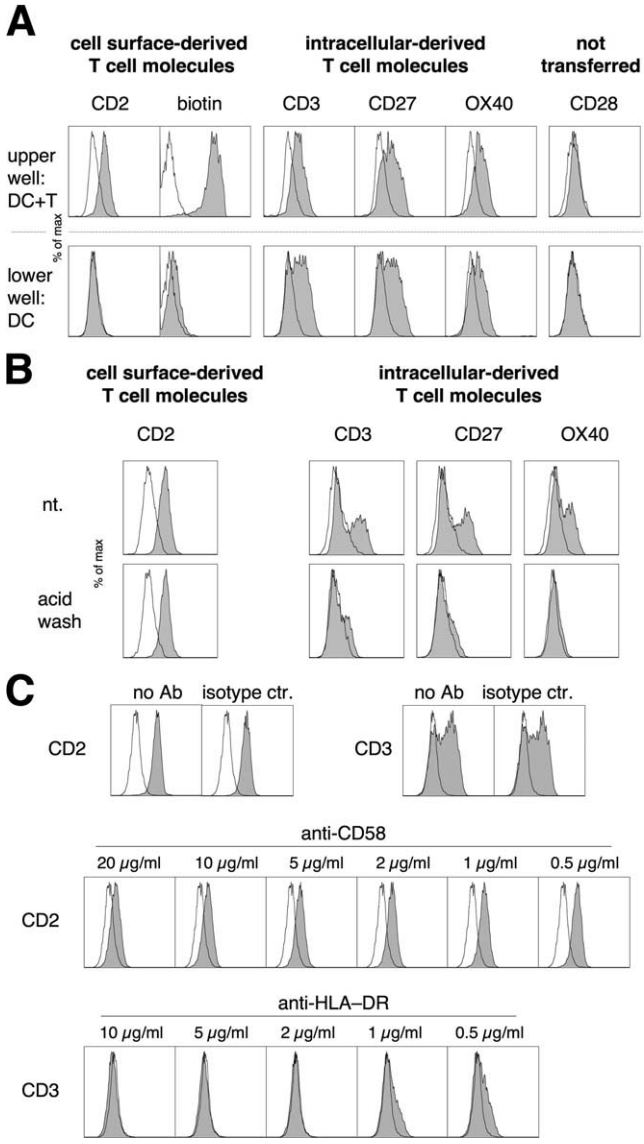

FIGURE 3. Transfer of cell surface-derived and intracellularly derived T cell molecules to DCs: different binding intensity and specificity of these molecules. *A*, Transfer of cell surface proteins from human $CD4^+$ T cells to DCs in a transwell system after 24 h of culture in the presence of SEB. The upper compartments contained $CD4^+$ T cells and DCs (*lower panels*), and the lower compartment contained DCs alone (*upper panels*). The compartments were separated by a semipermeable membrane of 0.4-μm pore size, thus enabling the exchange of small molecules but no cell transit. Data shown are taken from one out of seven experiments with similar results. *B*, After coculture in the transwell system as stated in A, cells from the upper (CD2) and the lower compartment (CD3, CD27, OX40) were either left untreated or treated by acid wash, comprising a brief 4-min incubation (at pH 3.3), to dissolve noncovalent protein-protein interactions and stained for indicated surface markers. Filled histograms represent DCs and T cells; open histograms represent DCs alone. Data shown are taken from one out of two experiments with similar results. *C*, For the transfer of cell surface-derived CD2 T cell molecules, human $CD4^+$ T cells and DCs were cocultured in the presence of SEB for 24 h with or without indicated anti-CD58 doses or isotype control (20 mg/ml) and analyzed for CD2 molecule transfer to DCs. The transfer of intracellularly derived CD3 T cell molecules was investigated by collecting supernatants from DC-SEB-activated T cells and adding those to fresh DCs for 24 h in the presence/absence of anti-HLA-DR or isotype control (20 μg/ml). Thereafter, CD3 molecule transfer to DCs was analyzed. Data shown are taken from one out of two experiments with similar results.

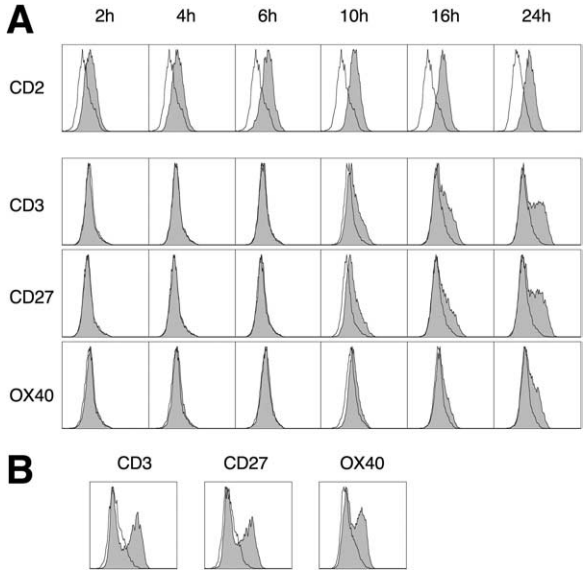

FIGURE 4. Transfer kinetics and stability of transferred T cell surface molecules. *A*, Cells were cultured in a transwell system containing human $CD4^+$ T cells and DCs in the upper compartment and DCs alone in the lower compartment in the presence of SEB. At the specified time points cells were washed, stained with indicated Abs, and processed for flow cytometry. *B*, After 24 h of coculture in the transwell system, DCs from the lower compartment were washed extensively and continued to culture with fresh medium for an additional 18 h. $CD4^+$ T cell molecules as CD3, CD27, and OX40 were still detected after this incubation. Filled histograms represent DCs and T cells; open histograms represent DCs alone. Data shown are taken from one out of two experiments with similar results.

(Fig. 3*C*), indicating that both surface-derived (i.e., CD2) and intracellularly derived (i.e., CD3) $CD4^+$ T cell molecule transfer is receptor-mediated.

Transfer kinetics: rapid transfer of cell surface-derived molecules; transfer of intracellularly derived molecules only after later T cell activation

The two observed transfer mechanisms do not only differ in their origin of the transferred molecules and their binding stability but also in their transfer kinetics. Whereas cell surface-derived molecules like CD2 were already transferred within 2 h, intracellularly synthesized proteins like CD3, CD27, or OX40 were detected not until 10–16 h after T cell activation on the DC surface (Fig. 4*A*). These findings further support our findings that T cell surface molecule transfer to DCs occurs via two entirely different mechanisms that might also differ in their biological function.

To assign a biological function to the molecules transferred onto DCs, it is important to know how long they stay on the DC surface even after the T cell has left. To this end, we cultured DCs in a transwell system containing DCs and $CD4^+$ T cells in the upper well and DCs alone in the lower well, leading to cell surface-derived molecule transfer of T cell surface molecules to DCs in the upper well and intracellularly derived molecule transfer in the lower well during a 24-h culture time. Thereafter, the upper well containing the activated T cells was removed, and the DCs in the lower well were thoroughly washed and cultured for an additional 18 h. After this additional 18-h culture, the intracellularly derived molecules CD3, CD27, and OX40 could still be detected on the DC surface, indicating they are long-lived and are not internalized (Fig. 4*B*).

Transfer of intracellularly derived surface proteins to DCs requires activation of the transferring cell

$CD4^+$ T cells only transferred the intracellularly derived T cell surface molecules CD3 or CD27 onto DCs after superantigen-induced activation, whereas resting T cells did not transfer significant amounts (Fig. 5*A*). However, transfer of the T cell surface-derived molecule CD2 was only marginally affected by the activation status of the $CD4^+$ T cells.

Since DCs also interact with NK cells in the periphery, we investigated whether, as for $CD4^+$ T cells, NK cells also transfer cell surface molecules to DCs. We employed the same transfer coculture system as described for $CD4^+$ T cells containing NK cell-DC coculture in the upper well and DCs alone in the lower well. Similar to $CD4^+$ T cells, which require TCR triggering to induce surface molecule transfer of the intracellularly derived T cell surface molecules, we observed surface molecule transfer of several NK cell surface receptors to DCs in the lower well only when NK cells were activated by IL-12 for 24 h (Fig. 5*B*, *lower panels*). Resting NK cells did not transfer any of the examined surface molecules to DC (Fig. 5*B*, *upper panels*). Transferred molecules included CD27, CD158b, and CD62L, whereas CD94 and CD2 where not transferred.

Thus, activation of the transferring cell seems to be crucial for the transfer of the intracellularly derived surface molecule, whereas surface-derived surface molecules are transferred independently of the activation status of the transferring cell.

To rigorously exclude the transfer of T cell molecules originating from apoptotic bodies or necrotic cell debris, DC-T cell coculture was established using apoptotic/necrotic T cells (Fig. 5*C*). Neither apoptotic nor necrotic $CD4^+$ T cells transferred T cell surface molecules onto DCs: cell surface-derived CD2 as well as intracellularly derived CD3 and CD27 molecules were undetectable on the DC surface after coculture, indicating that the T cells have to be intact to mediate molecule transfer.

CD3/TCR molecules are distributed over the entire DC surface

Because we observed a transfer of T cell-derived CD3/TCR complexes (Fig. 2) onto murine DCs, we expected the complexes to be bound Ag-specifically to their natural ligands (e.g., MHC II molecules). The strong fluorescence of CD3/TCR molecules on the DC surface suggests that most MHC II molecules are covered by these complexes. This observation was confirmed in the human, superantigen-induced transfer model. CD3 molecules were distributed over the entire DC surface to the same extent as were surface-localized HLA-DR molecules (Fig. 6*A*).

To investigate whether the T cell-derived CD3 molecules were transferred to the DC surface as single molecules or in the context of intracellularly released vesicles/exosomes, we subjected the cell- and debris-depleted supernatants of DC/SEB-activated $CD4^+$ T cells to a 100,000 $\times$ *g* ultracentrifugation step. Biochemical analysis of the pellets and supernatants after ultracentrifugation revealed large amounts of CD3 molecules in the pellet fraction, whereas CD3 was undetectable from pellet-depleted supernatants (Fig. 6*B*). This indicates that the transferred CD3 molecules are released in context of larger molecular mass structures such as exosomes, as single molecules cannot be pelleted at 100,000 $\times$ *g*. Another protein present in the pelleted fraction was annexin 1, a prominent marker present on exosomes of various cellular sources (17, 27).

Although one cannot exactly colocalize single CD3/TCR molecules to surface HLA-DR molecules due to resolution limitations of light microscopy, the distribution pattern of both molecules is the same, suggesting that all MHC II molecules were covered by

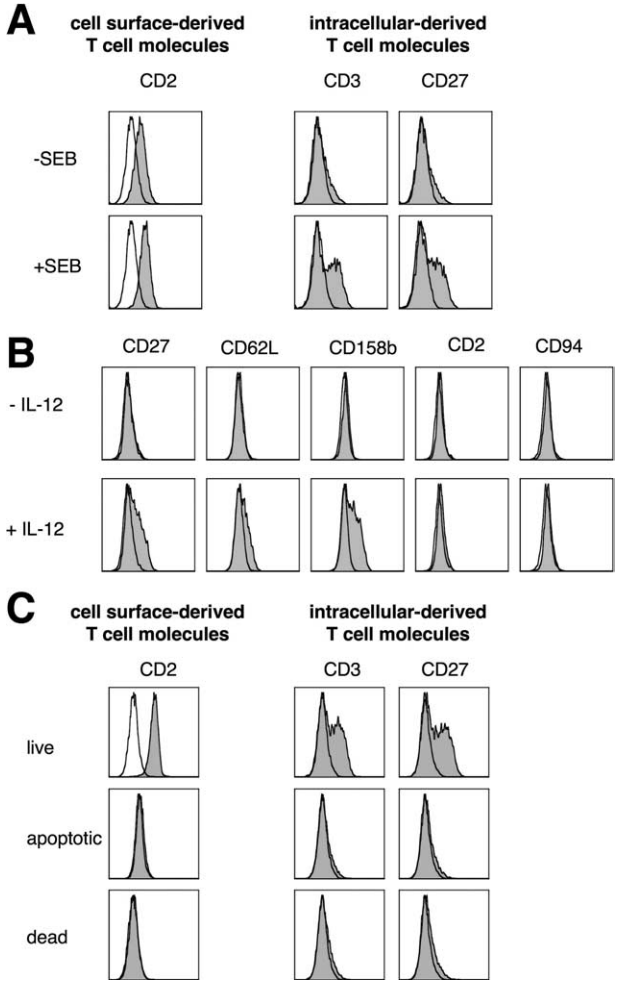

FIGURE 5. Only activated cells transfer surface molecules to DCs trough the transwell membrane. *A*, Human DCs were cocultured for 24 h with biotinylated $CD4^+$ T cells in the presence or absence of SEB in a transwell system including DCs and T cells in the upper compartment and DCs alone in the lower compartment. For surface-derived molecule transfer, cells from the upper well were analyzed (CD2); for transfer of intracellularly derived molecules, cells from the lower well were analyzed (CD3, CD27). T cell-DC aggregates in the upper well were excluded from the analysis by their strong streptavidin fluorescence. Data shown are taken from one out of three experiments with similar results. *B*, Human DCs were cocultured with NK cells in a transwell system including DCs and NK cells in the upper compartment and DCs alone in the lower compartment. Cells from the lower compartment were analyzed after 24 h of culture. No cytokines were added to leave the NK cells in a resting state (*upper panels*) or they were activated by the addition of IL-12 to the culture (*lower panels*). Filled histograms represent DCs in coculture; open histograms represent DCs alone. *C*, Human DCs were cocultured for 24 h with FarRed-labeled live, apoptotic or necrotic $CD4^+$ T cells in the presence of SEB in a transwell system including DCs and T cells in the upper compartment and DCs alone in the lower compartment. Apoptotic T cells were generated by UV irradiation; necrotic T cells were generated by freeze-thaw cycles. For surface-derived molecule transfer, cells from the upper well were analyzed (CD2); for transfer of intracellularly derived molecules, cells from the lower well were analyzed (CD3, CD27). T cell-DC aggregates in the upper well were excluded from the analysis by their strong FarRed fluorescence. Data shown are taken from one out of two experiments with similar results.

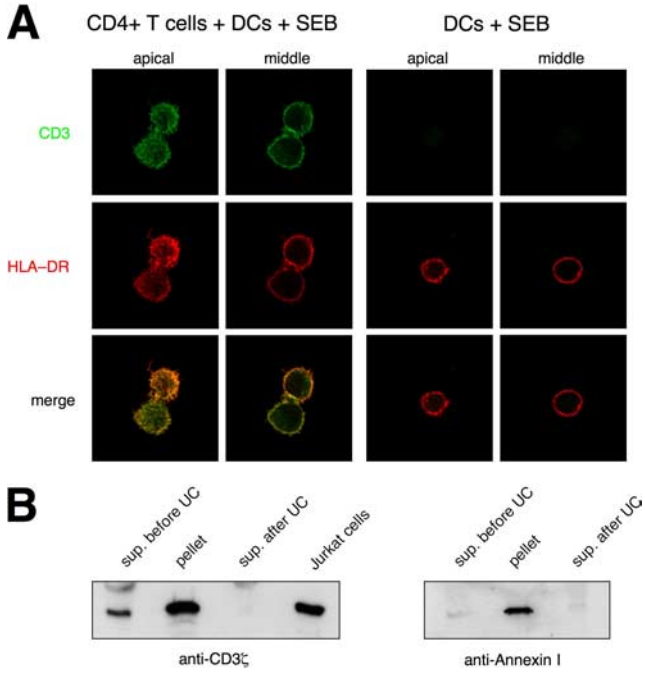

FIGURE 6. Colocalization of CD3 with HLA-DR and biochemical characterization of SEB-activated T cell supernatants. *A*, Human $CD4^+$ T cells and DCs were cocultured for 24 h with SEB in a transwell system containing DCs and T cells in the upper compartment and DCs alone in the lower compartment. Controls included DCs with SEB alone. After this incubation DCs from the lower compartment were collected, stained with indicated Abs for transferred T cell surface molecules, and examined by confocal microscopy. The cell monolayers were optically sectioned in 0.9-μm segments on the z-axis from the apical to the basolateral side and then examined by confocal microscopy as described in *Materials and Methods*. Shown is one section of the apical side (*left*) as well as one section from the middle of the cell (*right*). Fluorescence channels: CD3-FITC (green), HLA-DR-Alexa Fluor 647 (red). Overlapping areas are displayed in yellow. Data shown are taken from one out of two experiments with similar results. *B*, Human T cell blasts were cocultured with DCs in the presence of SEB for 24 h. Thereafter, DC/SEB-activated T cell supernatants were ultracentrifuged (UC) at 100,000 $\times$ g for 2.5 h, and resulting pellets and supernatants were subjected to biochemical analysis via Western blotting. Blots were probed with anti-CD3 (*left*) or anti-annexin 1 (*right*). For CD3 analysis, lysed Jurkat cells were used as a positive control.

CD3/TCR complexes. If the majority, if not even all, of the MHC II-peptide molecules are covered by Ag-specific CD3/TCR complexes, this could then hamper the Ag-presenting capacity of the covered DCs. To test this hypothesis, the Ag-presenting function of TCR-covered murine DCs to OVA-specific $CD4^+$ T cells was investigated in vitro.

Reduced activation of Ag-specific $CD4^+$ T cells by TCR-covered DCs

$CD4^+$ T cells of the same Ag specificity are reported to compete for access to the peptide-MHC II complex on the APC. It is thought that sterical hindrance or Ag extraction by previous T cells is the reason for this intraclonal competition (28, 29). Since CD3 molecules from activated T cells were distributed over the entire DC surface and appeared to be associated with HLA-DR (Fig. 6), we hypothesized that transferred surface molecules from $CD4^+$ T cells might mask MHC II-peptide complexes and thereby affect the ability of the DCs to subsequently stimulate $CD4^+$ T cells of the same specificity (intraclonal competition/feedback regulation). To test this hypothesis, we incubated DCs with OVA-specific OT-II T cells in the presence of OVA protein. After 24 h a great amount of transgenic TCR molecules from OT-II T cells could be detected on the DC surface (Fig. 7*B*). Thereafter, the DCs were sorted to high

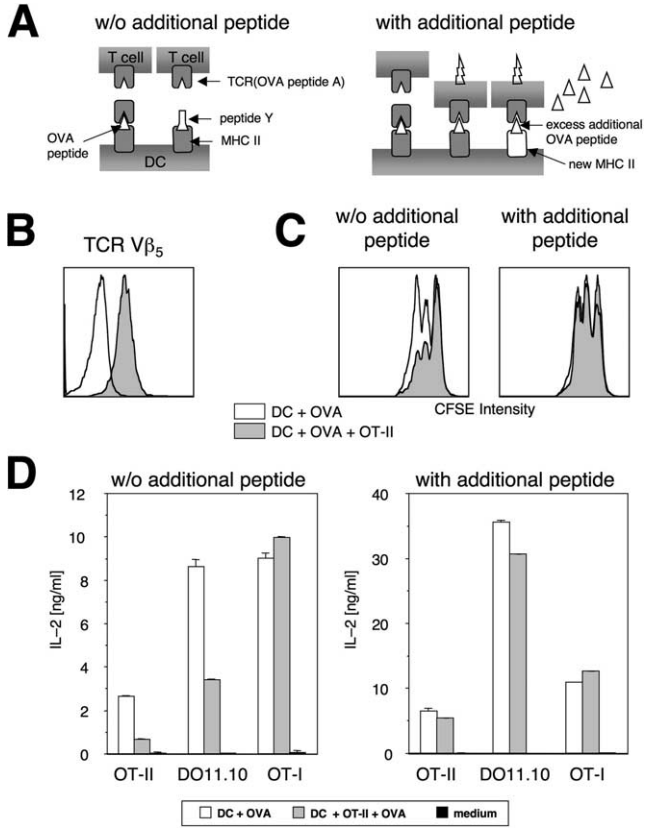

FIGURE 7. TCR-covered DCs have less capability in priming naive $CD4^+$ T cells. Murine splenic DCs from C57BL/6 mice (H-2^b) were cocultured with $OVA_{323–339}$-I-A^b-specific $CD4^+$ OT-II T cells in the presence of OVA protein for 24 h. Control DCs were pulsed with OVA in the absence of OT-II T cells. After coculture, DCs were sorted and used to stimulate naive TCR transgenic $CD4^+$ T cells of the same specificity ($OVA_{323–339}$-I-A^b-specific OT-II, $OVA_{323–339}$-I-A^b/$OVA_{323–339}$-I-A^d-specific DO11.10) or $CD8^+$ T cells ($OVA_{257–264}$-K^b-specific OT-I). *A*, As a control for DC functionality, additional OVA peptide was added during stimulation of $CD4^+CD8^+$ T cells ($OVA_{323–339}$ for OT-II/DO11.10, $OVA_{257–264}$ for OT-I). *B*, Transfer of transgenic TCR Vβ_5 on the surface of sorted DCs was verified by flow cytometry. *C*, Proliferation of naive DO11.10 T cells was measured after 72 h. *D*, IL-2 secretion by TCR transgenic $CD4^+CD8^+$ T cells was determined after 24 h coculture with sorted DCs. Data shown are taken from one out of two experiments with similar results.

purity (>98%) to exclude sterical hindrance by bound T cells and then incubated with fresh naive $CD4^+$ T cells of the same specificity or OVA-specific $CD8^+$ T cells.

Notably, DCs that had received CD3/TCR complexes through prior Ag-specific contact with OT-II T cells induced less proliferation (Fig. 7*C*, *left*) and considerably less cytokine production (OT-II, 4-fold less; DO11.10, 2.5-fold less; Fig. 7*D*) of naive OVA-specific OT-II/DO11.10 $CD4^+$ T cells. In contrast, fresh and "used" TCR-covered DCs showed similar capacity to stimulate naive OVA-specific OT-I $CD8^+$ T cells (Fig. 7*D*). This indicates that after cognate interaction of DCs and $CD4^+$ T cells, TCR molecules were only transferred to MHC II molecules, blocking $CD4^+$ T cell access without affecting access of $CD8^+$ T cells.

To provide further evidence that the lack of $CD4^+$ T cell stimulation was due to transferred TCR molecules and not due to a functional defect of the TCR-covered DCs, $OVA_{323–339}$ peptide was added to the sorted DCs in excess before coculture with naive OVA-specific $CD4^+$ T cells. In this setup, all available MHC II molecules were loaded with the high-affinity OVA peptide (Fig. 7*A*). TCR-covered DCs induced similar proliferation (Fig. 7*C*, *right*) and cytokine production (Fig. 7*D*, *right*) of naive $OVA_{323–339}$-specific $CD4^+$ T cells when they were re-pulsed with $OVA_{323–339}$ peptide. The capacity of OVA peptide-loaded TCR-covered DCs to stimulate OVA-specific naive $CD4^+$ T cells, as well as their ability to stimulate OVA-specific naive $CD8^+$ T cells in a comparable amount to that induced by DCs that were Ag-loaded in the absence of $CD4^+$ T cells, excludes a functional defect of these TCR-covered DCs.

Discussion

We herein show that $CD4^+$ T cells transfer particular T cell surface molecules including the CD3/TCR complex onto DCs after cognate interaction. This transfer had functional implications for the immune function of the receiving DCs. Specific binding of CD3/TCR complexes to peptide-loaded MHC II molecules prevented further stimulation of $CD4^+$ T cells of the same specificity but allowed activation of OVA-specific $CD8^+$ T cells. These findings suggest that transfer of T cell surface molecules regulates further DC-T cell interactions.

We observed the transfer of two groups of T cell surface molecules after the interaction of DCs with $CD4^+$ T cells. The first group of molecules (i.e., CD2) was derived from the T cell surface, was only transferred upon cell-cell contact, was transferred rapidly after 2 h of interaction, was relatively stably bound on the DC surface (possibly membrane-anchored), and was transferred independently of the T cell activation state; the other group of molecules, including the CD3/TCR complex, CD27, and OX40, was of intracellular origin, was transferred during the later activation phase after 10–16 h, was easily removable by acidic treatment, and was strictly dependent on Ag-specific T cell activation. This "two-class system" has not been reported to date.

Surface molecule exchange between cells can be conducted by trogocytosis, exosomes, or receptor shedding. Trogocytosis is a process that describes the active uptake of membrane fragments by interacting cells (30, 31). Exosomes are small vesicles that are secreted by fusion of multivesicular endosomal bodies with the plasma membrane (17, 26). Receptor shedding or enzymatic cleavage is primarily mediated by membrane-bound metalloproteinases (32). Publications about molecule transfer between various cell types describe only one particular type out of these described transfer mechanisms. The given information (e.g., the time that was required for the transfer from one cell to the other) might reflect the type of transfer observed by the investigators. The rapid transfer of HLA-C from target cells to NK cells is described to be an active process of the NK cell (33, 34). Furthermore, phosphoantigen recognition-initiated transfer of γδ T cell surface molecules after short contact with Daudi target cells is dependent on γδ T cell activation (11). Thus, these observations describe a rapid, active process of the receiving cell that points to a mechanism described as "nibbling" or trogocytosis (30). Because of its rapid nature, our observed transfer of surface-derived T cell molecules might be conducted by trogocytosis. However, this step might be accompanied by receptor shedding from the T cell surface. Since only live T cells transfer CD2 molecules onto the DC surface, but not apoptotic or dead cells (Fig. 5*C*), an active mechanism on the T cell side might be involved. The colocalization of biotinylated T cell surface proteins with CD2 (Fig. 1*B*) implies a common mechanism for the transfer of these biotinylated molecules and CD2. Interestingly, both biotinylated and CD2 molecules display a dotted distribution on the DC surface. This may indicate that they are concentrated in lipid rafts in the DC membrane, as it has been shown for transferred MHC II molecules (35).

Using the transwell system, our observation that considerably more biotin was transferred by direct T cell-DC contact in the

upper well in comparison to that from this coculture separated DCs in the lower well sheds light on the origin of the transferred molecules. Since biotinylated surface molecules were almost absent on DCs in the lower well, they must have originated from an intracellular T cell compartment. This might point to intracellularly formed molecules or vesicles by the activated T cells. Exosomes are originating from endosomes and contain intracellular proteins that would not be labeled by surface biotinylation. Consequently, there would be much less biotinylated protein transfer (as opposed to CD3, CD27, or OX40) to DCs by the cell-cell independent mechanism through the transwell membrane, which is supported by our observations. The fact that the intracellular ζ domain of CD3 was present in the 100,000 $\times$ *g* fraction of DC/SEB-activated T cell supernatants (Fig. 6*B*) further suggests that CD3 was bound as a complete molecule in the context of a structure of larger molecular mass rather than as a single ectodomain molecule from vesicle surfaces or cellular membranes. Together with the published data of exosome secretion by activated $CD4^+$ T cells bearing the TCR/CD3 complex (25), our data strongly suggest that transfer of intracellularly derived T cell surface molecules is mediated by exosomes. Notably, this transfer is only occurring if the T cells are activated; resting T cells do not release exosomes (25), thereby depending on the activation status of the transferring cell and not, as reported for trogocytosis, on the status of the receiving cell. In summary, we conclude that the rapidly surface-derived molecules might be transferred by trogocytosis, whereas the slower transfer of intracellularly derived molecules is most likely mediated by exosomes. However, identification of the exact cellular mechanisms underlying the transfer of these two sets of $CD4^+$ T cell surface molecules is currently under investigation.

Before entering secondary lymphoid organs, DCs can interact in the periphery with NK cells. Activated NK cells secrete great amounts of IFN-γ and they are thought to play an activating role upon interaction with DCs (36, 37). Our observation that activated NK cells leave some of their surface molecule behind after interaction with DCs may indicate a general mechanism for cells to change the phenotype and possibly the function of the cells they are leaving. NK cells might simply just mark the DCs, as it has been speculated for transferred NK cell molecules to target cells (38), or they may provide further signaling.

For $CD4^+$ T cells the two observed processes of surface molecule transfer might have essential functions during the Ag-specific activation of T cells: 1) the rapidly transferred, surface-derived molecules would be helpful for disengagement of DCs and T cells, and 2) during the later activation phase, the intracellularly derived molecules might be important for further immunomodulation. Our observations that transferred molecules like CD3 are bound to the DC surface for a long time (at least 18 h) strengthen the hypothesis that these transferred molecules indeed might have another function than just facilitating the dissociation of the two interacting cells. This seems to be different to APC-derived surface molecules, which are rapidly internalized by $CD8^+$ T cells (2) or DCs that take up allogenic DC-derived exosomes and internalize them rapidly to present the alloantigen (18). NK cells are also reported to rapidly internalize target cell HLA-C molecules (33).

Our findings contribute an additional important aspect to the current understanding of the process of $CD4^+$ T cell priming. $CD4^+$ T cell activation in vivo is initiated by a scanning phase that leads to transient interactions, followed by formation of stable DC-T cell clusters (1). The detachment of the $CD4^+$ T cell from the DC during the scanning phase could be facilitated by the transfer of surface-derived T cell surface molecules, thus facilitating maximal scanning events. Interestingly, after formation of stable DC-T cell clusters the T cells have been described to regain motility and "swarm" around DCs before they finally proliferate and exit the secondary lymphoid organ to the periphery (1). During this stage, T cells are visibly enlarged and migrate slowly among DCs in the local region with which they make dynamic serial interactions. During this stage they may deposit intracellularly formed T cell surface molecules on the DC surface.

We herein show that T cells modulate DC function in vitro after Ag-specific interaction by transferring T cell surface molecules such as costimulatory molecules and the TCR/CD3 complex to DCs. These T cell surface molecules might mask the corresponding MHC II-peptide molecules, leading to a diminished access of naive $CD4^+$ T cells of the same specificity to these modulated DCs and leaving the cell still accessible to Ag-specific $CD8^+$ T cells. This may play an important role in intraclonal competition and contraction of the Ag-specific immune response. $CD4^+$ T cells have been described to compete with other T cells of the same specificity for access to DCs (28, 29). Since these deposited molecules comprise the CD3/TCR complex, they might mask peptide-MHC II molecules and would thereby play an important role in intraclonal competition. Our observations strengthen this hypothesis since DCs that had acquired TCR molecules by Ag-specific interaction with $CD4^+$ T cells had reduced capacity to activate naive $CD4^+$ T cells of the same specificity even when the T cells from the first interaction had been sorted out (and were therefore not responsible for physical sterical hindrance). Furthermore, a diminished amount of MHC molecules detected on the DC surface after T cell interaction (9, 39) may be explained by MHC masking through transferred T cell surface molecules. The DC being the limiting factor during the priming of $CD4^+$ T cells of one specificity would be particularly meaningful in the dynamic model of Ag-specific T cell activation where the DC is thought to first interact with $CD4^+$ T cells, which leads to DC conditioning and subsequently recruits $CD8^+$ T cells (40–42). The $CD4^+$ T cell would be predominantly important for the DC conditioning to put it into a state ready for $CD8^+$ T cell activation. An exceeding activation of too many $CD4^+$ T cells, however, would not make sense. This could be achieved by masking MHC II molecules with T cell-derived CD3/TCR molecules rather than, as presumed so far, the physical presence of $CD4^+$ T cells (28).

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Disclosures

The authors have no financial conflicts of interest.

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