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Dendritic Cells Can Turn CD4$^+$ T Lymphocytes into Vascular Endothelial Growth Factor-Carrying Cells by Intercellular Neuropilin-1 Transfer[1]

Sarah Bourbié-Vaudaine,*†‡§ Nicolas Blanchard,¶ Claire Hivroz,¶ and Paul-Henri Roméo[2]*†‡§

Neuropilin-1 (NRP1) is a transmembrane protein expressed on neuronal and endothelial cells where it plays a crucial role in guiding axons and regulating angiogenesis. We have recently shown that NRP1 also is expressed on dendritic cells (DC) in the human immune system and have proposed a role for NRP1 in the first stages of the immune response. In these studies, we show that NRP1 can be transferred with a high efficiency from human DC to T lymphocytes by trogocytosis. The NRP1 transfer can occur independently of T lymphocyte activation; the amount of NRP1 transferred depends on the NRP1 expression level on APC and is enhanced when T cells are activated through the TCR. Moreover, the NRP1 transfer occurs between specific donor and recipient cells, because no NRP1 transfer is observed between endothelial cells and T lymphocytes or between APCs and CD34$^+$ hemopoietic cells. Finally, we show that a major NRP1 ligand, vascular endothelial growth factor (VEGF)$_{165}$, is secreted by mature human DCs and binds to NRP1 captured by T lymphocytes. These results show that NRP1 transfer to T lymphocytes during the immune synapse can convert T lymphocytes into VEGF$_{165}$-carrying cells. Together with the enhanced signaling of VEGF-R2 on endothelial cells in the presence, in *trans,* of the NRP1–VEGF$_{165}$ complex, our results suggest that the intercellular transfer of NRP1 might participate in the Ag-independent remodelling of the endothelial vessels in secondary lymphoid organs during inflammation. *The Journal of Immunology,* 2006, 177: 1460–1469.

Dendritic cells (DC)[3] can interact with naive T lymphocytes after infection in which DC-induced T cell priming results in a strong and highly specific immune response. DCs also can interact with naive T lymphocytes in the absence of exogenous stimulus thought to enhance T cell survival. In vivo, these interactions require DC–T cell contacts either of short duration (a few minutes in the absence of Ag) or long duration (at least 1 h in the presence of Ag), respectively, termed Ag-free or Ag-dependent immunological synapses (ISs) (1, 2). The formation of the IS involves a new distribution of membrane components at the area of contact. At the molecular level, the formation of the IS requires protein–protein interactions between APC and T lymphocytes, such as DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN/ICAM-3), CD2 and LFA-3, or ICAM-1–LFA-1 interactions (3, 4). The IS structure, once established, allows the exchange of information between APC and T lymphocytes, including Ag-dependent or Ag-independent signaling in T cells and cytokine secretion. Recently, another type of exchange has been identified that consists of the transfer of several membrane-bound molecules between APCs and T lymphocytes and has been termed trogocytosis (5, 6).

Trogocytosis is an active process that mediates transfer of membrane fragments between APC and T lymphocytes. Trogocytosis has been firstly described as a receptor-mediated unidirectional transfer of membranes from the APC to the lymphocytes, but several studies have now described spontaneous and/or bidirectional transfers between APC and T lymphocytes (7, 8). Among lymphocytes, trogocytosis has been reported in B lymphocytes (9), T lymphocytes (10–17), NK cells (18–21), and γδ T lymphocytes (22). Transfer to lymphocytes has been shown for lipids (11, 15, 16, 18, 22, 23) and plasma membrane proteins such as MHC (10–14, 16, 17, 24–26) and associated costimulatory molecules (10, 11, 14, 27–30), virus receptors (23, 31), and adhesion molecules (10, 13, 14). Several mechanisms have been proposed for this contact-dependent transfer of materials in T cell–APC conjugates, including exosomal release from APC and subsequent uptake by T lymphocytes (12, 13, 17), direct transfer of membranes while cells remain conjugated (10, 14, 16, 22), or mechanical withdraw of membrane fragments when the cells dissociate (32). Trogocytosis has been shown mainly to occur rapidly after TCR signaling and mainly with activated T lymphocytes (10, 11, 14, 16, 22, 24, 25). Functionally, trogocytosis may permit the acquisition of proteins usually not expressed by T cells, conferring new functions on these T cells. For example, the transfer of membrane-bound peptide–MHC complexes to Ag-specific T cell responders has been associated with the induction of tolerance (11, 26) or fratricide lysis at high Ag concentration (10). The transfer of the wild-type chemokine receptor CCR5 may lead to HIV infection of cells that do not express endogenous wild-type CCR5 (31), and the transfer of the OX40 ligand or CD80 can modulate the immune response (27, 29, 30). Other intercellular transfers have been described, such as capture

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[3] Abbreviations used in this paper: DC, dendritic cell; DCi, immature DC; DCm, mature DC; IS, immunological synapse; NRP1, neuropilin-1; Sema3A, semaphorin 3A; SEE, *Staphylococcus* enterotoxin E; CHX, cycloheximide; PFA, paraformaldehyde; MFI, mean fluorescence intensity; VEGF, vascular endothelial growth factor.

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of endothelial components by T lymphocytes during transendothelial migration (33) and capture from nonimmune cells (27, 34).

We have shown recently that neuropilin-1 (NRP1), a semaphorin 3A (Sema3A) and vascular endothelial growth factor $(VEGF)_{165}$ receptor, first characterized on neuronal and endothelial cells, is expressed on DC and plays a role in adhesion and clustering between DC and T lymphocytes (35). We now show that NRP1 can be efficiently transferred from APCs to activated or nonactivated $CD4^+$ T lymphocytes, that this trogocytosis requires specific donor and recipient cell, and finally, that this NRP1 trogocytosis could confer $VEGF_{165}$ *trans* presentation to $CD4^+$ T lymphocytes. Altogether, these data suggest a new role for NRP1 in the crosstalk between $CD4^+$ T lymphocytes and endothelial cells.

Materials and Methods

Cells

Cells were cultured in complete RPMI 1640 medium (GlutaMAX, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin; Life Technologies). Human PBMC were obtained from heparinized blood of healthy volunteers, after their informed consent, by density gradient centrifugation (Lymphoprep-Nyegaard). Human $CD4^+$ T lymphocytes and $CD14^+$monocytes were isolated by negative selection using, respectively, a $CD4^+$ T cell isolation kit II (human) and a monocyte isolation kit (Miltenyi Biotec). The purity achieved was always $\geq$97% for $CD4^+$ cells and $\geq$90% for $CD14^+$. Immature DC (DCi) were generated from monocytes cultured in complete RPMI 1640 medium supplemented with 100 ng/ml GM-CSF (Leucomax or PeproTech) and 20 ng/ml IL-4 (R&D Systems or PeproTech) for 5–6 days. Maturation was induced 1) for 24 h in the presence of 10 ng/ml TNF-α (R&D Systems) and 1 μg/ml PGE_2 (Sigma-Aldrich) or 2) for 48 h in the presence of 5 μg/ml LPS (Sigma-Aldrich). Superantigen-specific T cells blasts were obtained by activating PBMC with 0.1 mg/ml *Staphylococcus* enterotoxin E (SEE) for 9 days and with IL-2 20U/ml (R&D Systems) added at day 3. Superantigen loading on APC (pulsed APC) was performed at 37°C for 30 min at 1 mg/ml superantigen; SEE or a mix (SEA, SEB, SEC3, SED, and SEE) of superantigens were used. Primary human $CD34^+$ cells were obtained from cord blood as described previously (36). The purity achieved was always $\geq$90% of $CD34^+$ cells. HUVEC were isolated from umbilical vein as described previously (37).

Coculture of APC and T lymphocytes

T lymphocytes (or $CD34^+$ cells) were cocultured with APC (or HUVEC) at a ratio of 2:1 and a total cell concentration of 2×10^6/ml. For contact experiments, Transwells with pore size of 0.4 μm (Costar or Corning) were used.

Cycloheximide (CHX), paraformaldehyde (PFA), and PKH26 treatment

$CD4^+$ T lymphocytes were treated for 12 h with CHX at 20 μg/ml, then cocultured with DC without dilution of CHX (Sigma-Aldrich). DC were fixed for 10 min with 1% PFA (Electron Microscopy Sciences) at 4°C, then washed three times before coculture with T cells. Raji B cells were stained with red dye PKH26 (Sigma-Aldrich) according to the manufacturer's recommendations.

Analysis of surface NRP1 expression

Flow cytometric analysis were performed using a FACSCalibur flow cytometer (BD Biosciences). Data were acquired from at least 10,000 viable cells, using a forward/side scatter gate to select live cells. Immunofluorescence staining was performed for 30 min at 4°C. Before and after labeling, cells were washed and resuspended with PBS/EDTA 10 mM to minimize conjugates. The following Abs were used: anti-NRP1-PE, anti-allophycocyanin (BDCA4; Miltenyi Biotec); anti CD3-allophycocyanin, anti-FITC, anti-PE (Immunotech); anti CD4-FITC, anti-PE, anti-PC5 (Immunotech); anti CD19-allophycocyanin, anti-PE, anti-PC5 (Immunotech); anti CD25-FITC (Immunotech); anti CD69-PC5 (Immunotech). Controls were performed with IgG1-FITC, IgG1-PE, IgG1-PC5, IgG1-allophycocyanin (Immunotech), or IgG2a-PC5 (Immunotech). NRP1 expression on T cells was assessed using a forward/side scatter gate to select the smaller cells and/or gating on $CD3^+$ (or $CD4^+$ cells and/or gating on GFP-negative cells as shown in Figs. 1*C*, 2*A*, and 5*B*.

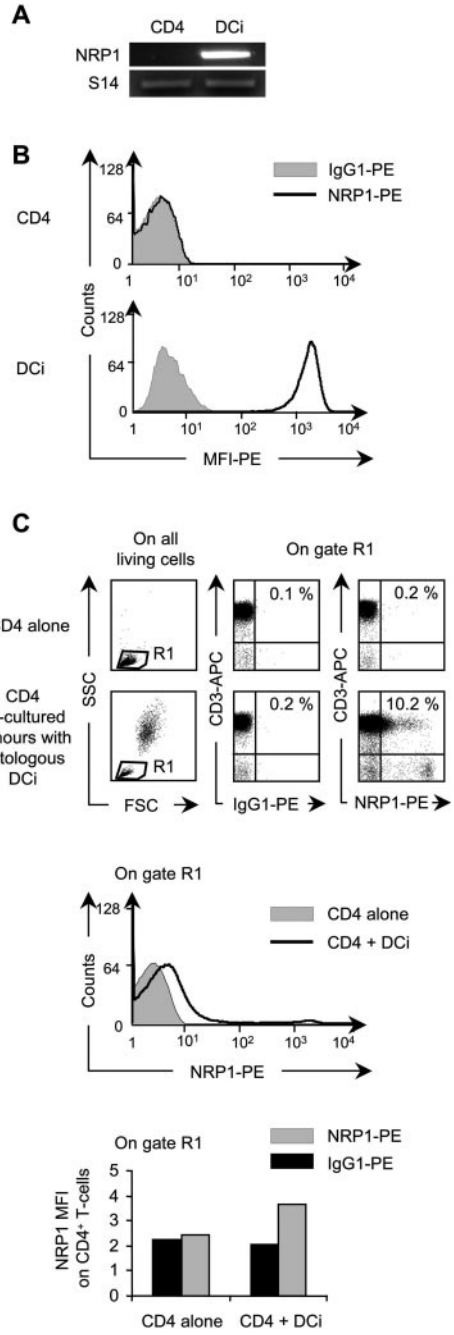

FIGURE 1. NRP1 is detected on human resting $CD4^+$ T lymphocytes after coculture with autologous DCi. Data presented are from one representative experiment ($n \geq 3$). *A*, NRP1 transcript levels in resting $CD4^+$ T lymphocytes (CD4) and in DCi from the same healthy donor were measured by semiquantitative RT-PCR analysis. Synthetized cDNA concentration was normalized between samples using the levels of the S14 gene product, and the NRP1-specific cDNA was detected using RT-PCR performed in the linear range. *B*, DCi or resting $CD4^+$ T lymphocytes (CD4) of the same healthy donor were stained with a PE-conjugated anti-NRP1 mAb (open histogram) or control mouse IgG1-PE Ab (filled histogram) and analyzed by flow cytometry. Mean of PE fluorescence is depicted. *C*, Resting $CD4^+$ T lymphocytes, alone or cocultured for 7 h with autologous DCi, were costained with a PE-conjugated anti-NRP1 mAb or control mouse IgG1-PE Ab and an allophycocyanin-conjugated anti-CD3 mAb, then studied by flow cytometry. Fluorescence was analyzed in the gate R1 containing T lymphocytes. *Top panel*, Results are presented in dot plots, and percentages of $NRP1^+$ $CD4^+$ T lymphocytes are shown. *Middle panel*, Mean of NRP1-PE fluorescence is depicted on CD4 (gate R1) cultured without (filled histogram) or with (open histogram) DCi. *Bottom panel*, Mean of PE fluorescence is depicted on CD4 cultured alone or with DCi using IgG1 (■) or NRP1 Abs (□).

NRP1-expressing constructs and cell lines

Human NRP1 cDNA was a gift from Dr. M. Klagsbrun (Children's Hospital and Harvard Medical School, Boston, MA) and was cloned into the lentiviral vector TRIPΔU3 EF1α IRES GFP (38). Rat NRP1 cDNA deleted of its stop codon was cloned in frame with GFP into pEGFP-N1 vector (Clontech Laboratories). Raji B cells were electroporated with a Gene Pulser (960 μF, 280 V, 8 $\times 10^6$ cells in 800 μl; Bio-Rad) with 40 μg of plasmid coding either for GFP or for the NRP1GFP fusion protein, then selected with neomycin (G418; Invitrogen Life Technologies) at 1 mg/ml before sorting of the GFP$^+$ cells. These Raji-tf-GFP and Raji-tf-NRP1GFP cell lines showed stable expression of GFP and NRP1GFP fusion protein, respectively.

RNA extraction, RT, and semiquantitative PCR

Total RNA was extracted from 10^6 cells with 1 ml of TRIzol reagent (Invitrogen Life Technologies). A second extraction with acidic phenol was done to increase RNA purity. RT was performed with 1.5 μg of RNA using SuperscriptII reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's recommendations. PCR was conducted in 50 μl using 1 μl of RT product and 2 U of *Taq* polymerase (ATGC). Synthetized cDNA concentration was normalized between samples using the levels of the S14 gene product. Linear response ranges were determined for each gene to semiquantify their expression. The following primers and conditions were used: S14 (150 bp, 64°C, 25–29 cycles), 5′-GGCAGAC CGAGATGAATCCTCA-3′ and 5′-CAGGTCCAGGGGTCTTGGTCC-3′; NPR1 (381 bp, 55°C, 29–33 cycles), 5′-TTCATCAGGATCTAC CCCGA-3′ and 5′-GAAGGTCTTGTGAGTGCCC-3′; VEGFR-1 (195 bp, 50°C, 30–35 cycles), 5′-CACCAAGAGCGACGTGTG-3′ and 5′-TTTT GGGTCTCTGTGCCAG-3′; VEGFR-2 (212 bp, 50°C, 30–35 cycles), 5′-CAGCTTCCAAGTGGCTAAGG-3′ and 5′-TCAAAAATTGTTTCTGG GGC-3′; and VEGF-A (v-121 442 bp, v-145 514 bp, v-165 574 bp, v-189 646 bp, v-206 687 bp, 60°C, 30 cycles), 5′-ATGAACTTTCTGCT GTCTTGGG-3′ and 5′-CACCGCCTCGGCTTGTCACAT-3′.

VEGF-A assay

VEGF-A in DC supernatants (5×10^5 cells/ml) was assayed by ELISA test with Quantikine (R&D Systems). Microcons (Amplicons) were used to concentrate supernatants.

Binding of biotinylated VEGF$_{165}$

Binding of biotinylated human VEGF$_{165}$ was assessed according to the manufacturer's recommendations (Human VEGF Biotinylated Fluorokine Kit; R&D Systems). Briefly, CD4$^+$ T cells were cocultured with Raji-GFP or Raji-NRP1 B cells for 24 h. T cells were then incubated with biotinylated VEGF$_{165}$ and avidin-FITC. Binding is proportional to FITC fluorescence measured by flow cytometry. Binding was blocked using a mAb against NRP1 (BDCA4, 1 μg/test; Miltenyi Biotec).

Results

NRP1 can be detected on CD4$^+$ T lymphocytes after coculture with DC

We investigated the NRP1 transcript levels in human DC and resting CD4$^+$ T lymphocytes from the same healthy donors. RT-PCR analysis showed that DC expressed a high level of NRP1 mRNA, whereas CD4$^+$ T lymphocytes did not express detectable levels of NRP1 mRNA under the same conditions (Fig. 1*A*). These results were supported by flow cytometric analysis showing high expression of NRP1 on DC and no detectable expression on the surface (Fig. 1*B*) or in the cytoplasm (data not shown) of resting CD4$^+$ T lymphocytes. However, when these CD4$^+$ T lymphocytes were cocultured with autologous DCi for 7 h, NRP1 expression was detected on 10% of CD4$^+$ T lymphocytes defined by their small size (Fig. 1*C*, gate R1, *upper panel*) and their CD3 expression (Fig. 1*C*, *upper panel*). A detailed analysis of the mean fluorescence intensity (MFI) linked to NRP1 expression revealed that the NRP1 signal was shifted in the whole population of CD4$^+$ T lymphocytes, indicating that all T cells expressed NRP1, but only 10% expressed NRP1 levels detectable by flow cytometry (Fig. 1*C*, *middle panel*). Altogether, these results demonstrated that NRP1 expression can be detected on resting CD4$^+$ T lymphocytes after coculture with autologous DC.

DC-induced expression of NRP1 on CD4$^+$ T lymphocytes is not dependent on lymphocyte activation

To define the different parameters that regulate the coculture-dependent expression of NRP1 on CD4$^+$ T lymphocytes, we first used an Ag-dependent system mimicking a strong immune response. Briefly, human activated T lymphocytes (T blasts) were obtained by stimulation of PBMC with the SEE superantigen, which mostly activates TCR CD4$^+$ subsets. At day 9, >95% of the cells were CD3$^+$, and >60% were CD4$^+$ (data not shown). CD4$^+$ T blasts were then obtained by depletion of the CD8$^+$ T lymphocytes and cultured with IL2 or reactivated with mature DC (DCm) loaded with the SEE superantigen. After 7 h of coculture with superantigen-pulsed DCm, the NRP1 cell surface expression was detected on >60% of CD4$^+$ T blasts (Fig. 2*A*, *upper panel*) and strongly enhanced in the whole population (Fig. 2*A*, *lower panel*), whereas CD4$^+$ T blasts cultured alone expressed no detectable NRP1 on their surface (Fig. 2*A*).

We then investigated whether NRP1 expression on CD4$^+$ T lymphocytes was dependent on Ag stimulation and T cell activation. We performed coculture experiments using different types of CD4$^+$ T lymphocytes (resting CD4$^+$ T lymphocytes or CD4$^+$ T blasts from the same donor) and different types of APC (B cells and DCi or DCm) in the presence or absence of superantigen. We first quantified NRP1 expression on the APC and showed that Raji B cells did not express detectable level of NRP1 at their surface, whereas both DCi and DCm expressed similar and high level of NRP1 (Fig. 2*B*). In the presence of SEE, Raji B cells, which do not express NRP1, were able to sustain reactivation of all T blasts or activation of the responding resting T cells (8–10%), as indicated by the expression of CD25 and CD69 activation markers (data not shown), but Raji B cells were unable to induce any NRP1 expression on those T lymphocytes after 7 h of coculture, whereas DCi and DCm induced a similar and high NRP1 expression on T lymphocytes (Fig. 2*C*). Furthermore, similar results were observed with autologous or heterologous DC (data not shown). Hence, NRP1 expression on T lymphocytes is not observed after coculture with any APC and is not related to the activation ability of the APC (39). We then compared the NRP1 expression on resting CD4$^+$ T lymphocytes and on CD4$^+$ T blasts in coculture with APC. DC-induced NRP1 expression on T lymphocytes was observed on both CD4$^+$ T cell types even in the absence of Ag. However, the presence of SEE significantly enhanced NRP1 expression (Fig. 2*C*, compare *gray* and *black columns*). In addition, CD4$^+$ T blasts expressed around two times more NRP1, in the percentage of positive cells and in MFI, than did resting CD4$^+$ T lymphocytes after coculture with DC (Fig. 2*C*, compare *upper* and *lower panels*). DC-induced NRP1 expression also was observed in the absence of activation, as we could detect CD69^{neg}NRP1$^+$ T cells among resting T cells cocultured with DCm or DCi in the absence of Ag (data not shown). Finally, the relationship between CD4$^+$ T blasts activation and NRP1 expression was studied using PFA that cross-links DC surface proteins. Superantigen-loaded DC, fixed with PFA before coculture with CD4$^+$ T blasts, failed to induce NRP1 expression on CD4$^+$ T blasts (Fig. 2*D*) but did not impair DC-mediated activation of CD4$^+$ T blasts (data not shown), dissociating CD4$^+$ T blasts activation from NRP1 expression. Altogether, these results demonstrated that NRP1 expression on T cells after APC coculture did not correlate with T cell activation and highlighted the importance of APC proteins membranes composition to observe NRP1 expression on T cells.

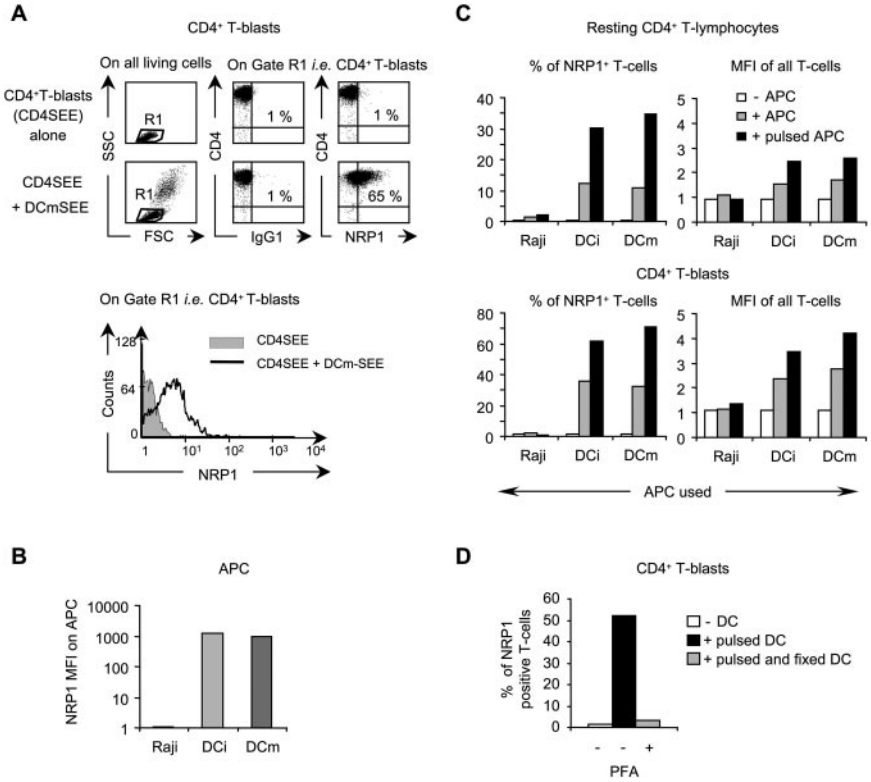

FIGURE 2. Resting CD4$^+$ T lymphocytes and CD4$^+$ T blasts express NRP1 after coculture with DCi or DCm. NRP1 expression on CD4$^+$ T cells was analyzed by flow cytometry using a PE-conjugated anti-NRP1 mAb or control mouse IgG1-PE Ab and gating in a forward/side-scatter window on T cells as shown in Figs. 1*C* and 2*A*. Data presented are from one representative experiment ($n \geq 3$). *A*, SEE-specific CD4$^+$ T blasts (CD4SEE) were cultured for 7 h alone or with mature SEE-pulsed DC (DCmSEE). *Upper panel*, Results are presented in dot plots, and percentages of NRP1$^+$ CD4$^+$ T cells are shown. *Lower panel*, Mean of NRP1-PE fluorescence is depicted on CD4$^+$ T cells when T cells are cultured without (filled histogram) or with (open histogram) DCmSEE. *B*, Expression of NRP1 on Raji, DCi, and DCm was assessed by flow cytometry. NRP1 MFI is depicted. *C*, Resting CD4$^+$ T lymphocytes (*upper panel*) or SEE-specific CD4$^+$ T blasts (*lower panel*) were cultured for 7 h with Raji B cells (Raji), DCi, or DCm pulsed (black column) or not pulsed (gray column) with SEE superantigen. CD4$^+$ T cells cultured without APC were used as a control (open column). Percentage of NRP1$^+$ CD4$^+$ T lymphocytes and MFI linked to NRP1 on all T cells are indicated. *D*, Pulsed DC were treated with PFA as described in *Materials and Methods*, and activated CD4$^+$ T lymphocytes were cultured for 7 h with these PFA-treated DC (gray column) or with untreated pulsed DC (black column). CD4$^+$ T blasts cultured alone were used as a control (open column). The percentage of NRP1$^+$ CD4$^+$ T lymphocytes is indicated.

DC-induced expression of NRP1 on CD4$^+$T lymphocytes is rapid and transient

We then investigated the kinetics of NRP1 expression on T cells cocultured with DC and the effect of cell concentration on this expression. Resting CD4$^+$ T cells were cocultured for 15 min or 4 h with an increasing concentration of superantigen-pulsed DCm and analyzed by flow cytometry for NRP1 expression. After 15 min of coculture, NRP1 expression was detected on a subset of T cells that increased while increasing the DC:CD4 ratio (Fig. 3*A*, *middle column*; and data not shown). In contrast, after 4 h of coculture, NRP1 expression on cocultured T cells was enhanced in the whole population and was independent of the DC:CD4 ratio (Fig. 3*A*, *right column*; and data not shown). Thus, DC-induced NRP1 expression on T cells is an early and saturable phenomenon depending on the DC:CD4 ratio at short times. Finally, we studied a longer kinetics of NRP1 expression on CD4$^+$ T blasts cocultured with SEE-pulsed DCm at a DC:CD4 ratio of 1:2. The percentage of T cells expressing NRP1 reached its maximum after 16–24 h, then slowly decreased and became undetectable at day 7 (Fig. 3*B*). Kinetics of NRP1 expression obtained with resting CD4$^+$ T lymphocytes displayed the same pattern (data not shown). Altogether, these results showed that NRP1 expression on T cells cocultured with DC was rapid and transient.

DC-induced expression of NRP1 on CD4$^+$ T lymphocytes does not require de novo protein synthesis

We showed that NRP1 expression on resting T cells could be detected 15 min after a coculture with DC, although resting T cells did not express detectable levels of NRP1 protein. To determine whether this NRP1 expression on T cells following coculture with DC was the result of acquisition of NRP1 by the T lymphocyte from the DCs or/and rapid de novo expression of NRP1, protein synthesis was inhibited in resting CD4$^+$ T lymphocytes by CHX treatment before and during the coculture with DC. The NRP1 MFI (data not shown) and the percentage of NRP1$^+$ T lymphocytes were not affected by CHX (Fig. 4, *upper panel*), although this treatment inhibited protein synthesis as shown by the inhibition of the increase in size of T lymphocytes in response to superantigen activation (Fig. 4, *lower panel*). Hence, DC-induced NRP1 expression on CD4$^+$ T lymphocytes did not require protein synthesis. Altogether, these results suggested that NRP1 was transferred from DC to CD4$^+$ T lymphocytes by trogocytosis.

Ectopic NRP1 can be transferred from APC to CD4$^+$ T lymphocytes

To get insights into NRP1 trogocytosis, NRP1 was stably expressed in Raji B cells, which represent efficient APC and do not

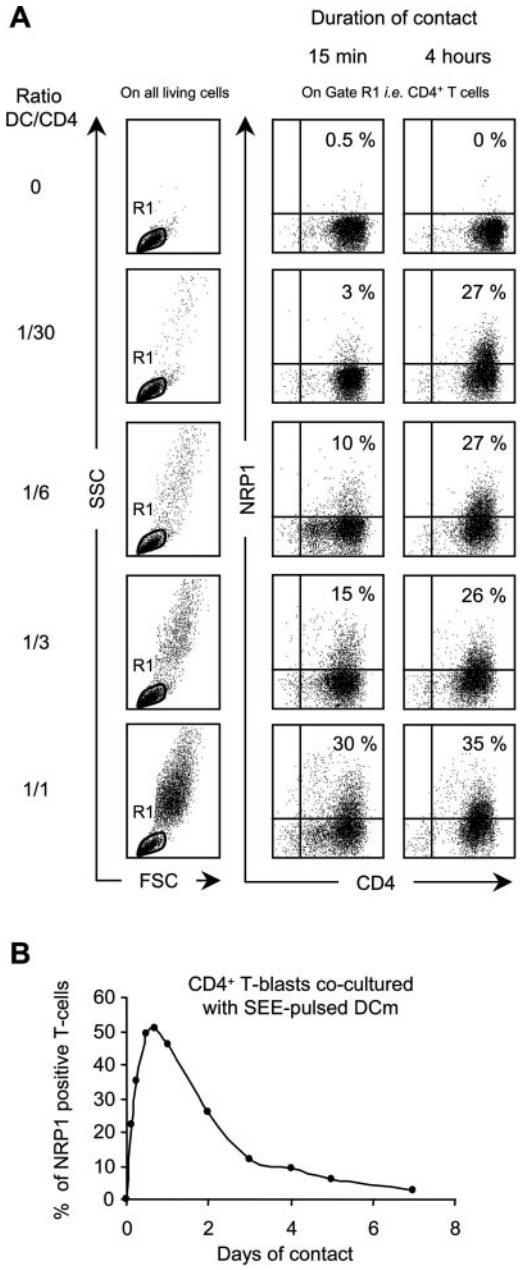

FIGURE 3. DC-induced expression of NRP1 on CD4+T lymphocytes is rapid and transient. NRP1 expression on $CD4^+$ T cells was analyzed by flow cytometry using allophycocyanin-conjugated anti-NRP1 mAb and a PE-conjugated anti-CD4 Ab gating on R1 as shown in *A*. Data presented are from one representative experiment ($n \geq 3$). *A*, Resting $CD4^+$ T cells were cocultured for 15 min (*center column*) or 4 h (*right column*) with increasing concentrations of pulsed DCm and analyzed for NRP1 expression. Results are presented in dot plots, and percentages of $NRP1^+$ $CD4^+$ T cells are shown. Ratios between number of DC and number of T cells are indicated on the left. *B*, Kinetics of NRP1 expression on $CD4^+$ T lymphocytes after various times of coculture with SEE-pulsed DCm. The percentage of $NRP1^+$ $CD4^+$ T lymphocytes is indicated.

express innate NRP1. Two lentiviruses, TRIPΔU3 EF1α NRP1 IRES GFP and TRIPΔU3 EF1α IRES GFP, coding for human NRP1 and GFP or for GFP alone, were engineered (Fig. 5*A*, *left panel*) and used to transduce Raji B cells. Different Raji B cell lines were obtained after transduction and sorting of the GFP^+ cells. As expected, nontransduced Raji expressed neither GFP nor NRP1, TRIPΔU3 EF1α IRES GFP-transduced Raji (hereafter Raji-GFP) expressed GFP but not NRP1, and TRIPΔU3 EF1α

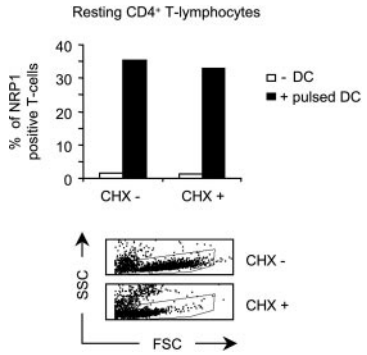

FIGURE 4. DC-induced expression of NRP1 on resting $CD4^+$ T lymphocytes does not require de novo protein synthesis. NRP1 expression on $CD4^+$ T cells was analyzed by flow cytometry using a NRP1 mAb and gating in a forward/side-scatter window on T cells as shown in Figs. 1*C*, 2*A*, and 3*A*. Data presented are from one representative experiment ($n \geq 3$). Resting $CD4^+$ T lymphocytes were either not treated (CHX^-) or treated with CHX^+ before any contact with pulsed DC, then cultured for 7 h with (black column) or without (open column) the pulsed DC, and analyzed for NRP1 expression (*upper panel*). The percentage of $NRP1^+$ $CD4^+$ T lymphocytes is indicated. Efficiency of the CHX treatment was monitored by forward/side-scatter analysis of $CD4^+$ T cells (*lower panel*).

NRP1 IRES GFP-transduced Raji (hereafter Raji-NRP1) expressed both NRP1 and GFP (Fig. 5*A*, *right panel*). Resting $CD4^+$ T lymphocytes were cocultured with the different Raji B cell lines, and NRP1 surface expression was monitored every day for 7 days in the absence of Ag. To analyze NRP1 expression on T cells, we gated on smaller cells that included $CD4^+$ T cells and excluded GFP^+ Raji B cells (Fig. 5*B*, *left panel*). Raji-NRP1 induced a transient NRP1 expression on T lymphocytes, while no NRP1 was detected on T lymphocytes cocultured with Raji-GFP (Fig. 5*B*, *right panel*). Kinetics of NRP1 expression on T cells observed with Raji-NRP1 were delayed, compared with those obtained with DC (compare Figs. 3*B* and 5*B*), and this difference may be partly explained by the difference of NRP1 surface expression levels on the two cell types (compare Figs. 2*B* and 6*D*, *upper panel*). Then, $CD4^+$ T lymphocytes were cocultured 24 h with Raji-NRP1 B clones expressing different levels of NRP1 at their surface and analysis of NRP1 expression on these T lymphocytes showed a dose-dependent transfer of NRP1 to T cells (Fig. 5*C*). These results demonstrated that ectopic NRP1 expression at the surface of Raji B cells is sufficient to induce NRP1 expression on T lymphocytes and that the amount of NRP1 transferred on T lymphocyte is directly correlated to the NRP1 expression level on Raji-NRP1 B clones. Finally, we investigated the effect of superantigen on the transfer of NRP1 to $CD4^+$ T lymphocytes and compared resting to activated $CD4^+$ T lymphocytes responses after 24 h of contact. Similar to the experiments performed with DC, NRP1 transfer occurred in the absence of T cell activation, as shown by the absence of activation markers such as CD69 and CD25 on $NRP1^+$ T lymphocyte in the absence of Ag, but was enhanced within activated T lymphocytes (in MFI and in percentage of $NRP1^+$ T cells) and in the presence of superantigen (Fig. 5*D* and data not shown). These results showed that ectopically expressed NRP1 at the surface of Raji B cells and endogenous NRP1 at the surface of DC can be similarly transferred to $CD4^+$ T lymphocytes. We then investigated whether a full-length transmembrane NRP1 and not a cleaved fragment of NRP1 was transferred from Raji B cells to $CD4^+$ T lymphocytes. $CD4^+$ T blasts were cocultured with two Raji B cell lines expressing GFP (hereafter Raji-tf-GFP) or the NRP1 protein fused at its intracytoplasmic COOH terminus to

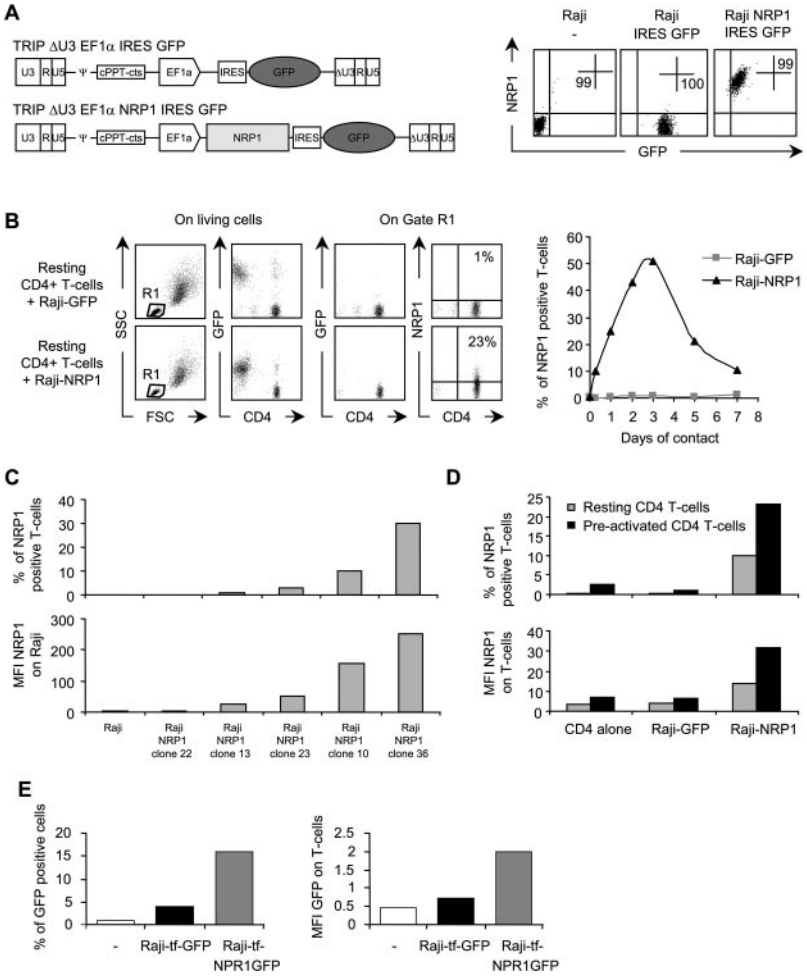

FIGURE 5. Ectopic expression of NRP1 on Raji B cells is sufficient to observe NRP1 transfer onto CD4$^+$ T lymphocytes. NRP1 expression on CD4$^+$ T cells was analyzed by flow cytometry using a NRP1 mAb and gating on smaller cells and CD4$^+$ cells as shown in Fig. 4*B*, *left panel*. Data presented are from one representative experiment ($n \geq 3$). *A*, Schematic representation of the two lentiviruses used to transduce Raji B cells (*right panel*). Untransduced and transduced Raji B cells were analyzed by flow cytometry for GFP and NRP1 expression (*left panel*). Percentages of cells in the corresponding quadrant are indicated. *B*, *Left panel*, Flow cytometric analysis of FSC, SSC, GFP, CD4, and NRP1 on resting CD4$^+$ T cells cocultured 24 h with Raji-GFP or Raji-NRP1. Percentages of NRP1$^+$ T cells are indicated. *Right panel*, Percentages of NRP1$^+$ CD4$^+$ T lymphocytes after various times of contact with Raji-GFP or Raji-NRP1 B cells. *C*, Various Raji B cells clones expressing increasing amounts of NRP1 as determined by the NRP1 MFI (*lower panel*) were used in coculture with CD4$^+$T lymphocytes for 24 h and the percentage of NRP1$^+$ CD4$^+$ T lymphocytes was determined by flow cytometry (*upper panel*). *D*, Resting (gray column) or activated CD4$^+$ T lymphocytes (black column) were cultured for 24 h with the indicated Raji B cells. The percentage of NRP1$^+$ T cells (*upper panel*) and the MFI of NRP1 on T cells (*lower panel*) were analyzed by flow cytometry. *E*, Activated CD4$^+$ T lymphocytes were cultured for 24 h without Ag with Raji transfected stably with GFP (gray column) or with fusion protein of NRP1 and GFP (black column) and analyzed by flow cytometry. The percentage of GFP$^+$ T cells (*left panel*) and MFI GFP on T cells (*right panel*) cultured with these Raji B cells or alone are represented.

GFP (hereafter Raji-tf-NRP1GFP). Fusion protein could be followed by GFP fluorescence or NRP1 Ab staining. CD4$^+$ T cells cocultured with Raji-tf-NRP1GFP strongly expressed NRP1, whereas CD4$^+$ T cells cultured alone or with Raji-tf-GFP did not (data not shown). Moreover, 16% of the T blasts cocultured with Raji-tf-NRP1GFP were GFP$^+$ (Fig. 5*E*, *left panel*), and the MFI GFP in the whole population was increased four times, compared with MFI GFP of CD4$^+$ T blasts cultured alone or with Raji-tf-GFP (Fig. 5*E*, *right panel*). Altogether, these results indicated that NRP1 could be transferred without cleavage of the protein from NRP1 expressing APC to T lymphocytes.

NRP1 transfer is cell contact dependent and associated with membrane dye transfer

To study the contact requirement for NRP1 transfer, Raji-NRP1 B cells were separated from T lymphocytes by a 0.4-μm pore-size membrane. The diameter of the pores was large enough to let microparticles or soluble factors reach T cells but small enough to prevent diapedesis of Raji B cells. As shown in Fig. 6*A*, no NRP1 was detected on resting CD4$^+$ T lymphocytes unable to encounter Raji B cells, even in the presence of superantigen, indicating that a physical contact between resting T lymphocytes and APC is critical for NRP1 transfer. We then uniformly labeled Raji membranes with PKH26, a red fluorescent dye that incorporates into lipidic regions of the membranes. Resting CD4$^+$ T lymphocytes cocultured for 24 h with labeled Raji-NRP1 B cells were analyzed for NRP1 expression and PKH26 fluorescence. Most T lymphocytes (>80%) were either double positive or double negative for NRP1 expression and PKH26 fluorescence (Fig. 6*B*), indicating a correlation between NRP1 and PKH26 transfer from Raji-NRP1 B cells to T lymphocytes. Although we could not exclude that the dye itself, extracted from the lipidic regions of Raji membranes, was captured by CD4$^+$ T cells, these results strongly suggested that CD4$^+$ T lymphocytes captured pieces of APC membranes together with NRP1 during contact between T lymphocyte and APC.

NRP1 transfer occurs between specific donor and recipient cells

We then tested the specificity of recipient and donor cells in this NRP1 transfer. First, we compared the ability of CD34$^+$ hemopoietic cells and resting CD4$^+$ T lymphocytes to capture NRP1 at the surface of Raji-NRP1 B cells. Unlike resting T cells, CD34$^+$ hemopoietic cells cocultured with Raji-NRP1 B cells could not capture NRP1, indicating a specificity of recipient cells for the NRP1 transfer (Fig. 6*C*). Second, we compared the percentage of NRP1$^+$ CD4$^+$ T lymphocytes after coculture with Raji-GFP, Raji-NRP1, or HUVEC, which express endogenous NRP1 and present twice more NRP1 receptors at their surface than Raji-NRP1 (Fig. 6*D*, *upper panel*). HUVEC were unable to transfer NRP1 to cocultured T lymphocytes, demonstrating a specificity of NRP1 donor cells (Fig. 6*D*, *lower panel*). Altogether, these results demonstrated that NRP1 transfer is a phenomenon that requires specific donor and recipient cells.

Captured NRP1 expressed on T lymphocytes can bind VEGF$_{165}$

We first investigated the presence of two major NRP1 ligands, Sema3A and VEGF$_{165}$, during DC/T lymphocyte contact. Ligands

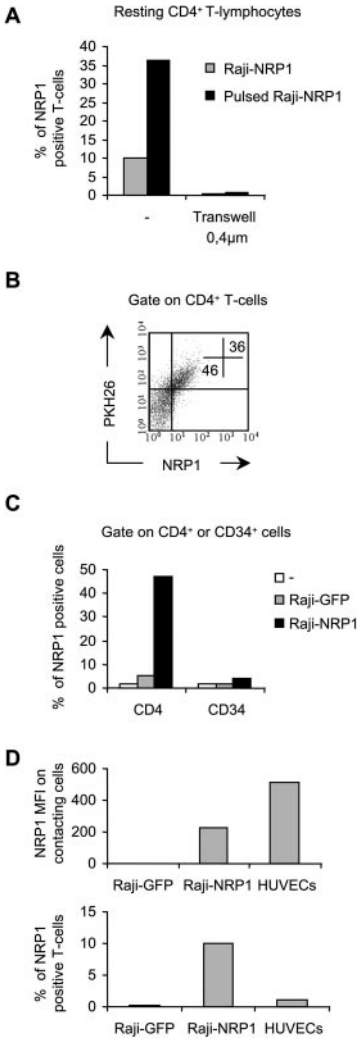

FIGURE 6. Transfer of NRP1 to CD4$^+$ T lymphocytes requires contact with the APC and requires specific donor and recipient cells. Data presented are from one representative experiment ($n \geq 3$). NRP1 expression on CD4$^+$ T cells or CD34$^+$ hemopoietic cells was analyzed by flow cytometry using a NRP1 mAb and gating on smaller cells and CD4$^+$ or CD34$^+$ cells. *A*, Raji-NRP1 B cells pulsed or not with a mix of superantigens were cultured for 24 h with CD4$^+$ T lymphocytes separated or not separated by a 0.4-μm pore-size Transwell membrane. The percentage of NRP1$^+$ CD4$^+$ T lymphocytes was determined by flow cytometry. *B*, PKH26, a red fluorescent dye, was used to uniformly label the Raji-NRP1 membranes. CD4$^+$ T lymphocytes were cocultured with PKH26-labeled Raji B cells for 24 h and analyzed by flow cytometry, for simultaneous expression of NRP1 and PKH26 fluorescence. The percentages of cells in the corresponding quadrant are indicated. *C*, CD4$^+$ T lymphocytes and CD34$^+$ hemopoietic cells were cultured for 24 h alone or with Raji-GFP or with Raji-NRP1 B cells. NRP1 expression by CD4$^+$ or CD34$^+$ cells was determined by flow cytometry. *D*, Resting CD4$^+$ T lymphocytes were cultured for 24 h in the presence of Raji-GFP B cells, Raji-NRP1 B cells, or endothelial HUVECs. *Upper panel*, NRP1 expression on transduced Raji B cells and HUVECs. *Lower panel*, The percentage of NRP1$^+$ cells among CD4$^+$ T cells.

expression was studied in DCi and DCm, resting CD4$^+$ T lymphocytes, and HUVEC as a control. Sema3A transcripts were weakly detected in all cell types, and Sema3A protein was not detectable in supernatants (data not shown). Therefore, we focused our studies on $VEGF_{165}$. VEGF-A transcripts were detected in DC, T lymphocytes, and HUVEC, but DCm expressed higher levels of VEGF transcripts (Fig. 7*A*). ELISA analysis on DCi and DCm supernatants showed that VEGF-A was strongly detected in supernatants from DCm and not in DCi (Fig. 7*B*). These results demonstrated that DCm are VEGF-A secretory cells and can be a source of $VEGF_{165}$ for surrounding T cells.

We then quantified the levels of the three $VEGF_{165}$ receptors (NRP1, VEGFR1, and VEGFR2) transcripts in resting CD4$^+$ T lymphocytes and in superantigen-pulsed Raji-GFP or Raji-NRP1 cultured without (−) or with (+) resting CD4$^+$ T lymphocytes. We did not detect any $VEGF_{165}$ receptor transcript in resting CD4$^+$ T cells. NRP1 mRNA was the only $VEGF_{165}$ receptor transcript that could be detected in the RNAs isolated from T cells/Raji B cells cocultures but only when Raji-NRP1 B cells were used (Fig. 7*C*). These results indicated that the only $VEGF_{165}$ receptor that can be expressed on T cells cocultured with Raji B cells is NRP1 uptaken on Raji-NRP1 B cells surface. Finally, we investigated whether human biotinylated $VEGF_{165}$ could bind NRP1 transferred to CD4$^+$ T lymphocytes. Resting T cells were cocultured 24 h with Raji-GFP or Raji-NRP1, and binding of biotinylated $VEGF_{165}$ was measured using avidin-FITC by flow cytometry, gating on T cells. CD4$^+$ T cells cocultured with Raji-NRP1 bound 3.5 times more $VEGF_{165}$ than did T cells cocultured with Raji-GFP (Fig. 7*D*, *left panel*). This binding was blocked by an Ab against NRP1, showing the specificity of binding (Fig. 7*D*, *right panel*). Altogether, these results indicated that CD4$^+$ T lymphocytes expressing exogenous NRP1 can bind $VEGF_{165}$ that can be secreted by surrounding or contacting DCm.

Discussion

In these studies, we have shown that NRP1 can be transferred from APC to resting or activated CD4$^+$ T lymphocytes and have demonstrated that this transfer requires specific donor and recipient cells. Finally, we have found that the transferred NRP1 can efficiently bind one of its natural ligand, $VEGF_{165}$, secreted by DCm.

NRP1 capture is independent of CD4$^+$ T lymphocyte activation

We have shown that NRP1 expression was very low at the mRNA level and undetectable by flow cytometry on resting CD4$^+$ T lymphocytes but could be greatly enhanced, at the protein level, after a coculture with NRP1-expressing APCs. Moreover, this increased expression on CD4$^+$ T cells was the result of NRP1 uptake from the surface of the APC. Although, the capture of membrane-associated proteins from APC by CD4$^+$ T lymphocytes has been previously described and termed trogocytosis, it was mainly described with activated T lymphocytes and essentially, in the presence of Ag. In this study, we extended the phenomenon to any coculture between a CD4$^+$ T lymphocyte and a NRP1-expressing APC, because we showed that NRP1 can be efficiently transferred from NRP1-expressing APC to resting CD4$^+$ T lymphocytes in the absence of activation and/or Ag. Thus, NRP1 transfer could occur independently of TCR signaling, independently of TCR–ligand interaction and possibly during the transitory contacts observed between DCi and resting T cells in the absence of Ag (40).

NRP1 transfer was greatly enhanced in the presence of superantigen or when blast T cells were used instead of resting CD4$^+$ T lymphocytes. Two nonexclusive hypotheses may explain this increase. First, the surface of contact between a T cell and an APC is larger in the presence of Ag (41), allowing more membrane uptake by the T cell. Second, the ability of capture depends on the reactivity of the T cell that we define as its ability to respond to extracellular signals. Indeed, resting CD4$^+$ T cells could capture more NRP1 if they were treated with a calcic ionophore (our unpublished data) and activated T cells capture more NRP1 than resting T cells. In support of this hypothesis, the actin cytoskeleton

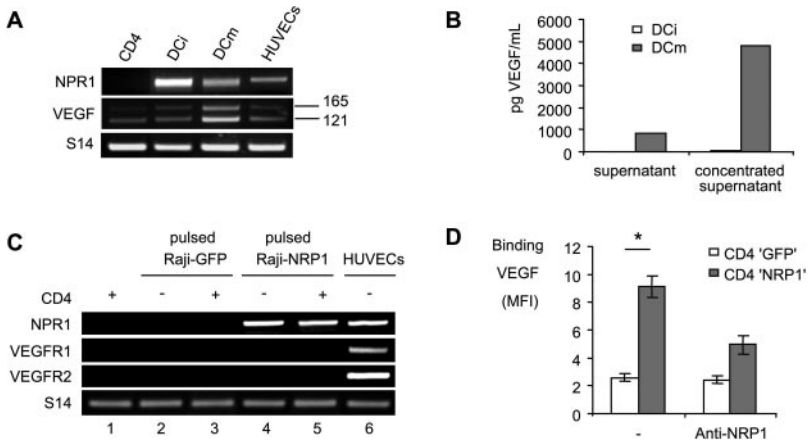

FIGURE 7. NRP1 transfer onto $CD4^+$ T lymphocytes can convert these $CD4^+$ T lymphocytes into $VEGF_{165}$ carrying cells. The data presented in *A–C* are from one representative experiment ($n \geq 3$). *A*, NRP1, $VEGF_{165}$, and $VEGF_{121}$ mRNA levels were determined in resting $CD4^+$ T lymphocytes, DCi, and DCm and HUVEC endothelial cells by semiquantitative RT-PCR. Synthetized cDNA concentration was normalized between samples using the levels of the S14 gene product and the NRP1- and VEGF-specific cDNAs were detected using RT-PCR performed in the linear range. *B*, VEGF-A expression was quantified in the supernatants or in 8-fold concentrated supernatants of DCi or DCm by ELISA. *C*, $VEGF_{165}$ receptors (VEGFR1, VEGFR2, and NRP1) mRNA levels were analyzed by semiquantitative RT-PCR in resting $CD4^+$ T cells (*lane 1*), superantigen-pulsed Raji-GFP (*lane 2*), coculture (24 h) of superantigen-pulsed Raji-GFP and resting $CD4^+$ T cells (*lane 3*), superantigen-pulsed Raji-NRP1 (*lane 4*), coculture (24 h) of superantigen-pulsed Raji-NRP1 and resting $CD4^+$ T cells (*lane 5*) and HUVEC endothelial cells as a positive control (*lane 6*). All RT-PCRs were performed as in *A*. *D*, Resting $CD4^+$ T cells were cocultured for 24 h with Raji-GFP or Raji-NRP1. Binding of human biotinylated $VEGF_{165}$ was analyzed by flow cytometry using avidin-FITC in the absence or presence of an anti-NRP1 Ab. Results shown are average from five independent experiments, and SE of the mean are shown. Differences are statistically significant as assessed by Mann-Whitney *U* test (*, $p = 0.0325$).

of T cells was shown to be crucial for absorption and internalization of ligands from APC (13).

Kinetics of NRP1 trogocytosis

Trogocytosis is described as a very rapid phenomenon occurring within minutes of contact. In agreement with this observation, we showed that NRP1 could be detected on T cells as early as after 15 min of coculture and that the transfer rate in short cocultures (10–60 min), but not in long cocultures (4 h), could be enhanced when T cell–APC interaction was favored by increasing cell concentration, modifying APC:CD4 ratio or by a low-speed centrifugation (data not shown). These results could be analyzed in terms of probability of contact between APC and T cells. After a few minutes, the probability for a T cell to encounter an APC is weak and greatly enhanced by increasing the number of DC, whereas after a few hours, each T cell could have contacted a DC and nibbled its membrane even at a DC:T cell ratio of 1:30.

Raji B cells as a model to study NRP1 transfer

Our Raji B cell model turned out to be a powerful tool to study NRP1 transfer. First, in the NRP1 transfer with DC, we could not exclude that T cells captured only soluble NRP1. Soluble isoforms of NRP1 are not a cleavage product of transmembrane NRP1 but are encoded by mRNA produced from the primary NRP1 transcript through alternative splicing and polyadenylation sites (42, 43). Because transcripts encoding soluble NRP1 were expressed at the same levels as transmembrane NRP1 mRNA in DCi and DCm, we could not discriminate between soluble and transmembrane NRP1 transfer. Using Raji B cells transduced with a lentivirus driving the transmembrane NRP1 expression only, we showed a transfer of NRP1 in the absence of soluble NRP1. Second, the Raji B cell model demonstrated the exogenous origin of NRP1 on T cells by comparing NRP1 expression on T cells after a contact with Raji-GFP or Raji-NRP1 and by the transfer of the transmembrane fusion NRP1GFP protein. Finally, we showed that trogocytosis of NRP1 was dependent on NRP1 expression level on Raji B cells and was enhanced in the presence of superantigen. Hence, ectopically expressed NRP1 on Raji is sufficient to induce NRP1 transfer on T cells in a dose-dependent manner, as shown with CD80 transfer (29, 30).

Putative mechanisms of transfer

The flow cytometric analysis of NRP1 transfer on $CD4^+$ T lymphocytes indicated that, in contrast with TCR-MHC transfer (13, 14), NRP1 expressed by T lymphocytes was not internalized immediately after transfer. Moreover, NRP1 on T cells exhibited a correct spatial orientation, because it is detected by Abs directed against the extracellular part of NRP1, suggesting that the extracellular domain of NRP1 also is accessible for its ligands. We also demonstrated that NRP1 was transferred together with a membrane dye and that CD19, a molecule that is specifically expressed by B cells, also was captured by $CD4^+$ T cells cocultured with Raji B cells (data not shown).

Transfer of NRP1 could be the consequence of APC membrane nibbling by T cells or release of small exosome-like APCs derived vesicles (44), which contained NRP1 among other molecules and are captured by T cells. Our studies demonstrated that physical contact was crucial to observe transfer, favoring the nibbling theory. However, some of our results suggested that vesicles also might participate in the transfer of material. Indeed, when $CD4^+$ T lymphocytes/Raji B cells conjugates were analyzed by electronic microscopy, we observed many small vesicles released by Raji B cells; some of them looked like stuck onto T cells membranes, suggesting that these vesicles may then fused with the T lymphocytes membranes (data not shown). Another important aspect of NRP1 transfer was to analyze whether NRP1 could be integrated in the T cell plasma membrane and be fully functional. The fact that we could not detect any NRP1-mediated signaling on the NRP1-loaded $CD4^+$ T lymphocytes (data not shown) suggested that NRP1 is not integrated in the T cells membrane. However, this result also may be due to an insufficient number of integrated receptors or absence of the appropriate coreceptor to transduce the

signal. It also is worth noticing that when CD4$^+$ T cells were sorted after coculture with Raji-GFP or Raji-NRP1 B cells, NRP1 expression on T cells was three times weaker than before sorting and did not diminish when these nondividing T cells were cultured the following days. These results suggested that the total amount of NRP1 loaded on T cells was not integrated, because part of NRP1 expression is lost during cell sorting, and also suggested that a part of NRP1 is integrated in the T cell plasma membrane, because NRP1 expression on T cells is stable after sorting. Altogether, our data suggest that NRP1 transfer result from both membranes nibbling and vesicles capture, and that a part of NRP1 captured may be integrated in T cells membranes.

NRP1 transfer does not occur between all types of cells

We found that NRP1 transfer is cell-type specific, because human CD34$^+$ hemopoietic cells cocultured with NRP1-expressing APC did not acquire NRP1 and because HUVEC, expressing high levels of NRP1, could not transfer this protein to resting CD4$^+$ T lymphocytes. Transfer of endothelial proteins to T cells has already been described during transendothelial migration (33) or when activated T cells were cocultured with HUVEC and could capture OX40L (27). However, transendothelial migration is not comparable with mere cocultures, and membranes transferred from HUVEC to T cells may contain OX40L, but not NRP1, because these two proteins might be localized in different membrane compartments.

VEGF binding and cross-talk between CD4$^+$ T lymphocytes and endothelial cells

Finally, we studied the binding of NRP1 ligands to the NRP1-loaded T cells. We demonstrated, by comparing CD4$^+$ T cells cocultured with Raji-GFP to CD4$^+$ T cells cocultured with Raji-NRP1, that NRP1 captured by T cells could bind $VEGF_{165}$. In the presence of DCm, which express NRP1 and secrete $VEGF_{165}$, capture of NRP1 by T cells and binding of $VEGF_{165}$ allows T cells to carry and vehicle VEGF through the body. Many studies have highlighted the crucial regulating role of $VEGF_{165}$ on endothelial cells permeability and proliferation and have shown that $VEGF_{165}$ signaling was strongly enhanced when $VEGF_{165}$ was presented in *trans* by NRP1 (45–49). Moreover, T cells are numerous highly motile cells, traveling mainly through blood vessels where they can interact with endothelial cells. It is thus tempting to speculate that $VEGF_{165}$ carried by T cells may participate in the cross-talk between CD4$^+$ T lymphocytes and endothelial cells during infection or inflammation. We hypothesize that, during inflammation, nonresponding and responding T cells, which have contacted DCm in the lymph node, leave this secondary organ transporting $VEGF_{165}$ and can then activate endothelial cells contributing, for instance, to the increasing vascularity of lymph node, a hallmark of inflammatory immune responses (50). T cells are more likely to deliver $VEGF_{165}$ to these endothelial cells than DCm that secrete $VEGF_{165}$, because DCm are mostly resident cells in the draining lymph node. NRP1 trogocytosis might act as an important biological event in the control of the endothelial microenvironment by the immune system.

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Disclosures

The authors have no financial conflict of interest.

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