European Journal of Immunology

Measles virus modulates dendritic cell/T-cell communication at the level of plexinA1/neuropilin-1 recruitment and activity

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Measles virus (MV)-infected DC fail to promote T-cell expansion, and this could explain important aspects of measles immunosuppression. The efficiency of the immune synapse (IS) is determined by the formation of stable, stimulatory conjugates involving a spatially and timely controlled architecture. PlexinA1 (plexA1) and its co-receptor neuropilin (NP-1) have been implicated in IS efficiency, while their repulsive ligand, SEMA3A, likely acts in terminating T-cell activation. Conjugates involving MV-infected DC and T cells are unstable and not stimulatory, and thus we addressed the potential role of plexA1/NP-1 and semaphorins (SEMAs) in this system. MV does not grossly affect expression levels of plexA1/NP-1 on T cells or DC, yet prevents their recruitment towards stimulatory interfaces. Moreover, MV infection promoted early release of SEMA3A from DC, which caused loss of actin based protrusions on T cells as did the plexA4 ligand SEMA6A. SEMA3A/6A differentially modulated chemokinetic migration of T cells and conjugation with allogeneic DC. Thus, MV targets SEMA receptor function both at the level of IS recruitment, and by promoting a timely inappropriate release of their repulsive ligand, SEMA3A. To the best of our knowledge, this is the first example of viral targeting of SEMA receptor function in the IS.

Keywords: Actin dynamics · DC · Measles virus · Semaphorin receptor

Introduction

Modulation of myeloid DC functions has been attributed an important role in viral immunosuppression, and for many systems analyzed this is reflected by the inability of infected DC to promote allogeneic T-cell expansion [1–3]. There are so far few examples relating this phenomenon to alterations of immune synapse (IS) stability, and these include, in addition to HIV and RSV, measles virus (MV) [4, 5]. For the latter, infection of $CD11c^+$ DC *in vivo* has been directly documented and this supports the notion of $CD11c^+$ DC being the Trojan horses in viral transport to secondary lymphatic tissues [6–8]. DC mobilization from peripheral tissues relies on pattern recognition receptor signalling to promote DC maturation. Accordingly, MV acts as DC-SIGN and TLR2 agonist [7, 9] and induces phenotypic maturation (including upregulation of MHC and co-stimulatory molecules and cytokine release), morphodynamic changes and enhanced motility of infected DC on fibronectin (FN) supports [10]. In contrast, CCR5/CCR7 switching, MHCII upregulation, and IL-12 production are less efficiently induced by MV as compared to other maturation stimuli [11, 12]. These differences do, however, not explain the inability of MV-infected DC (MV-DC) to promote T-cell expansion *in vitro* [12–14]. Rather, ligation of an as yet unknown surface receptor by the MV glycoprotein (gp) complex (displayed on the surface of MV-DC) interferes with TCR-stimulated activation of the phosphatidylinositol-3(PI3)/Akt kinase pathway. This efficiently abrogates activation of downstream effectors essential for actin cytoskeletal reorganization and cell cycle entry (reviewed in [15–17]). MV contact induced activation of sphingomyelinases in T cells which accounts for its

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interference with cytoskeletal dynamics [18], yet molecules and mechanisms actively conferring IS instability to MV-DC/T-cell conjugates are poorly characterized.

The mature IS segregates molecules involved in peptide recognition and TCR signalling from surrounding molecules also including those involved in stabilization and adhesion. It is an area of highly active cytoskeletal rearrangement, which essentially controls centripetal movement of TCR microclusters, but also receptor segregation including that of integrins, which regulate both TCR microcluster confinement and stability of the DC/T-cell conjugate (for a recent review [19]).

Initially described as guidance factors regulating axonal pathfinding during neuronal development, the general ability of semaphorins (SEMAs) to act as adhesion/repulsion cues has meanwhile highlighted the importance of these molecules in diverse physiological functions also including vascular growth, cell migration, and immune cell regulation [20–23]. SEMAs share a common "SEMA" domain and are divided into eight subclasses, and those expressed in vertebrates are membrane associated (class IV-VII) or secreted (class III, SEMA3 species). Class VIII summarizes virally encoded, secreted SEMAs with similarity to SEMA7A, and modulate immune activation by acting on monocytes [21, 24, 25]. Most membrane-resident SEMAs use members of the plexin family for binding and signalling, while most SEMA3 molecules require neuropilins (NP-1 or -2) as obligate binding receptors for initiating cellular responses through plexins. In addition to using these receptors, certain SEMAs (SEMA7A and SEMA4A and 4D) also signal to their immune effector cells by interaction with integrins, CD72, or TIM-2 [23, 26].

SEMA receptor signalling targets small GTPases and thereby actin dynamics and integrin activation, and adhesion [22, 27, 28]. Thus, plexinA1 (plexA1) and NP-1 were implicated in DC migration through endothelial layers and lymphatic entry [29], yet also in T-cell activation by murine or human DC [30–32], though neither their co-segregation at the IS nor their ligands there clearly identified. In contrast, the plexA1/NP-1 complex relays repulsive signals when exposed to soluble SEMA3A thereby causing loss of thymocyte adhesion, impairing actin cytoskeletal reorganization and activation of essential components of TCR signalling, or controlling Fas-mediated apoptosis [33–37]. Apparently, timely regulated IS recruitment and the respective interaction molecule essentially determine the ability of plexA1/NP-1 to promote or terminate T-cell activation. In line with this hypothesis, repulsive SEMA3A is produced only late in DC/T-cell co-cultures [34].

The role of plexA1/NP-1 and their ligands in viral immunomodulation has not yet been addressed. Based on the hypothesis that signalling to conjugating T cells might contribute to MV interference with IS stability and function, we addressed the role of plexA1/NP-1 and their ligand SEMA3A in this system. We found that levels of plexA1/NP-1 on MV-exposed T cells or MV-infected DC did not differ from those measured on controls. In T cells, however, contact to the viral gps abrogated translocation of both plexA1 and NP-1 towards stimulatory interfaces as required for their ability to enhance IS efficiency. As a second level of IS interference, MV-DC released high levels of repulsive SEMA3A early after infection and this accounted for loss of filamentous actin and actin-based protrusions of T cells, altogether indicating that MV affects plexA1/NP-1 signalling in the IS.

Results

MV contact abrogates recruitment of plexA1 and NP-1 to stimulatory interfaces in T cells

PlexA1/NP-1 supports IS stability and function, both of which are impaired if these involve MV-infected DC (MV-DC), or T cells pre-exposed to the MV gp complex. To analyze the role of plexA1/NP-1 in destabilization of the MV-DC/T-cell IS, we first analyzed whether MV affected surface expression of these molecules within the experimental conditions used throughout our study. These involved MV-infected DC (to evaluate effects of direct infection as occurring *in vivo* [6]) and T cells exposed to UV-inactivated MV to mimic T-cell surface contact-dependent signalling elicited by the viral gp complex (displayed by MV-infected DC) in the presence of fusion inhibitory peptide (to avoid MV uptake). In line with the published data, both plexA1 and NP-1 were expressed to very low levels on freshly isolated human primary T cells, and this was not altered upon UV-MV exposure (or mock exposure; both applied for 2 h) (Fig. 1A). In permeabilized T cells, especially plexA1 was efficiently detected indicating it mainly resides in intracellular compartments (not shown here, and Fig. 2C). The importance of plexA1/NP-1 in IS function has as yet mainly been addressed in murine cells [30, 31], and corroborating these findings, pretreatment of T cells and allogeneic LPS-matured DC (mDC) with plexA1-specific antibodies reduced – with differential efficiency – allogeneic T-cell expansion to levels seen in co-cultures between unmodified T cells and immature DC (iDC) (Fig. 1B). Next, plexA1 expression was siRNA ablated in T cells to evaluate its importance for their expansion driven by allogeneic mDC. Though the scrambled RNA also reduced to some extent the efficiency of proliferation, this was much more pronounced upon plexA1 silencing (Fig. 1C, and inset for RNA silencing control). Similarly, ectopic expression of dominant negative, but not full length plexA1 (nor that of an unrelated eGFP-expression plasmid), efficiently abrogated allogeneic T-cell expansion though transfection efficiencies were around 25% only as detected by flow cytometry for the VSV-G-tag of the respective constructs (Fig. 1D, and inset for expression control).

To relate their functional requirement to subcellular localization, we analyzed redistribution of plexA1/NP-1 in fixed allogeneic DC/T-cell conjugates (Fig. 2). CD3 and plexA1 inefficiently translocated towards interfaces in the rarely detectable iDC/T-cell conjugates (Fig. 2A, exemplified in the upper row). In about 80% of mDC/T-cell conjugates, however, interface recruitment of plexA1, and there, co-localization with CD3 were observed (Fig. 2A, exemplified in the bottom row, and in Fig. 2B, fourth panel). PlexA1 interface accumulation was similarly efficient in autologous conjugates involving superantigen(SA)-

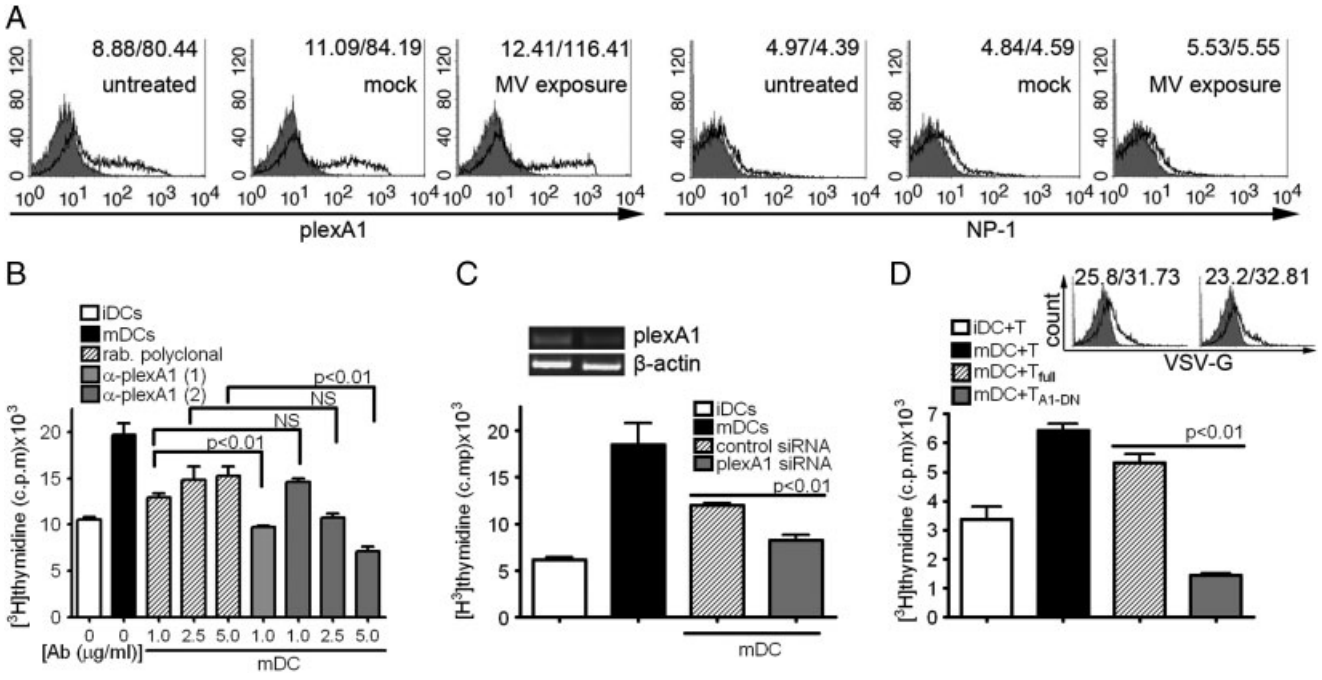

Figure 1. Expression of plexA1 on T cells is important for their expansion by allogeneic DC. (A) Surface expression levels of plexA1 (left panels) or NP-1 (right panels) were analyzed on live primary human T cells left untreated (each left panel), or exposed to a mock extract (each middle panel) or UV-inactivated MV (each right panel) after 2 h by flow cytometry using specific (open histograms) or isotype-matched (filled histograms) antibodies. The percentages of plexA1 or NP-1 T cells were determined after staining purified peripheral blood leucocytes. Mononuclear cells were gated on the basis of forward and side scatter information, and $CD3^+$/plexA1 or $CD3^+$/NP-1 cells were determined in T cells. Stainings were routinely controlled using an isotype control antibody. Expression levels are indicated in % positive cells/mfi. (B) MLR assays were performed using T cells co-cultured with mDC (left untreated (black bar), both exposed to a rabbit polyclonal antiserum (hatched bars) or plexA1-neutralizing antibodies (grey bars) at the concentrations indicated. (C) T cells were transfected with scrambled (inset RT-PCR, left lane; and graph, hatched bar) or plexA1-specific siRNA (inset RT-PCR, right lane; and graph grey bar) 48 h prior to co-culture with allogeneic mDC (D) T cells were used for co-cultures with mDC 6 h following transfection of plasmids encoding for GFP (black bar), or VSV-G tagged full length (hatched bar, and upper left graph) or DN plexA1 (grey bar, and upper right graph) (see inset for flow cytometry using VSV-G by specific (open histograms) or an isotype (filled histograms) with the respective percentage of expression/mfi indicated). (B–D) iDC co-cultured with unmodified T cells (each white bar) were included into the MLRs where T-cell proliferation was determined by ^{3}H-thymidine incorporation. Data show mean+SEM ($n = 3$) and are representative of three independent experiments. *p*-Values (student's unpaired *t*-test) are indicated. NS: not significant.

loaded mDC (not shown). As reported earlier [32], a fraction of NP-1 was also detected within allogeneic mDC/T-cell interfaces (an example is shown in Fig. 2B). Collectively, these data indicate that plexA1 and, to a more limited extent, NP-1 are components of the IS. The instability of MV-DC/T-cell conjugates prevented direct analyses of potential alteration of plexA1/NP-1 redistribution [10].

Since IS recruitment of plexA1 specifically in T cells was not yet reported, we confirmed redistribution of this molecule and NP-1 towards stimulatory interfaces by replacement of mDC by αCD3/CD28-coated beads (Fig. 2C). In line with our flow cytometry data, especially plexA1 was mainly detected in intracellular compartments from where it was effciently recruited towards the bead interfaces in about 50% of conjugating T cells (Fig. 2C, upper row and right graph), and this also referred to NP-1 (Fig. 2C, bottom row and right graph). Pre-exposure to MV dramatically decreased the percentage of T cells that are able to polarize these molecules towards the interface (Fig. 2C graphs). This was dependent on the interaction of T cells with the MV gp complex since translocation was recovered in the presence of antibodies directed against the MV H protein. Moreover, plexA1/NP-1 efficiently translocated towards the interfaces in T cells exposed to a recombinant MV expressing VSV-G protein instead of the MV gps (MGV) (Fig. 2C). Altogether these findings indicate that plexA1 and NP-1 are functionally important IS component recruitments of which is largely abrogated on MV contact with T cells.

PlexA1 and NP-1 surface expression on maturing DC is regulated by endocytosis

Aberrant signalling by DC is thought to account for MV T-cell silencing during immunosuppression. To analyze as to whether in addition to prevent plexA1/NP-1 IS recruitment on T cells, MV infection of DC impairs T-cell activation at the level of SEMA receptors as well, we first analyzed expression profiles of plexA1/NP-1 on DC. Expectedly, NP-1 [32](in around 75%) and, so far only described to be expressed on murine DC [30], plexA1 was readily detectable on the surface of about 20% of iDC (with MFIs of 25 and 42, respectively), and both were downregulated within 24 h on LPS maturation (Fig. 3A). Interestingly, mock or MV-infection caused moderate (for plexA1) or no (for NP-1)

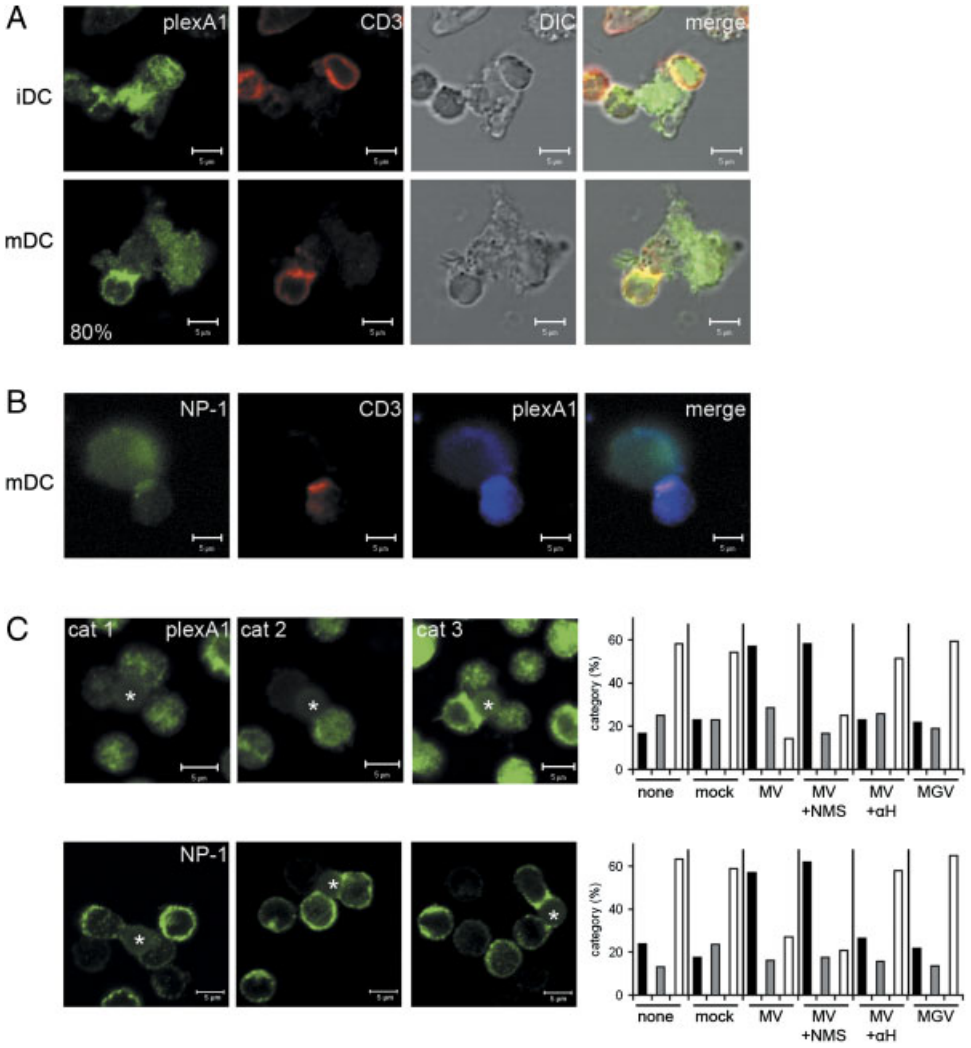

Figure 2. Recruitment of plexA1 and NP-1 to stimulatory interfaces are affected by MV exposure to T cells. (A) PlexA1 (green) and CD3 (red) were co-detected in allogeneic iDC/T cell (upper row) or mDC (bottom row) fixed after 30 min. The frequency of conjugates revealing IS translocation of plexA1 in mDC/T-cell conjugates was 80% of a total of 35 conjugates analyzed. (B) Co-detection of NP-1 (green), CD3 (red) and plexA1 (pseudocoloured in blue) in allogeneic mDC/T-cell conjugates fixed after 30 min. One out of 20 cells analyzed is shown. (C) αCD3/CD28 beads (indicated by asterisks) were added for 30 min to T cells left untreated or preexposed to mock or UV-MV, UV-MV preincubated with normal mouse serum (NMS) or with an anti-MV-H-specific antibody (both: 50 μg/mL), or UV-MGV for 2 h at 4°C, and plexA1 (upper row and right graph) or NP-1 (bottom row and right graph) were detected. Cells were scored into three categories with no (cat1, black bars), partial (cat2, grey bars) or predominant (cat3, white bars) recruitment of the respective molecule towards the interface. At least, 30 cells were evaluated for each condition. (A–C) scale bars 5 μm, magnification 40×.

downregulation confirming earlier observations that DC maturation by MV may not be complete [12]. To address the mechanisms underlying LPS-dependent plexA1 and NP-1 downregulation, we co-detected markers for endo/lysosomal compartments iDC and mDC. In iDC, plexA1 and NP-1 localized both at the cell surface and in cytosolic compartments not labelled by lysotracker (Fig. 3B, upper row). In mDC, NP-1, but not plexA1, efficiently co-localized with lysotracker indicating that its surface downregulation may involve lysosomal degradation (Fig. 3B, second row). In line with this hypothesis, chloroquine (CQ) present during LPS maturation partially rescued surface detection of NP-1 as detected also by flow cytometry (in a typical example, percentages of iDC, mDC, and mDC+CQ were 57, 17, and 28%, respectively). In contrast, partial co-localization of plexA1 with CD71 in iDC was strongly enhanced in mDC, indicating surface expression of plexA1 is regulated by shuttling through recycling endosomal compartments (Fig. 3C). Thus, inclusion of phenylarsine oxide (PAO) stabilized and slightly enhanced surface expression of plexA1, but not NP-1, on mDC (27, 6, 63% on iDC, mDC, and mDC+PAO, respectively). In line with the flow cytometry data, mock and MV-DC resembled iDC with regard to NP-1 expression, and caused only marginal internalization of plexA1 (Fig. 3B and C, each third and fourth rows). Altogether, LPS but not MV infection efficiently downregulates surface expression of both plexA1 and NP-1 on DC by endocytosis.

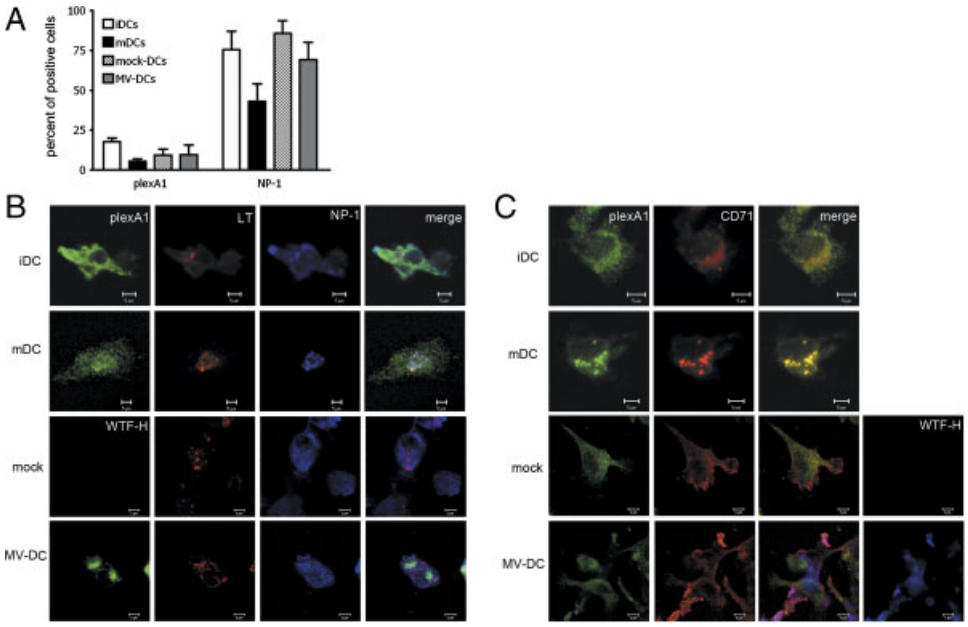

Figure 3. PlexA1 and NP-1 are downmodulated on DC maturation. (A) Surface expression of plexA1 and NP-1 on iDC, mDC mock or MV-DC (indicated in %). Data show mean+SEM pooled from three independent experiments. (B) NP-1 (pseudocoloured in blue), plexA1 or MV-H (green) and lysotracker (LT, red) were co-detected in iDC (upper row) or mDC (second row), or mock DC (third row) or MV-DC (bottom row). (C) PlexA1 (green), CD71 (red), and MV-H (pseudocoloured in blue) were co-detected on iDC (upper row) or mDC (second row), or mock DC (third row) or MV-DC (bottom row). (B and C) Scale bar, 5 μm. Magnification, 40 ×. Representative examples out of each three independent experiments are shown.

Upregulation and early secretion of SEMA3A through MV-DC

The plexA1/NP-1 ligand SEMA3A, released late after activation of T cells or DC or in DC/T-cell cocultures, acts to block T-cell proliferation, and has thus been proposed to avoid overactivation or to terminate T-cell responses [34]. Supernatants from iDC, LPS-matured or MV-infected cultures were used for immunoprecipitation to determine levels of secreted SEMA3A. Strikingly, detection of the repulsive 110 kDa SEMA3A species was confined to supernatants of MV-DC within the observation period of 48 h (Fig. 4A). To reveal if early SEMA3A secretion also occurred in co-cultures involving MV-DC, these (as were the control DC cultures) were extensively washed prior to addition of allogeneic T cells and levels of SEMA3A were determined over time. Indeed, SEMA3A was detectable already 6 h after onset of the MV-DC/T-cell co-culture, and continuously accumulated until 48 h where it entered a plateau phase, while, in agreement with published observations, release of SEMA3A in LPS-DC/T-cell cultures was seen only after 72 h (Fig. 4B). In addition and in line with previous observations [38, 39], a lower molecular-weight species was also detected, the activity of which is unknown as yet. For unknown reasons, the mock preparation also caused some SEMA3A production from DC in these co-cultures, which was not detected in DC cultures (Fig. 4A and B). Collectively, MV infection of DC promotes release of a repulsive plexA1/NP-1 ligand, which, in co-cultures with T cells, occurs very early and to concentrations exceeding at least fivefold those described to inhibit TCR-stimulated T-cell expansion *in vitro* [33, 34].

SEMA3A and SEMA6A cause transient loss of F-actin and microvillar extensions in T cells

Its interference with TCR polarization and early signal transduction indicated SEMA3A-dependent inhibition of actin cytoskeleton reorganization [34]. To corroborate these findings, we exposed FN seeded T cells for various intervals to IgG (included for control) or recombinant SEMA3A (SEMA3A-Fc) and analyzed their F-actin content. SEMA3A, but not IgG significantly decreased the average mean intensity of F-actin in T cells within 15 min, which then returned to normal within 60 min (Fig. 5A upper row, and graph). Strikingly, T cells exposed to recombinant SEMA6A (SEMA6A-Fc), initially included as a further negative control, also revealed a transient loss in F-actin, identical to that induced by SEMA3A (Fig. 5A, bottom row and graph). SEMA6A binds plexA4 rather than plexA1, and in line with its biological effect in our system, we readily detected expression of plexA4 on a substantial fraction of primary human T cells (Fig. 5A, bottom left panel). In contrast to SEMA3A, SEMA6A was not produced from MV- or LPS-DC on RNA or protein level (not shown). Surprisingly, exposure of T cells to SEMA3A or SEMA6A did not detectably abrogate their ability to aquire a front-rear polarity on FN as assessed by double detection of F-actin and CD43 after 15 or 60 min (Fig. 5B). This is in line with observations made by scanning EM, where also no effects of both compounds on T-cell polarization on FN were seen (Fig. 5C, upper right graph). However, in accordance with their loss of F-actin (Fig. 5A), the integrity of their microvillar extension was effectively lost within 15 min, which fully recovered within 60 min (Fig. 5C, bottom right graph.). Thus, ligation of SEMA3A and -6A receptors on

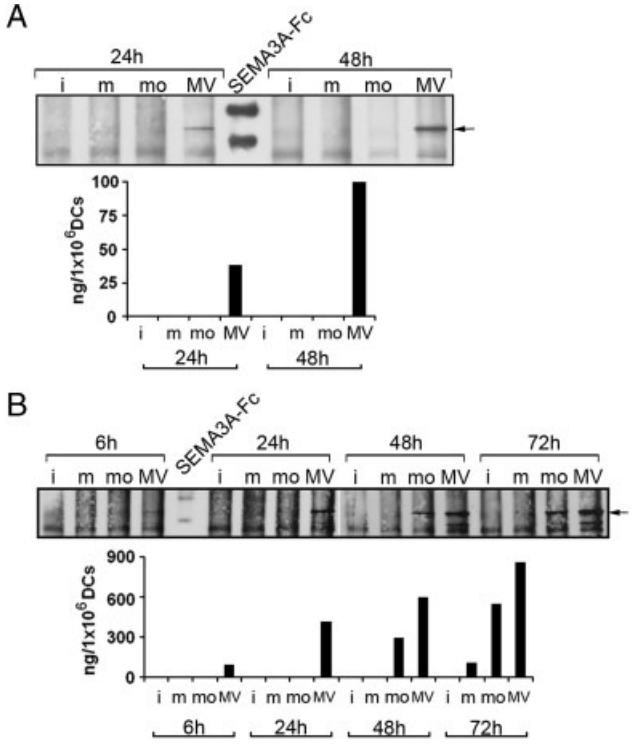

Figure 4. MV infected DC promote early production of soluble SEMA3A, particularly in the presence of allogeneic T cells. (A) SEMA3A (110 kDa; indicated by the arrow) was detected in immunoprecipitates of pooled supernatants (three different donors) of the different DC species (MV infection was 10% after 48 h). Quantification of SEMA3A secretion was determined by comparison of band intensities to a defined SEMA3A-Fc-fusion protein. (B) SEMA3A (110 kDa; indicated by the arrow) was detected in immunoprecipitates of pooled supernatants of three independent co-cultures of the indicated allogeneic DC species and T cells. Infection levels for MV-DC ranged between 60 and 80% after 48 h. Data were quantified as in A. (A, B) Lanes represent iDC alone (each lane 1), or exposed to LPS (mDC; each lane 2), mock (each lane 3) or MV (each lane 4) analyzed at the time intervals indicated. In lanes 5, SEMA3A-Fc is loaded for quantification of the signals, which were done using the AIDA software (each bottom graphs).

T cells affects actin turnover and dynamics in T cells transiently causing loss of membrane protrusions, yet not of front-rear polarization on FN.

SEMA receptor signalling affects T-cell motility, DC/T-cell conjugate formation and CD3 IS recruitment

To address SEMA receptor ligation on T cells in functional terms, we analyzed their ability to migrate in response to a mixture of CCL19 and CCL21 after a 15-min pre-exposure to SEMA3A or 6A (or, for control, human IgG) in under-agarose assays where chemotactic motility is dependent on actin flow [40, 41] (see graph Fig. 6A, upper right for schematic representation). As revealed by tracking of a statistically relevant number of cells *per* sample (between 30 and 90 cells were analyzed, representative examples are shown in Fig. 6A), both SEMA6A and SEMA3A affected T-cell motility. For SEMA3A, this did, however, not receive statistical relevance as compared to the IgG control (Fig. 6A, bottom right panel). The ability of exogenous SEMA3A, but not SEMA6A to cause reduction of allogeneic T-cell expansion in MLRs by approximately 30% has been reported earlier [34], and we thus reasoned that these compounds might interfere with IS efficiency at the level of conjugate formation. To analyze this directly, DC and allogeneic T cells were pre-labelled prior to co-cultures and the frequency of conjugates formed in the presence of SEMA3A, -6A or IgG was determined by flow cytometry (Fig. 6B). Both SEMAs detectably reduced conjugate frequencies measured after 20 and 30 min (Fig. 6B, left panel, for 30 min shown in Fig. 6B, right panel) and this almost numerically matched with the data published on MLR inhbition by SEMA3A [34]. As already evident from the migration experiment, SEMA6A more effiently interferred with conjugate formation, and this could not be compensated for by increasing SEMA3A doses (Fig. 6B, and not shown). Corroborating our hypothesis of SEMA3/6A directly interferring with T-cell activation at the IS level, pre-exposure to SEMAs, yet not to IgG (included as a control) largely abolished recruitment of CD3 to the interface (Fig. 6C). Though we repeatedly tried, we were unable to increase conjugate frequencies in MV-DC/T-cell co-cultures by neutralization of SEMA3A, and this is most likely due to the presence of the MV gp complex in the interface previously shown to largely account for IS destabilization in these cultures [10]. Altogether, these findings support the interpretation that of SEMA receptor ligation by SEMA3A and -6A affect motility and, at IS level, activation of T cells and thus, modulations in kinetic and levels of their expression or subcellular redistribution of their receptors by MV infection would be expected to contribute to immunosuppression.

Discussion

Measles pathogenesis is marked by the paradoxon of a coincident efficient virus-specific immune activation and generalized immunosuppression. The latter is characterized *in vivo* by lymphopenia and cytokine imbalance reflected by an early switch to a Th2-dominated response, while *ex vivo*, a failure of PBMCs to expand in response to mitogenic stimulation is observed (recently reviewed in [42]). The frequency of infected PBMCs is, however low, indicating that indirect mechanisms, such as soluble mediators (which have not been revealed), or contact-mediated signalling causing inappropriate propagation of activation signals may account for the observation. In the latter scenario, the interaction of T cells with MV-infected DC (which were recently identified *in vivo* in infected rhesus macacques [6]) in secondary lymphatics received particular attention with regard to qualitative and quantitative alterations of T-cell activation [43]. Our work has specifically focused on the interaction of MV-DC with T cells at the level of the IS, which proved to be only short lived and unable to support sustained Ca^{2+} fluxing [10]. The MV gp complex displayed on the MV-DC/T-cell interface essentially, yet not fully determined IS destabilization and thus, other molecules, potentially including SEMA receptors are likely to be involved also. The important role of the plexA1/NP-1 complex in regulating immune functions has been documented because their

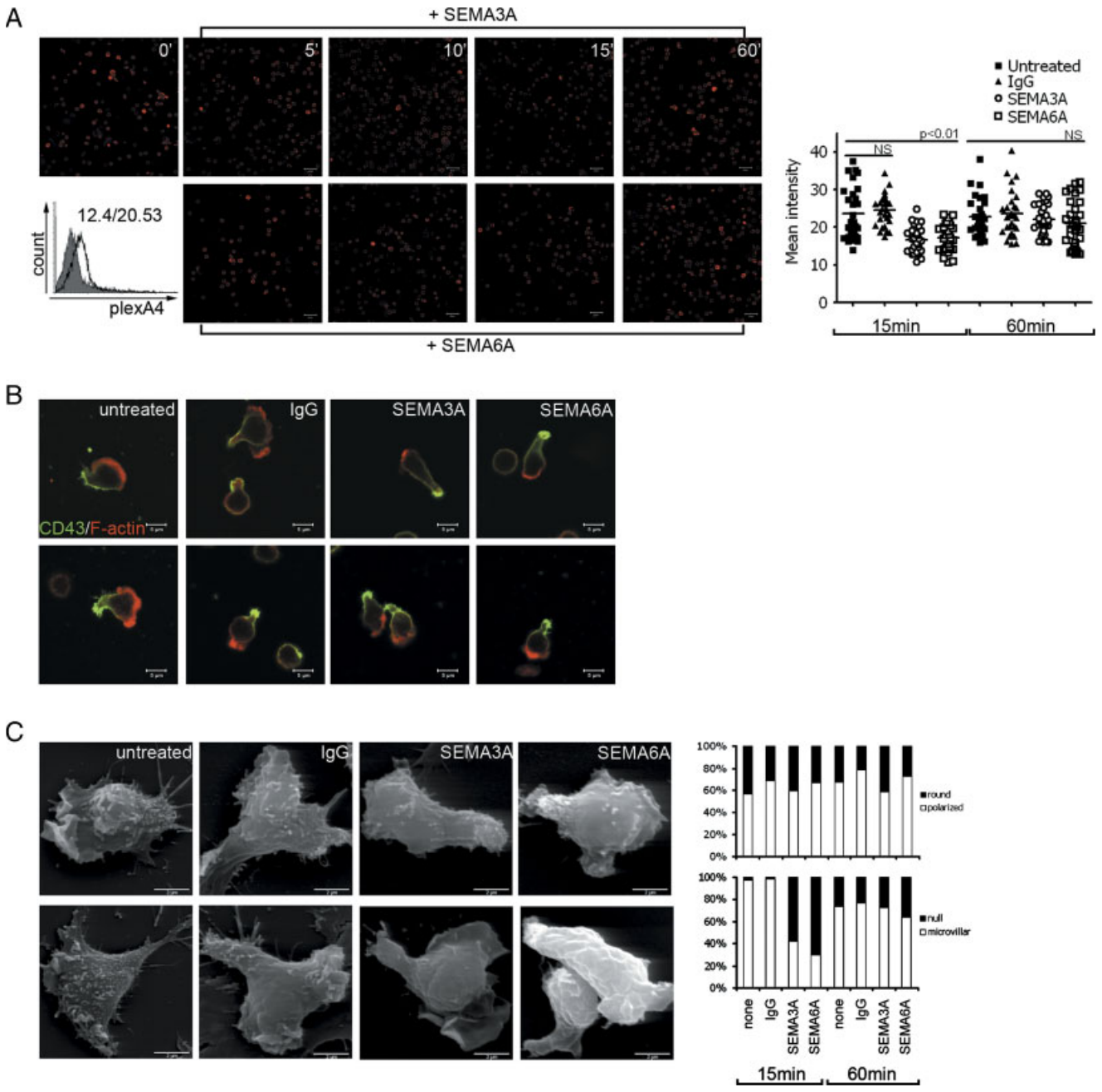

Figure 5. SEMA3A and SEMA6A cause transient loss of F-actin and microvillar extensions in T cells. (A) T cells seeded onto FN-coated slides were exposed to SEMA3A (left panel, upper row), SEMA6A (left panel, bottom row) or, for control, IgG, for the time intervals indicated, fixed and stained for F-actin using phalloidin-Alexa 594. Scale bar, 20 μm. Magnification, 40×. In addition plexA4 was detected on living, primary T cells by flow cytometry (filled histogram: isotype control, open histogram showing the specific antibody staining; left bottom panel). Quantification of F-actin intensity was performed using the ImageJ software, function Measure (right panel). At least, 30 cells were counted for each experiment. *P*-Vvalues from Student's unpaired *t*-test are indicated. (B) One hour following seeding onto FN, T cells were left untreated (left panels) or exposed to IgG (second panels), SEMA3A (third panels) or SEMA6A (right panels) for 15 (upper row) or 60 min (bottom row) prior to fixation and detection of F-actin and CD43. Representative examples of each 20 analyzed are shown. Magnification, 63×, scale bar, 5 μm. (C) T cells seeded onto FN-coated slides were fixed after 15 min (left panels and right graph) or 60 min exposure to SEMA3A, SEMA6A or IgG (right graph) and analyzed by scanning EM. Cells (examples are shown in the left panels) were scored into morphological categories (round *versus* polarized and null *versus* microvillar). Each 100 cells were recruited into the analysis (right graph). Scale bar, 2 μm. Magnification, 5000×.

ligands determine whether they functionally support (by self-interaction) or rather contribute to termination of (by SEMA3A interaction) the IS [22, 23, 44]. The importance of the ligand-binding NP-1 in the IS has been established in murine and human systems [32, 45], and we now confirmed that, similar to the murine system, plexA1 is an important component of IS function (Fig. 1) and redistributes to the interface between human T cells and DC or stimulator beads (Fig. 2). T-cell exposure to

MV-affected surface expression levels of neither plexA1 nor NP-1 (which remained very low and, in agreement with previous observations, is not a marker for human Tregs [46]). LPS-driven maturation promoted downregulation of these molecules from the DC surface (Fig. 3) which, for NP-1, is in contrast to what has been observed for that induced by proinflammatory cytokines ([32] and also own observations, not shown). As DC matured by inflammatory cytokines are effective at stimulating T-cell expansion, it remains unclear as to whether full or partial retention of NP-1 and plexA1 by MV infection are important in MV-induced alterations of DC functions.

Given the importance of plexA1 in T-cell activation, our finding that its recruitment to interfaces with stimulator beads is impaired is likely to interfere with IS efficiency as well. The inability of MV-exposed T cells to organize a correct synapse architecture has previously been described by us and the established interference of MV signalling with actin cytoskeletal dynamics expectedly accounts for aberrant sorting of receptors probably also including plexA1/NP-1 to this structure [18, 47]. This could, however, not directly be confirmed in conjugates between MV-DC and T cells because the majority of these is highly unstable [10].

In axon guidance, NP-1/SEMA3A signalling modified the growth cone cytoskeleton by causing retraction of filopodia and lamellopodia and localized rearrangement of the actin cytoskeleton [22]. Though it has not been directly addressed, interference with cytoskeletal dynamics might also account for the NP-1/SEMA3A-mediated loss of human thymocyte adhesion to thymic epithelial cells or their ECM-driven migration [35]. In line with our observations, inhibition of actin cytoskeletal rearrangement on NP-1 interaction with SEMA3A (the latter produced from mesenchymal stem cells or DC late after activation) has been linked to human T-cell immunosuppression [33, 34]. A role for SEMA3A in termination of DC/T-cell interactions by repulsive destabilization of the conjugates on NP-1 interaction has been proposed [34], and in line with this, SEMA3A was produced only late after onset of allogeneic MLRs ([34] and Fig. 4B). In contrast, SEMA3A production from MV-DC alone or in co-cultures with allogeneic T cells raised within few hours, indicating that this might contribute to destabilization of the IS as described to occur in these cultures earlier [10] and as evidenced by lower frequencies of stable conjugates on exogenous addition of SEMA3A (and also SEMA6A)(Fig. 6B). Notably, amounts of SEMA3A released from MV-DC/T-cell co-cultures several fold exceeded those determined to actively inhibit T-cell expansion stimulated allogeneic LPS-DC [34] or on αCD3/CD28 ligation [36]. In line with previous reports [38, 39], we repeatedly detected especially in the co-cultures, at least two SEMA3A species (Fig. 4B), the generation of may involve intracellular or surface proteolytic processing, *e.g.* furin or membrane-resident metalloproteases [48]. Whether production of two species in the MV-DC/T-cell cocultures relates to higher infection levels (as compared to the MV-DC only, Fig. 4A) or to the presence of allogeneic T cells remains to be resolved. While abrogation of NP-1/SEMA3A interaction reportedly signficantly improved allogeneic T-cell expansion driven by LPS-DC [34], this and conjugate stability in MV-DC/T-cell co-cultures could not detectably be rescued by SEMA-neutralizing antibodies (not shown). This is, however, not surprising since the presence of the MV gp complex on the DC surface within the DC/T-cell interface has previously been linked to IS destabilization and contact-mediated inhibition of T-cell expansion [10, 47, 49, 50]. It is also because MV particles displaying the inhibitory complex were likely present in conditioned supernatants of MV-DC or MV-DC/T-cell co-cultures containing high levels of SEMA3A that we did not directly prove their activity on αCD3/CD28-stimulated T-cell expansion.

In contrast to earlier studies [34, 36], SEMA6A was at least as efficient at interferring with IS stability and function as SEMA3A (Fig. 6B). As the IgG control always included at comparable levels did not have any effect on all parameters determined except for T-cell motility (Fig. 6A), and ligation of murine plexA4 by SEMA6A is known to negatively regulate T-cell responses [51], we consider the activity of SEMA6A in the assay as specific and thus, the obvious discrepancy cannot be explained at present, and needs further experimentation which would, as the identification of the cellular source of SEMA6A, exceed the present study.

As reported earlier [34], SEMA3A (and, in our hands, SEMA6A) transiently abrogated the integrity of the actin cytoskeleton as reflected by the loss of actin content (Fig. 5A), microvillar extensions (Fig. 5C) and, for SEMA6A only, motility in T cells (Fig. 6A). Interestingly, SEMA-mediated cytoskeletal interference did not affect the overall β1-integrin-stimulated front-rear polarization or receptor-segregation (Fig. 5B and C) thereby essentially differing from actin cytoskeletal paralysis induced on MV exposure of these cells [18, 47]. In line with hypothesis, induction of ceramides as found relevant for MV actin interference [18] was not detectable on SEMA3A/6A exposure of T cells (not shown) indicating the SEMA-induced signalling may not involve SMase activation.

In addition to adding to the current view on the role and regulation of human SEMA receptors in the IS in general (such as plexA1 IS recruitment and its importance for IS function in T cells, plexA4 expression in human T cells, plexA1/NP-1 turnover in maturing DC, SEMA3A and SEMA6A in regulation of T-cell protrusions and chemokinetic migration), our study to the best of our knowledge is the first to address regulation of those by a pathogen and their importance in the established MV interference with IS function. Recruitment to and concentration of SEMA receptors to the IS might, however, also be of relevance for viral transmission there as indicated by the function of NP-1 as physical and functional partners of HTLV env proteins during transmission in the virological synapse [32, 52].

Materials and methods

Ethics statement

Primary human cells were obtained from the Department of Transfusion Medicine, University of Würzburg, and analyzed

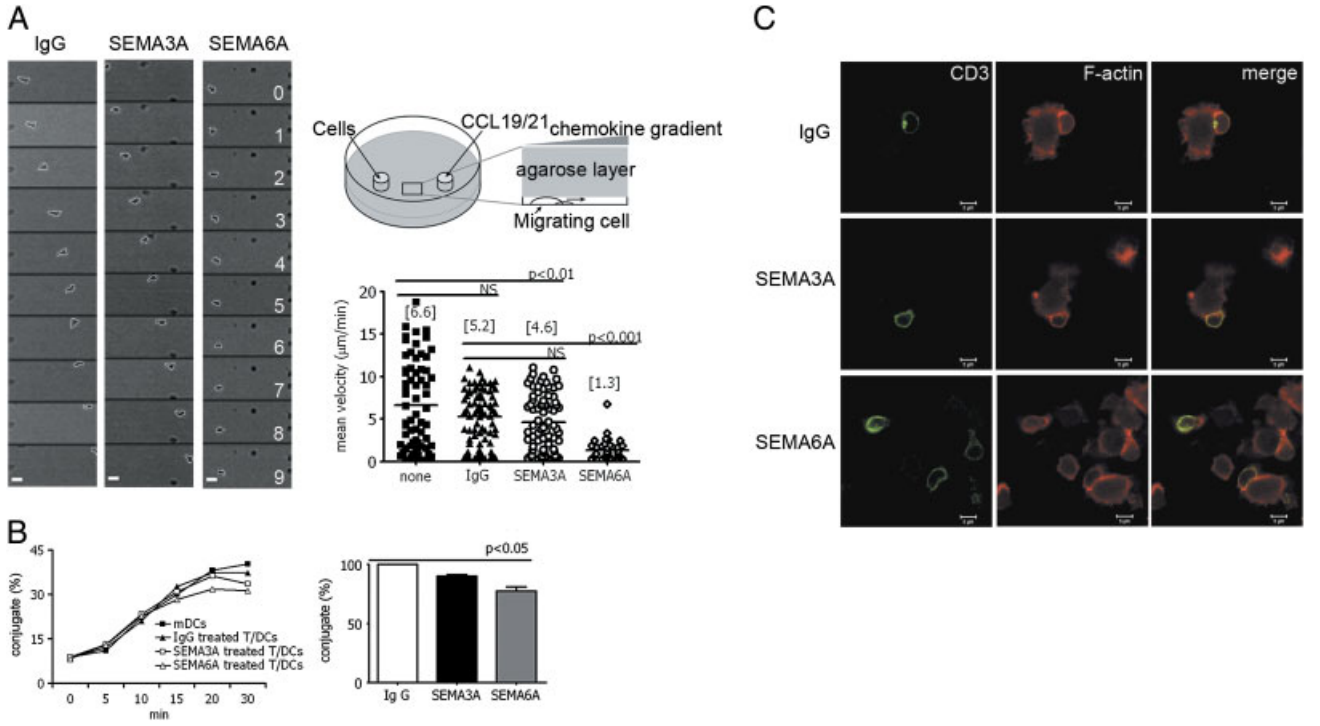

Figure 6. SEMA3A and SEMA6A affect DC/T-cell conjugate formation and migration of DC but not T cells. (A) Montage micrographs obtained in under-agarose migration assays (schematically depicted and adapted from [41]) depicting migrating T cells left untreated (bottom right panels, black squares) or pre-exposed to IgG (bottom panels, black triangles), SEMA3A (bottom right panels, open circles) or SEMA6A (bottom right panels, open diamonds) for 15 min under agarose within the indicated time schedule (min) in response to CCL19/21. Cells (at least, 30 to 90 cells for each experiment) were traced using ImageJ, plugin Manual tracking, and mean velocities were calculated using ImageJ, plugin Chemotaxis tool. Graphs showed the speed of tracked T cells (bottom graph), with mean velocities indicated (*X*) in μm/min. One representative out of three independent experiments is shown. Scale bar, 10 μm. Magnification, 10×. (B) Conjugate formation between CFSE-labelled T cells and R18-labelled DC pre-exposed to for 15 min and in the presence of IgG (left panel, black triangles; right panel, white bar), SEMA3A (left panel, open squares, right panel, black bar) or SEMA6A (left panel, open triangles, right panel, grey bar) for the time intervals indicated was determined after fixation by flow cytometry (gating of double-positive DC) over time (left panel) or after 30 min (right panel, where IgG treated controls were set to 100%). Data show mean ± SEM pooled from three independent experiments. *p*-Values (Student's paired *t*-test) are indicated. (C) CD3 (green) and F-actin (red) were co-detected in allogeneic mDC/T-cell conjugates exposed for 15 min to IgG (upper row), SEMA3A (middle row) or SEMA6A (bottom row) prior to and during conjugate formation. Examples of each 30 conjugates analyzed are shown. Scale bar, 5 μm. Magnification, 40×.

anonymized. All experiments involving human material were conducted according to the principles expressed in the Declaration of Helsinki and ethically approved by the Ethical Committee of the Medical Faculty of the University of Würzburg.

Cells and infection experiments

Primary human T cells were enriched from peripheral blood obtained from healthy donors by Ficoll gradient centrifugation followed on nylon wool columns and maintained in RPMI1640/10% FBS. Immature DC (iDC) were generated from monocytes in RPMI 1640/5% FBS by culture with GM-CSF (500 U/mL; Strathmann) and 250 U/mL IL-4 (250 U/mL; Promocell) and, when indicated, exposed to LPS (100 ng/mL) (LPS-DC) or a mock preparation obtained by freeze/thawing and subsequent low-speed centrifugation of human lymphoblastoid BJAB cells (kept in RPMI1640/10% FBS)(mock-DC) for 24 h. The MV WT strain WTF and the MVrecombinant MGV (expressing VSV-G protein instead of the MV gps [53]) were grown on human lymphoblastoid BJAB cells and titrated on marmoset lymphoblastoid B95a cells (kept in RPMI1640/10% FBS). For exposure experiments, MV was purified by sucrose gradient ultracentrifugation as was the mock control from uninfected BJAB cells. T cells were co-cultured with MV (at a multiplicity of infection (m.o.i.) of 0.5) or a mock preparation (used at a corresponding protein/volume concentration) for 2 h in the presence of a fusion inhibitory peptide Z-D-Phe-L-Phe-Gly-OH (Bachem, Heidelberg, Germany; 200 μM in DMSO) to prevent infection of T cells throughout the experiment. When indicated, MV was UV-inactivated prior to application. Before they were cocultured with T cells, DC were captured on chamber slides coated with poly-L-lysine (PLL) (0.01% w/v in water; Sigma, München, Germany) and loaded with superantigen (SA) (*Staphyloccocus aureus* Cowan Strain enterotoxins A and B, 1 μg/mL each) (Sigma) in RPMI containing 10% FBS. Co-cultures were performed in the absence of the fusion-inhibiting peptide.

Human recombinant SEMA3A fused to human Fc fragment (SEMA3A-Fc), SEMA6A-Fc (both: R&D Systems) and human IgG (Invitrogen) (dissolved in PBS) were applied onto cells in serum-free medium RPMI (final concentration: 150 ng/mL) for the time intervals indicated. F-actin was detected following fixation of cells in BSA containing 2% paraformaldehyde (PFA) and extensive washing.

Scanning EM, flow cytometry and immunostaining

For scanning EM, cells were seeded onto FN-coated slides (20 μg/mL in PBS; Sigma) for 1 h at 37°C and fixed by addition of 6.25% glutaraldehyde in 50 mM phosphate buffer (pH 7.2) for 30 min at room temperature and subsequently at 4°C overnight. After a washing step in phosphate buffer, samples were dehydrated stepwise in acetone, critical point dried, and sputtered with platin/paladium before scanning EM analysis (Zeiss DSM 962).

Living cells were analyzed by flow cytometry analysis after incubation with primary and secondary antibodies (each for 30 min at 4°C)(FACS Calibur, Becton Dickinson). Lysotracker® Red DND-99 (Invitrogen) was dissolved in DMSO and directly applied to living cells at a final concentration of 0.5 μM for 5 min at 37°C. CQ/PAO (Sigma) was dissolved in water/DMSO and applied at a final concentration of 50 μM and 0.1 μg/mL, respectively for 24 h at 37°C. For immunostainings, DC were captured on FN-coated chamber slides, and, when indicated, allogeneic or autologous T cells (if not indicated otherwise, DC/T-cell ratios were 1/4) were added for 30 min at 37°C prior to fixation in PBS containing 4% PFA prior to staining with antibodies (diluted in PBS/1% BSA). For pseudo-IS formation analysis, 10^7 T cells were stimulated using 2×10^5 Dynabeads® Human T-Activator CD3/CD28 (Invitrogen) for 30 min at 37°C, captured onto a poly-L-lysine-coated chamber slides for 30 min at 4°C and fixed at room temperature for 20 min. After washing and a blocking step (1% w/v BSA in PBS for 30 min at 4°C), cells were stained in PBS containing 1% BSA for 1 h at 4°C using primary and secondary or directly conjugates antibodies (see below). For immunodetection on chamber slides (Ibidi), Alexa594-conjugated phalloidin (Molecular Probes), and the following antibodies were used: Alexa488-, Alexa594-, or Alexa633-conjugated goat α-mouse- or goat α-rabbit- (both: Molecular Probes), FITC-, or PE-conjugated goat α-mouse- or mouse-α-CD3 (clone UCHT1), -α-CD11c (clone B-ly6), -α-CD80 (clone MAB104), -α-CD86 (clone 2331) (all: Becton-Dickinson Biosciences), -α-HLA-DR (clone B.8.12.2, Beckman Coulter), -α-CD71 (clone PAL-M1), -α-CD3ζclone 6.B10.2) (all: Santa Cruz), rabbit polyclonal α-plexA1 or -A4 (Abcam), mouse α-VSV-G (Sigma), mouse α-MV H protein (K83, produced in our laboratory) and mouse α-NP-1 (clone AD5-17F6, Miltenyi). For double stainings with mouse monoclonal antibodies, α-NP-1 was directly conjugated according to the manufacturer's protocol (Zenon, Molecular Probes/Invitrogen). After final washing steps in PBS, fluorochrome G (Southern Biotech, Eching, Germany) was used as the mounting medium and cells were analyzed by confocal laser scanning microscopy (Laser Scan Microscope, LSM510 Meta, Software version 3.0; Axiovert 200 microscope, objective: 100×; NA = 1.4 Plan Apochromat).

Transfection, RT-PCR, and Western Blot analysis

T cells were nucleofected with 2 μg plasmid encoding for DN-plexA1 (kindly provided by L. Tamagnone, Milano) [54] following the manufacturer's protocol (Amaxa). For silencing of plexA1, human T cells were transfected with a two-day interval according to the manufacturer's protocol (DharmaFECT, Thermal Scientific) with 100 nM siRNA targeting plexA1 (Santa Cruz) or, for control, a scrambled siRNA (Sigma). Before cells were recruited into the respective experiments, aliquots were harvested for nucleic acid extraction (Qiagen, RNAeasy Kit) and subsequent RT-PCR analyses. Forward 5′-ctgctggtcatcgtggctgtgct and reverse 5′-gggcccttctccatctgctgcttga primers were used for specific amplification of *plexA1*. Signals obtained after electrophoresis were digitalized and quantified using the AIDA software program (Raytest, Straubenhardt, Germany).

Immunoprecipitation

Supernatants of DC or DC/T-cell co-cultures were harvested at the time intervals indicated and immunoprecipitated using 2 μg/mL rabbit polyclonal anti-SEMA3A antibody (H300, Santa Cruz). Immune complexes were washed in PBS containing 0.5 M LiCl and 1% v/v Triton X100, and analyzed by Western blot using an anti-SEMA3A mAb (R&D Systems) followed by an anti-mouse HRP-conjugated antibody (Dianova, Hamburg, Germany). Signals obtained after ECL development were digitalized and quantified (recombinant SEMA3A-Fc was included for reference) using the AIDA software program.

DC/T-cell conjugate analyses and MLR

For conjugate analyses, DC were labelled with 1 μM R18 dye for 20 min and T cells with 1 μM CSFE (both: Invitrogen) for 5 min (each in RPMI-5% FBS at 37°C). DC and T cells (exposed to SEMA3A/6A or human IgG (150 ng/mL each) for 15 min at 37°C) were co-cultured directly in an FACS tube for the time intervals indicated, fixed with PFA (final concentration of 2% w/v in PBS), washed once with FACS buffer (low-speed centrifugation (400 rpm)) and subsequently analyzed by flow cytometry. The double-positive population representing conjugates was determined, and percentages were calculated using one sample *t*-test with hypothetical value set as 100 for the IgG-treated controls.

Under-agarose assay

Under-agarose assays were performed as described elsewhere [41]. Briefly, 2.5% UltraPure agarose (Invitrogen) in distilled water was heated, mixed with 56°C pre-warmed RPMI/20% FBS and 2× Hank buffered salt solution (Sigma-Aldrich) at a 1:2:1 ratio (final agarose concentration: of 6.25 mg/mL) and 2 mL was cast in 3.5-cm cell-culture dishes (BD Falcon). After polymerization, a mixture 1:1 of CCL19 and CCL21 (both: Preprotech) (1.2 μg/mL each in PBS) was applied into a punched attractor hole, and following a 30-min equilibration period at 37°C, 2×10^4 T cells (in 2 μL) were injected beneath the agarose with a fine pipette tip at a 5 μm distance from the attractor hole and moving cells were immediately recorded and tracked (once/20 s for 30 min) using the ImageJ software (http://rsb.info.nih.gov/ij/), plug-in Manual tracking. Tracked data were transformed and speeds were calculated using plug-in Chemotaxis tool. Mean-velocity graphs were performed using unpaired student *t*-test.

Statistical analysis

All statistics were performed using the Graphpad 4.0. Unpaired student *t*-test was applied, if not indicated otherwise.

Acknowledgements: The authors thank Harry Harms and Georg Krohne for their invaluable assistance in confocal and scanning electron microscopical image acquisition, Evelyn Gassert, Michael Sixt, Peter Friedl, Marie-Christine Dabauvalle, and Jürgen Schneider-Schaulies for helpful discussions, Luca Tamagnone, University of Milano for providing the DN-plexA1 plasmid, the Department for Transfusion Medicine of the University Clinic, Würzburg, for providing healthy donor cells, and the Interdisciplinary Center for Clinical Research, Würzburg and the Deutsche Forschungsgemeinschaft (SPP1175) for financial support. H. T.-V. was supported by a grant of the German Excellence Initiative to the Graduate School of Life Sciences, University of Würzburg.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **CQ:** chloroquine · **FN:** fibronectin · **gp:** glycoprotein · **iDC:** immature dendritic cell · **IS:** immune synapse · **mDC:** mature DC · **MGV:** MV gps · **MV:** measles virus · **MV-DC:** MV-infected DC · **NP-1:** neuropilin · **PAO:** phenylarsine oxide · **PFA:** paraformaldehyde · **plexA1:** plexin A1 · **SEMAs:** semaphorins

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Received: 19/7/2010
Revised: 29/9/2010
Accepted: 25/10/2010
Accepted article online: 8/12/2010

