

Semaphorin 3A induces cytoskeletal paralysis in tumor-specific CD8+ T cells

Mike B Barnkob¹, Yale S Michaels², Violaine André¹, Philip S Macklin³, Uzi Gileadi¹, Salvatore Valvo⁴, Margarida Rei¹, Corinna Kulicke¹, Ji-Li Chen¹, Vitul Jain⁵, Victoria Woodcock¹, Huw Colin-York¹, Andreas V Hadjinicolaou¹, Youxin Kong⁵, Viveka Mayya⁴, Joshua A Bull⁶, Pramila Rijal¹, Christopher W Pugh³, Alain R Townsend¹, Lars R Olsen⁷, Marco Fritzsche^{1,4}, Tudor A Fulga², Michael L Dustin⁴, E Yvonne Jones⁵, Vincenzo Cerundolo¹.

¹ MRC Human Immunology Unit, MRC Weatherall Institute of Molecular Medicine, University of Oxford, Headley Way, Oxford OX3 9DS, UK.

² MRC Weatherall Institute of Molecular Medicine, University of Oxford, Headley Way, Oxford OX3 9DS, UK.

³ Nuffield Department of Medicine, University of Oxford, Nuffield Department of Medicine Research Building, Roosevelt Drive, Oxford OX3 7FZ, UK.

⁴ Kennedy Institute of Rheumatology, University of Oxford, Roosevelt Dr, Oxford OX3 7FY, UK.

⁵ Division of Structural Biology, Wellcome Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford, OX3 7BN, UK.

⁶ Wolfson Centre for Mathematical Biology, Mathematical Institute, University of Oxford, Radcliffe Observatory Quarter, Woodstock Road, Oxford, OX2 6GG, UK.

⁷ Department of Health Technology, Technical University of Denmark, Ørsteds Plads, Building 345C, 2800 Kgs. Lyngby, Denmark.

Corresponding author: Vincenzo Cerundolo (V.C.), vincenzo.cerundolo@imm.ox.ac.uk.

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32

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36 **ABSTRACT**

37 Semaphorin-3A (Sema3A) regulates tumor angiogenesis, but its role in modulating anti-
38 tumor immunity is unclear. We demonstrate that Sema3A secreted within the tumor
39 microenvironment (TME) suppresses tumor-specific CD8+ T cell function via Neuropilin-1
40 (NRP1), a receptor that is upregulated upon activation with T cells' cognate antigen.
41 Sema3A inhibits T cell migration, assembly of the immunological synapse, and tumor killing.
42 It achieves these functional effects through hyper-activating the acto-myosin system in T
43 cells leading to cellular paralysis. Finally, using a clear cell renal cell carcinoma patient
44 cohort, we demonstrate that human tumor-specific CD8+ T cells express NRP1 and are
45 trapped in Sema3A rich regions of tumors. Our study establishes Sema3A as a potent
46 inhibitor of anti-tumor immunity.

47 INTRODUCTION

48 Cytotoxic CD8⁺ T cells are often restricted to certain areas within tumors or completely
49 excluded from the tumor microenvironment (TME) (1). We hypothesized that cell guidance
50 cues involved in developmental processes may also play a role in T cell restriction in the
51 tumor microenvironment. The secreted protein Sema3A is known to guide both endothelial
52 cells and neurons during embryogenesis through the cell-surface receptor family Plexin-A
53 (2, 3). Sema3A binding to Plexin-A requires the co-receptor NRP1 (4, 5). In axonal growth
54 cones, Sema3A signaling leads to profound changes in filamentous actin (F-actin)
55 cytoskeletal organization (6), an effect that is thought to be dependent on myosin-IIA activity
56 (7). Sema3A can also be produced by cancer cells (8) and recent evidence indicates that
57 NRP1, like PD-1, is upregulated on dysfunctional tumor-specific CD8⁺ T cells and can
58 modulate their anti-tumor response (9–11). However, there is no consensus on whether the
59 Sema3A-NRP1 axis is immunosuppressive (8, 12) or supportive of CD8⁺ T cells' response
60 to tumors (13). Furthermore, due to Sema3A's anti-angiogenic effects (14), several groups
61 have proposed utilizing Sema3A to inhibit tumor growth (13, 15). It is therefore critical to
62 examine the role of Sema3A in anti-tumor immunity more closely.

63 RESULTS

64 Tumor-specific CD8⁺ T cells upregulate NRP1 and Plexin-A1

65 To establish whether Sema3A can affect CD8⁺ T cells, we first examined NRP1 expression
66 of its cognate receptor, NRP1 on naive and stimulated T cells. NRP1 was upregulated on
67 human NY-ESO-1-specific HLA-A2 restricted CD8⁺ T cells, as well as on murine OT-I CD8⁺
68 T cells (OT-I T cells), upon stimulation with their cognate peptides, NY-ESO-1₁₅₇₋₁₆₅ and
69 Ovalbumin₂₅₇₋₂₆₄ (Ova), respectively (**Figure 1A-B**). Analysis of transcriptional data from the
70 Immunological Genome Project Consortium (16) of naive and effector CD8⁺ T cells
71 corroborated these findings (**Supplementary Figure 1A**). We examined whole OT-I T-cell
72 protein lysate and found that two NRP1 isoforms exist in murine T cells, with the larger NRP1
73 protein being the dominant form following T cell activation (**Supplementary Figure 1B**). To
74 examine NRP1 regulation in CD8⁺ T cells, we utilized antigenic Ova peptides with varying
75 affinities for the OT-I TCR (17), namely SIINFEKL (N4), SIIQFEKL (Q4) and SIITFEKL (T4)

76 and found that NRP1 expression was correlated with both peptide concentration and affinity
77 of TCR engagement (**Figure 1C**).

78

79 NRP1 is a co-receptor for a number of cell-surface receptors, including TGF β receptors 1
80 and 2 (TGF β R1-2) (18), VEGF receptor 2 (VEGFR2) (19) and Plexin-A1, -A2, -A3 and -A4
81 receptors (20), and its function is highly dependent on the availability of these receptors for
82 downstream signaling. We therefore screened OT-I T cells for expression of NRP1 partner
83 receptors. Stimulated, but not naive, OT-I T cells expressed Plexin-A1 but little to no Plexin-
84 A2, TGF β R1, TGF β R2 or VEGFR2 (**Supplementary Figure 1C-E**). Plexin-A4 was
85 expressed at low levels on both unstimulated and stimulated cells. Analysis of Plexin-A3
86 expression was not included because antibodies specific to Plexin-A3 could not be found.
87 Having identified NRP1 and Plexin-A1 receptors on stimulated, but not naive T cells, we
88 expected Sema3A ligation to the former (5). Indeed, flow cytometric analysis confirmed that
89 only stimulated OT-I T cells could bind recombinant murine Sema3A_{S-P} (**Figure 1D**).
90 Confocal imaging further indicated that Sema3A was internalized upon binding to T cells
91 (**Figure 1E**).

92

93 We next explored whether NRP1 and Plexin-A1 expression would be retained by CD8+ T
94 cells during infiltration in the TME. We adoptively transferred congenically marked and
95 activated OT-I T cells into syngeneic C57BL/6 mice bearing either B16.F10 or OVA
96 expressing B16.F10 cells (B16.F10.Ova) in opposing flanks. While few NRP1 expressing
97 OT-I T cells infiltrating B16.F10 control tumors were NRP1 positive, the majority of OT-I T
98 cells residing within B16.F10.Ova tumors expressed NRP1 (**Figure 1F**) and Plexin-A1
99 (**Figure 1G, right**) up to eleven days after adoptive transfer. Of note, endogenous
100 CD4+CD25+FoxP3+ T cells found within the tumor expressed both NRP1 and Plexin-A1 as
101 well as TGF β R1-2 (**Supplementary Figure 1E**), indicating that this subset of T cells might
102 be modulated differently from CD8+ T cells. Collectively, these data show that NRP1 and
103 Plexin-A1 receptors are upregulated on CD8+ T cells in a TCR-dependent manner, that they
104 are expressed on tumor-specific OT-I T cells, and that recombinant Sema3A can bind
105 directly to activated CD8+ T cells.

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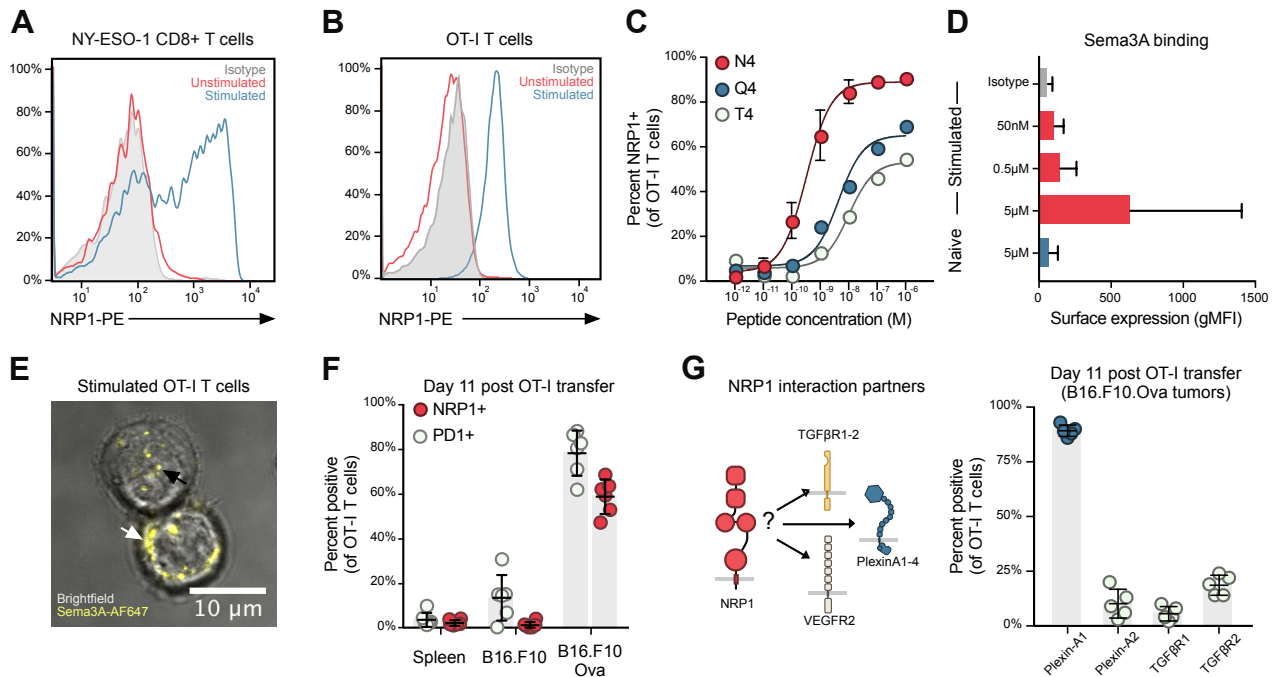


Figure 1. Tumor-specific CD8+ T cells up-regulate NRP1 and Plexin-A1 allowing for Semaphorin 3A binding.

A-B. Representative histogram of flow cytometric analysis of surface NRP1 expression on human NY-ESO-1-specific HLA-A2 restricted CD8+ T cells and murine OT-I CD8+ T cells following 48 hours stimulation with cognate peptides. Cells are gated on CD45, CD8 and TCR β . Experiment repeated three times. **C.** Analysis of NRP1 up-regulation using peptides with varying TCR affinities. Cells are gated on CD45.1, CD8 and TCR β . Cells from 3 mice per group, experiment was performed once. Data indicate mean \pm SD. **D.** Quantification of surface binding of Semaphorin 3A-S-P on naive and 48 hour stimulated OT-I T cells. Cells are gated on CD8 and CD3. Experiment was repeated three times. Data indicate mean \pm SD of representative experiment. **E.** Confocal imaging of 48 hour stimulated OT-I T cells stained with AF647-labelled Semaphorin 3A-S-P shows that the protein can bind to the cell membrane (white arrow) and within the cell (black arrow). **F.** Flow cytometric analysis of PD-1 and NRP1 expression on OT-I T cells 11 days after adoptive transfer in spleen, non-antigen expressing tumor (B16.F10) and antigen-expressing tumor (B16.F10.Ova) (n=6). Data representative of two independent experiments and indicate mean \pm SD of six mice per group. **G.** Schematic of NRP1 interactions partners (left). Flow cytometric analysis of expression of selected NRP1 interactions partners on OT-I T cells 11 days after adoptive transfer (n=5) (right). Experiment was performed once. Data indicate mean \pm SD. Abbreviations: gMFI, geometric mean fluorescence intensity. N4, SIINFEKL. Q4, SIIQFEKL. T4, SIITFEKL.

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109 **Sema3A negatively regulates CD8+ T cell adhesion, motility and migration through** 110 **NRP1**

111 Sema3A is known to restrict neuronal migration (4), but can have opposing effects on
112 immune cell motility. While both thymocyte (21) and macrophage (22) migration can be
113 inhibited, Sema3A has also been shown to increase dendritic cell (DC) migration (23). We
114 therefore undertook a number of *in vitro* experiments designed to dissect the effect of
115 Sema3A on CD8+ T cell adhesion experiments and motility. We first utilized interference reflection
116 microscopy (IRM) to assess T cell contact and adhesion (24). This was done on plates
117 coated with ICAM-1 and the chemokine ligand C-X-C motif chemokine ligand 12 (CXCL12,
118 SDF-1a) in order to emulate the environment found on endothelial cells and extracellular
119 matrix within the TME (25). When Sema3A_{S-P} was coated on plates, T cell adhesion was
120 significantly weakened (**Figure 2A**), an effect that was present from initial attachment until
121 at least 10 minutes later (**Figure 2B**). In addition T cells displayed a reduced polarized

122 morphology (**Figure 2C, Supplementary Figure 2A**). T cell motility was also affected, as
123 both distance and velocity were reduced when Sema3A_{S-P} was present, an effect that could
124 be reverted by pre-treating T cells with a blocking anti-NRP1-antibody (**Figure 2D-E**).
125 Extravasation into tumors requires T cells to first adhere to endothelial cells and then
126 transmigrate into the underlying parenchyma. To model this, we performed two experiments.
127 First, we perfused T cells across surfaces with ICAM-1 and CXCL12 with or without
128 Sema3A_{S-P}, and found that under a range of external flow rates, Sema3A decreased the
129 number of cells able to display rolling or tight adhesion (**Figure 2F-G**). At flow rates of 80
130 $\mu\text{m}/\text{sec}$, many T cells had a migration path similar to laminar flow indicating little ability to
131 adhere (**Figure 2F, lower right figure**). Secondly, using a transwell assay, we found that
132 Sema3A strongly inhibited transmigration (**Figure 2H**). We wondered if these effects were
133 mediated through changed expression levels of integrins or selectins involved in adhesion
134 and extravasation. However, flow cytometric analysis did not reveal any down-regulation of
135 CD11a (part of LFA-1), CD49d or CD162 (**Supplementary Figure 2B**), suggesting that
136 Sema3A signaling does not affect expression of these archetypal adhesion receptors on
137 CD8⁺ T cells. These data illustrate that Sema3A strongly inhibits activated CD8⁺ T cell
138 adhesion and motility, an effect that can be modulated using anti-NRP1-blocking antibodies.
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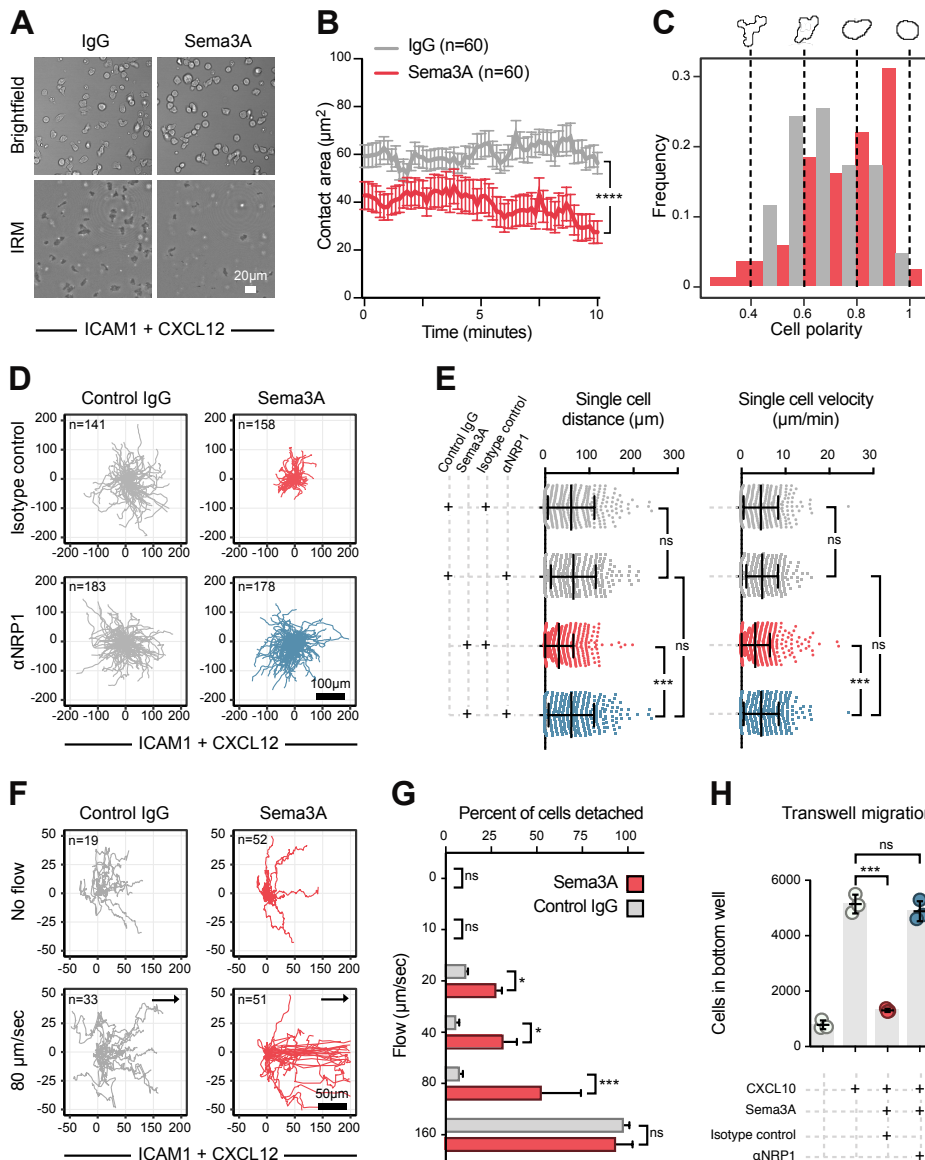


Figure 2. Sema3A negatively regulates CD8+ T cell adhesion, motility and migration through NRP1.

A. Representative brightfield and IRM images of 48 hour stimulated OT-I T cells adhering to ICAM-1 and CXCL12 coated plates with either Sema3AS-P or IgG immobilized. **B.** Quantification of contact area per single cell using live-cell microscopy for 10 minutes after OT-I T cells were added to plate (n=60 cells). Data representative of three independent experiments and indicate mean \pm SEM. **** = $P < 0.0001$ by Student's t-test. **C.** Relative frequency of cell polarity from brightfield images. A polarity of 1 indicates a shape of a perfect circle, 0 a rectangular shape. Representative images of OT-I T cells illustrated above graph. **D.** Representative spider plots showing the migration paths of individual T cells pre-treated with either a NRP1 blocking antibody or isotype control antibody on similar plates as in (A). **E.** Graph of single cell distance (left) and single cell velocity (right) in same experiment as (D). (n=314-744 cells per group). Data combined from five independent experiments indicate mean \pm SD. *** = $P < 0.001$, ns = not significant by Kruskal-Wallis test. **F.** Representative spider plots showing the migration path of individual OT-I T cells on similar plates as in (A), with flow rates at 0 or 80 $\mu\text{m}/\text{sec}$. Arrows indicate flow direction. **G.** Quantification of percent of OT-I cells that detach in same experiment as (F) (n=20-73 cells per condition). Data representative of two independent experiments and indicate mean \pm SEM. * = $P < 0.05$, *** = $P < 0.001$, ns = not significant by two-way ANOVA. **H.** Representative graph of number of stimulated OT-I T cells able to transmigrate through 3 μm Boyden chamber with CXCL12 in bottom chamber, with or without Sema3AS-P in top-chamber. OT-I T cells were pre-treated with either a blocking NRP1 antibody or isotype control antibody. Data representative of two independent experiments and indicate mean \pm SD. *** = $P < 0.001$, ns = not significant, by two-way ANOVA. Abbreviations: IRM, interference reflection microscopy. Sec, second.

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142 Sema3A negatively regulates CD8+ T cells' immunological synapse formation and 143 cell-cell contact

144 Given the strong effects of Sema3A on CD8+ T cell adhesion and motility, we investigated
145 whether Sema3A also affects the formation of the immunological synapse (IS). We first
146 tested the ability of CD8+ T cells to form close contacts with an activating surface displaying
147 immobilized ICAM-1 and anti-CD3 antibodies. To mimic an environment in which Sema3A
148 had been secreted, T cells were added and allowed to settle in medium containing either
149 Sema3AS-P or control IgG, while the size and spreading speed of contact areas was
150 measured using time-lapse IRM. T cells added to Sema3A-rich medium formed fewer and

151 smaller contact zones (**Figure 3A, left, Movie S1-2**). We noticed that cells in Sema3A-rich
152 medium did not spread as much and were slower to adhere (**Figure 3A, right**). Indeed,
153 when analyzing contact zones over time, many cells in Sema3A-rich medium could not form
154 large contact areas (**Figure 3B, top**) and spread at a reduced velocity (**Figure 3B, bottom**).
155 These results were reminiscent of the effects seen when T cells were added to plates coated
156 with ICAM-1, CXCL12 and Sema3A_{S-P} (**Figure 2A**) and indicated that T cells' ability to form
157 IS could be compromised as well.

158
159 To more closely examine the effects of Sema3A on IS formation, we utilized supported lipid
160 bilayers containing ICAM-1, CD80 and H-2 K^b-Ova pMHC monomers. Stimulated OT-I T
161 cells were pretreated with fluorescently-labelled Sema3A_{S-P-I}, washed to ensure that residual
162 protein did not interfere with the bilayer, and IS formation visualized using time-lapse total
163 internal reflection fluorescence (TIRF) microscopy. T cells with none to little Sema3A-binding
164 were seen to form classical IS containing a CD80-clustered central supramolecular
165 activation cluster (cSMAC) and an outer ICAM-1-rich peripheral supramolecular activation
166 cluster (pSMAC), while T cells that had strongly bound Sema3A_{S-P-I} were unable to spread
167 and appeared incapable of engaging with CD80 and ICAM-1 on the bilayer (**Figure 3C,**
168 **Movie S3**). To quantify the extent of this defect, we turned to a recently developed high-
169 throughput method to quantify relevant IS parameters (26), where T cells are first fixed on
170 the bilayer, then washed to remove non-adherent cells. Nearly two-thirds of stimulated T
171 cells were either washed away, could not cluster CD80, or form pSMACs when pre-treated
172 with Sema3A_{S-P-I} compared to untreated T cells (**Figure 3D-E**). Diminished IS formation in
173 the presence of Sema3A mirrored a scenario where OT-I T cells were presented to an
174 irrelevant pMHC-ligand, H-2 K^d-gp33, on the bilayer (**Supplementary Figure 3A**). Among
175 the Sema3A-treated T cells that formed IS, there was a discernible reduction in CD80
176 accumulation and in the radial symmetry of the synapse (**Supplementary Figure 3B-D**),
177 indicating that CD8⁺ T cells can be rendered non-responsive to their cognate antigen
178 through Sema3A signaling. We confirmed these findings by examining T cell binding to live
179 cancer cells. Stimulated OT-I T cells and B16.F10.Ova cells were co-incubated in the
180 presence of control IgG, Sema3A_{S-P} or a mutated Sema3A protein, in which the NRP1
181 interaction site on Sema3A has been mutated to substantially reduce the binding affinity (5),

182 followed by enumeration of OT-I T cell:B16.F10.Ova cell-cell conjugates. We noticed a 50%
 183 reduction in number of OT-I cells capable of binding to antigen-expressing cancer cells in
 184 the presence of Sema3A, but not with the control or mutant Sema3A (**Supplementary**
 185 **Figure 3E**). These results thus demonstrate that Sema3A signaling leads to profound effects
 186 on a majority of CD8+ T cells' abilities to adhere to target cells and form an IS.
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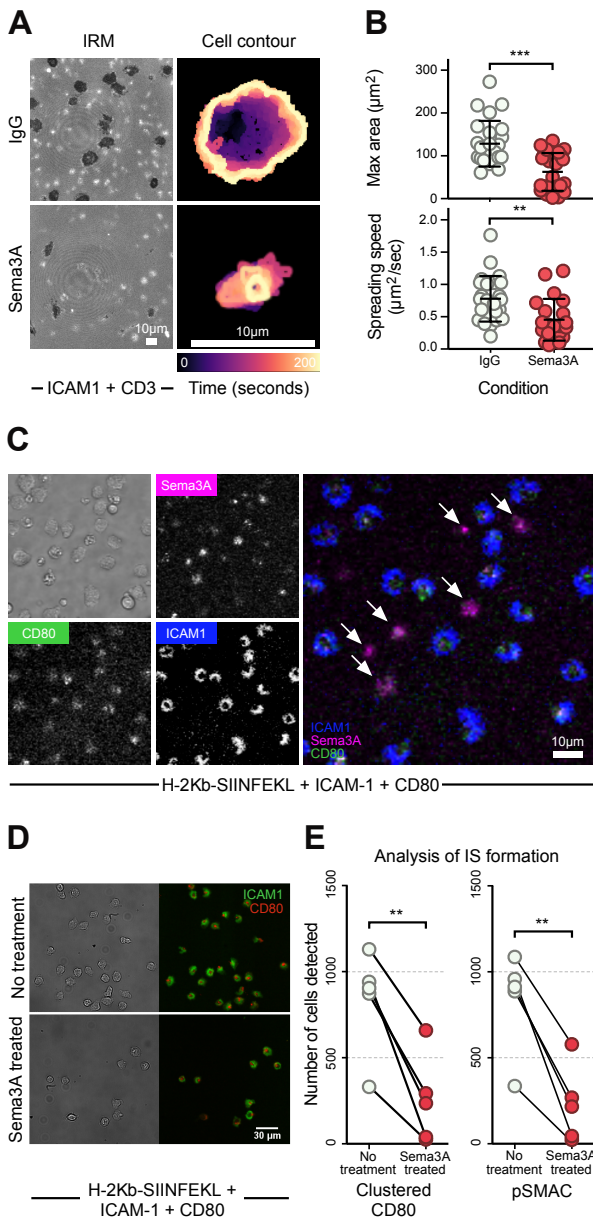


Figure 3. Sema3A negatively regulates CD8+ T cells' immunological synapse formation.

A. Live-cell imaging visualizing surface interface using IRM of stimulated CD8+ T cells dropped on an activating surface with immobilized ICAM-1 and CD3 and Sema3AS-P or IgG present in medium (left). Cell contour of representative cells from either condition (right). Color of contour indicates time from 0 to 200 sec as denoted on scalebar. **B.** Quantification of maximum size of cell contact area (top) and spreading speed from initial contact to maximum contact area (bottom) (n=25 cells per group) in same experiment as (A). Data combined from three independent experiments and indicate mean \pm SD. ** = $P < 0.01$, *** = $P < 0.001$, by Mann-Whitney test. **C.** Live-cell imaging of activated T cells pre-treated with Sema3AS-P-I-AF647 and allowed to form synapses on supported lipid bilayers with ICAM-1, CD80 and H-2Kb-SIINFEKL. Arrows in merged image indicate cells that have bound Sema3A and do not form immunological synapses. **D.** Representative image from high-throughput analysis of immunological synapses on supported lipid bilayers as in (C) with OT-I T cells pre-treated with Sema3A or not. **E.** Quantification of immunological synapses with or without Sema3AS-P-I pre-treated OT-I T cells. Data from six independent experiments (n=90-1100 cells per mouse per group). ** = $P < 0.01$, by paired t-test. Abbreviations: IRM, interference reflection microscopy. Sec, seconds.

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190 **Sema3A affects T cell actin dynamics through actomyosin II activity**

191 Class 3 semaphorins have been shown to have various effects on the cytoskeleton in
192 hematopoietic cells, including thymocytes (27), dendritic cells (23) and T cells (12), however
193 the precise nature of these effects in CD8⁺ T cells is not well characterized. Since
194 cytoskeletal F-actin remodeling is necessary for T cell binding to target cells (28) as well as
195 lamellopodium (24) and IS formation (29, 30), we examined F-actin content and dynamics
196 in T cells during Sema3A_{S-P} exposure. We first treated stimulated OT-I T cells with Sema3A_{S-}
197 _P at varying durations and examined F-actin content using flow cytometry. Surprisingly, no
198 actin depolymerization was observed up to 30 minutes after Sema3A_{S-P} treatment (**Figure**
199 **4A**). To better visualize F-actin dynamics before and after Sema3A_{S-P} treatment, we crossed
200 LifeAct-eGFR (31) mice with OT-I mice to generate LifeAct-OT-I T cells. Mice developed
201 normally and generated Ova-specific T cells with GFP-labelled F-actin. Stimulated T cells
202 formed an active lamellopodium that undulated across an activating surface containing CD3
203 and ICAM-1, allowing for close inspection of F-actin dynamics using time-lapse confocal
204 microscopy. When Sema3A_{S-P} was added to cells during this undulating phase, T cell
205 morphology changed and took a more irregular and roughened appearance (**Figure 4B**).
206 During this phase, F-actin content at the surface interface did not change, but lamellopodia
207 formation stopped and F-actin became non-dynamic and immobile (**Figure 4C, 4F, Movie**
208 **S4**). We therefore analyzed F-actin velocity along the cell edge using kymographs (**Figure**
209 **4D**). Sema3A_{S-P} profoundly inhibited F-actin dynamics (mean velocity was 1.34 $\mu\text{m}/\text{min}$ after
210 treatment versus 3.8 $\mu\text{m}/\text{min}$ before) (**Figure 4E**). Next, we treated T cells with mutant
211 Sema3A and found no difference in F-actin dynamics after treatment (**Figure 4E**), confirming
212 that the effect of Sema3A on F-actin in the lamellopodia is NRP1-dependent. We assessed
213 if this stark effect was due to localized F-actin depolymerization at the interface. Consistent
214 with our flow cytometric analysis of global F-actin abundance (**Figure 4A**) however, the
215 fluorescence intensity of LifeAct at the interface did not change, although the F-actin network
216 contracted, and the cell width shrank substantially following treatment with Sema3A_{S-P}
217 (**Figure 4F-G**). Because these effects on the actin cytoskeleton suggested that F-actin
218 turnover dynamics could be affected, we tested whether Jasplakinolide treatment would
219 phenocopy the effects of Sema3A_{S-P}. However, this instead led to constant shrinking of the
220 cells' F-actin network, not the immobilizing effects Sema3A produced (**Figure 4G**).

221

222 Sema3A signaling through Plexin-A1 inactivates the small GTPase Rap1A (32), which in
223 turn modulates myosin-IIA activity in diverse cell types (33, 34). The effects on the T cell
224 cytoskeleton we observed in the presence of Sema3A_{S-P} appeared consistent with increased
225 myosin-IIA activity. We therefore visualized and quantified the contact area of undulating T
226 cells before and after Sema3A_{S-P} treatment followed by treatment of the myosin-II inhibitor
227 Blebbistatin. As the border of IRM and F-actin signal overlay completely (**Supplementary**
228 **Figure 3F**), we quantified IRM area to avoid phototoxic effects and inactivation of
229 Blebbistatin, which would be caused by exciting LifeAct (35). When Sema3A was added, T
230 cell contact area contracted significantly and cells became immobilized, in line with our
231 analysis of F-actin (**Figure 4F-G**). However, when Blebbistatin was added, T cells started
232 undulating and regained their former size (**Figure 4H, Movie S5**). Conversely, when cells
233 were pre-treated with Blebbistatin followed by Sema3A_{S-P}, they retained their shape and
234 activity (**Figure 4I, Movie S6**). We therefore conclude that Sema3A inhibits F-actin dynamics
235 in CD8+ T cells, through hyper-activation of myosin-IIA.

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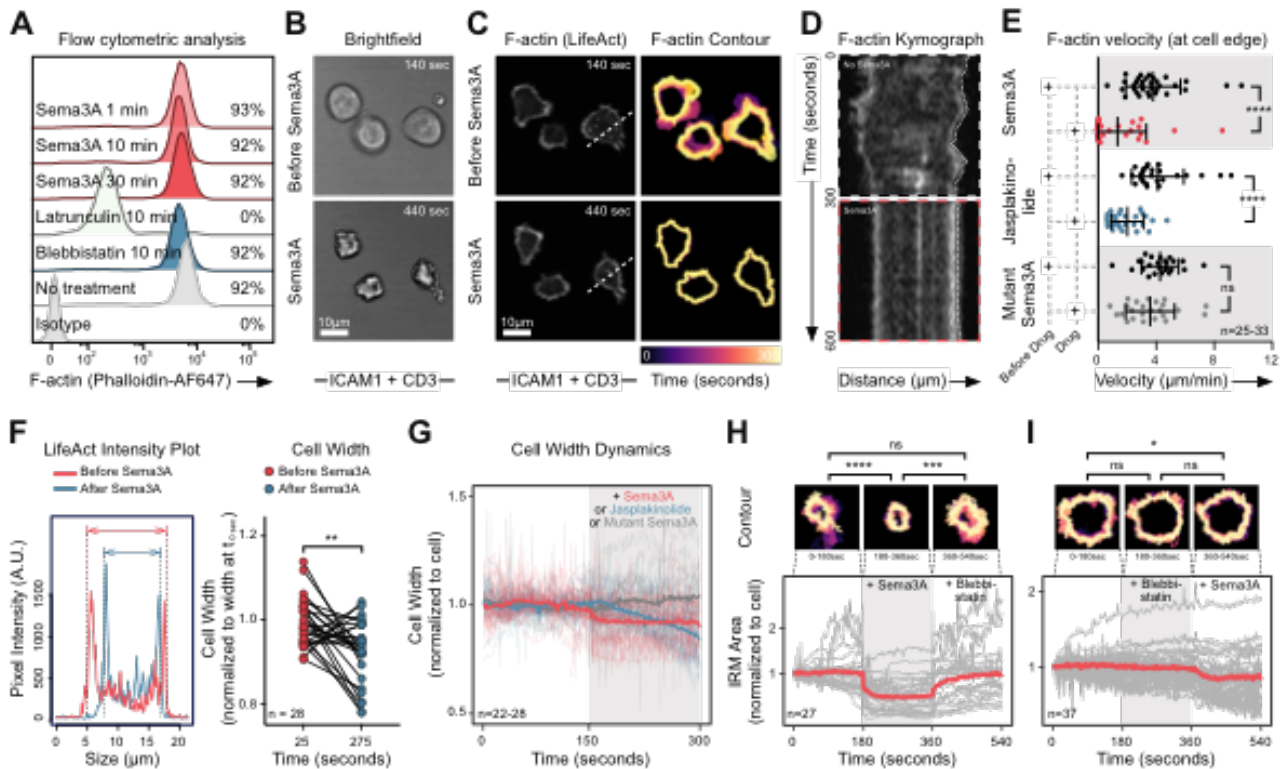


Figure 4. SEMA3A affects T cell actin dynamics through actomyosin II activity.

A. Representative flow cytometric analysis of F-actin content with no or varying exposure to SEMA3AS-P treatment in 48 hour stimulated OT-I T cells as measured by Phalloidin-staining. Percentage indicate positive cells in each condition. Data representative of two independent experiments. **B.** Representative brightfield images of 48 hour stimulated LifeAct OT-I T cells adhering to ICAM-1 and CD3 coated plates before and after SEMA3AS-P added to medium. **C.** Representative confocal images of LifeAct in OT-I T cells (left) and their contour plots (right) from same experiment as in (B). Image taken at cell-surface interface. Dashed white line indicate area used for (D). Color of contour indicates time from 0 to 300 sec as denoted on scalebar. **D.** Kymograph before (top) and after (bottom) SEMA3AS-P added to medium on area indicated with white dashed line in (C). Dotted line along edge of cell denoted example of data used for calculating data in (E). **E.** Quantification of F-actin velocity at cell edge before and after treatment with either SEMA3AS-P, Jasplakinolide or mutant SEMA3A ($n=25-33$ cells per group) using same experimental setup as in (B). Data combined from three independent experiments and indicate mean \pm SD. **** = $P < 0.0001$, ns = not significant, by paired t-test. **F.** Intensity plot of LifeAct signal before and after SEMA3AS-P treatment of a single OT-I T cell (left) or quantified on multiple cells exposed to SEMA3AS-P (right) using same experimental setup as in (B). Arrows indicate measured cell width. ** = $P < 0.01$, by paired t-test. **G.** Cell width dynamics measured like (F) over time before (white background) or after (grey background) SEMA3AS-P, Jasplakinolide or mutant SEMA3A addition to medium. **H.** Quantification of IRM area of individual OT-I T cells (grey lines) or average for group (red line) over time, with no treatment (leftmost white background), under treatment with SEMA3A (grey background) and then Blebbistatin (rightmost white background). Above representative contour plots of single cell under different treatments, with color denoting time (150 sec total). Cells were allowed to settle, and form contact for 3-5 min before data acquisition. Area normalized to cell area at $t = 0$ sec. Data combined from three independent experiments ($n=27$ cells). *** = $P < 0.001$, **** = $P < 0.0001$, ns = not significant by two-way ANOVA at time-points 90, 270 and 450 sec. **I.** Quantification of IRM area of individual OT-I T cells and representative contour plots as in (H), but with treatment with Blebbistatin (grey background) before SEMA3AS-P (rightmost white background). Data combined from three independent experiments ($n=37$ cells). * = $P < 0.05$, ns = not significant by two-way ANOVA at time-points 90, 270 and 450 sec. Abbreviations: Min, minutes. Sec, seconds. t, time.

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239 Nrp1-deficiency enhances anti-tumor activity of CD8+ T cells against SEMA3A-rich 240 tumors

241 To investigate the functional importance of SEMA3A in suppressing T cell migration and IS
242 formation *in vivo*, we pursued two complementary lines of enquiry. During development,
243 CD8+ T cells express CD4 molecules during a CD4-CD8 double positive stage (36). Thus
244 we crossed LoxP-flanked (Flox) Nrp1 mice with CD4-Cre mice to generate CD4-Cre X
245 Nrp1^{+/+}, CD4-Cre X Nrp1^{Flox/+} and CD4-Cre X Nrp1^{Flox/Flox} mice (hereafter referred to as
246 Nrp1^{+/+}, Nrp1^{Flox/+} and Nrp1^{Flox/Flox}, respectively), to generate Nrp1-deficient T cells.
247 Disruption of Nrp1 expression on stimulated CD8+ T cells was confirmed by flow cytometric

248 analysis (**Supplementary Figure 4A**), thereby generating mice with T cells insensitive to
249 Sema3A ligation. Mice bred normally, had no gross anatomical differences, grew at similar
250 rates and showed no sign of splenomegaly (**Supplementary Figure 4B-C**). Analysis of
251 thymocyte subsets and differentiated T cell memory subsets in the spleen revealed no
252 differences between genotypes (**Supplementary Figure 4D-E**), suggesting that NRP1 is
253 not involved in thymocyte development or T cell homeostasis in non-inflamed conditions.
254 CD8⁺ T cells from mice of all genotypes expressed similar levels of effector cytokines
255 following CD3/CD28 stimulation (**Supplementary Figure 4F**). We next set out to establish
256 the role of NRP1 on CD8⁺ T cell priming and activation by infecting mice with the A/PR/8/34-
257 derived pseudotyped influenza virus H7 (Netherlands/2003) N1 (England/2009) (here called
258 H7N1 S-Flu). This virus is capable of triggering strong H-2 D^b-restricted influenza
259 nucleoprotein (NP)-specific CD8⁺ T cell responses, but due to suppression of the
260 hemagglutinin (HA) signal sequence cannot replicate or generate anti-HA specific
261 neutralizing antibodies (37). This allowed us to specifically consider T cell responses. Mice
262 were infected intranasally with H7N1 S-flu and weighed daily. No differences in weight
263 between genotypes was observed (**Supplementary Figure 4G**). We detected no
264 differences in percentage or absolute number of H-2 D^b NP-tetramer positive CD8⁺ T cells
265 in lungs, draining lymph nodes (dLN) or spleen, ten days post-infection (**Supplementary**
266 **Figure 4H-I**). Examining the phenotype of CD8⁺ T cells in the lung, we found that infected
267 mice from all genotypes had an expansion of effector T cells as compared to uninfected
268 mice (**Supplementary Figure 4J**). Consequently, we conclude that NRP1 is dispensable
269 for CD8⁺ T-cell priming and activation.

270
271 We then challenged *Nrp1*^{+/+}, *Nrp1*^{Flox/+} and *Nrp1*^{Flox/Flox} mice with B16.F10 and Lewis lung
272 carcinoma (LL/2) cells. The poor immunogenicity of both cell-lines has been overcome using
273 combination therapies that augment immune responses, such as anti-PD1 and anti-4-1BB
274 (38, 39), and we therefore considered them good models for examining T cell anti-tumor
275 activity. Notably, *Nrp1*^{Flox/Flox} mice had significantly delayed tumor growth and increased
276 survival when challenged with either B16.F10 or LL/2 (**Figure 5A-B, Supplementary Figure**
277 **4K**). We confirmed that this effect was dependent on CD8⁺ T cells, as antibody-mediated
278 depletion of CD8⁺ T cells allowed B16.F10 cells to grow unperturbed in *Nrp1*^{Flox/Flox} mice

279 **(Figure 5C, Supplementary Figure 4L)**. When examining levels of tumor-infiltrating
280 lymphocytes (TILs) in $Nrp1^{+/+}$, $Nrp1^{Flox/+}$ and $Nrp1^{Flox/Flox}$ mice, we noticed a significant
281 increase in the numbers of CD8⁺ T cells within tumors in $Nrp1^{Flox/Flox}$ mice, but not of CD4⁺
282 T cells **(Figure 5D, Supplementary Figure 4M)**. Bone-marrow (BM) chimeric mice,
283 containing mixed $Nrp1^{Flox/+}$ and $Nrp1^{Flox/Flox}$ BM, confirmed that the increased levels of
284 infiltration were intrinsic to CD8⁺ T cells themselves and not dependent on unspecific effects
285 by other cell types **(Figure 5E)**.

286
287 We hypothesized that the reason T cell immunity was enhanced by NRP1-deficiency in our
288 tumor models, but not against H7N1 S-flu, was an increased availability of Sema3A in the
289 former. Indeed, we did not find high levels of Sema3A on either epithelial cells, leukocytes
290 or endothelial cell-subsets in the lung before, during or after infection with H7N1 S-flu
291 **(Supplementary Figure 5A-B)**. Conversely, aggressively growing tumors such as B16.F10,
292 often generate a hypoxic TME (40) which itself can induce Sema3A production (22). We
293 cultured B16.F10 cells in normoxic or hypoxic conditions and performed RT-qPCR. As
294 expected, hypoxic conditions led to upregulation of known hypoxic response genes,
295 including Pdk1, Bnip3 and Vegfa, in addition to upregulation of Sema3A transcript
296 **(Supplementary Figure 5C)**. Flow cytometric analysis of B16.F10 cells grown for 11 days
297 *in vivo* confirmed expression of Sema3A within the TME **(Supplementary Figure 5D-E)**. In
298 order to better control the level of Sema3A within the TME, we therefore generated
299 B16.F10.Ova cells that either overexpress or lack Sema3A, upon gene disruption by
300 CRISPR/Cas9 (here referred to as Sema3A OE and Sema3A KO, respectively). Deep-
301 sequencing, RT-qPCR for Sema3a transcript, and analysis by flow cytometry, confirmed
302 that cells lacked or over-expressed Sema3A **(Supplementary Figure 5F-H)**. Sema3A OE
303 and Sema3A KO cell-lines grew at similar rates compared to wild-type B16.F10.Ova cells
304 under both normal growth conditions and in the presence of the proinflammatory cytokines
305 IFN γ and TNF α *in vitro* **(Supplementary Figure 5I)**. Importantly, when we injected Sema3A
306 OE and KO cell lines into opposite flanks of C57BL/6 mice, tumors grew at similar rates
307 **(Figure 5F)**, thus confirming that the cell-lines had a comparable phenotype and growth
308 potential. However, when we adoptively transferred stimulated OT-I T cells into these mice,
309 Sema3A KO tumor growth was significantly delayed compared to Sema3A OE tumors

310 **(Figure 5G)**. These results demonstrate that Sema3A overexpression within the TME was
311 sufficient to effectively suppress tumor-specific killing. Furthermore, significantly fewer OT-I
312 T cells had infiltrated tumors that overexpressed Sema3A, compared to Sema3A KO tumors
313 **(Figure 5H)**. Taken together, our data underscores the functional significance of Sema3A
314 within the TME as a potent inhibitor of CD8+ T cell migration, and thereby anti-tumor
315 immunity, via interaction with NRP1.
316

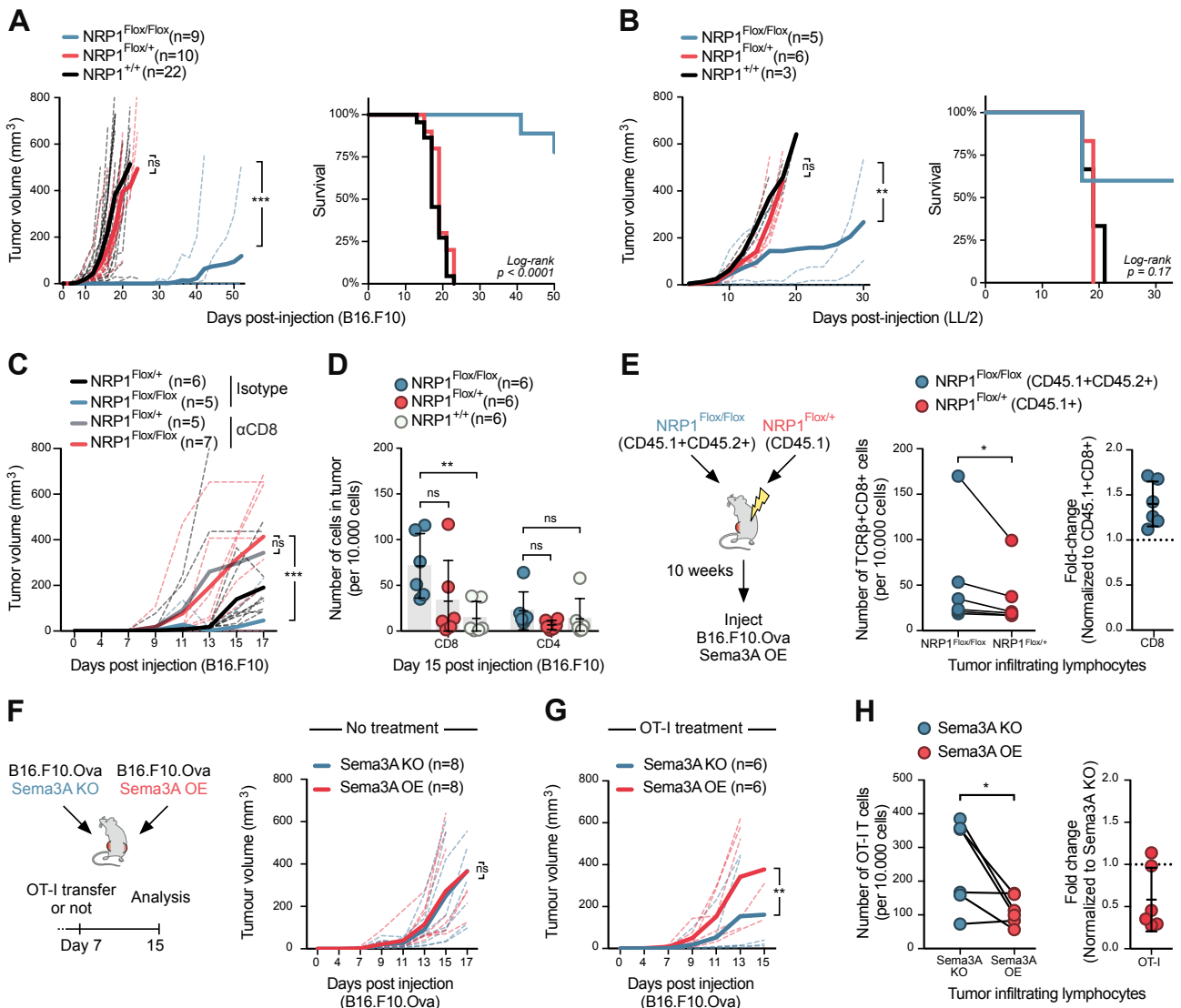


Figure 5: Nrp1-deficiency enhances anti-tumor migration and activity of CD8+ T cells.

A. Growth curve of B16.F10 cells in NRP1^{+/+}, NRP1^{Flox/+} and NRP1^{Flox/Flox} mice (left) and Kaplan-Meier survival curve (right). Dashed lines indicate growth in individual mice, bold line average for group. Combined data from 4 independent experiments with 3-6 mice per group. *** = P < 0.001, ns = not significant by two-way ANOVA. **B.** Growth curve of LL/2 cells in NRP1^{+/+}, NRP1^{Flox/+} and NRP1^{Flox/Flox} mice (left) and Kaplan-Meier survival curve (right) (n=3-6 mice per group). Dashed lines indicate growth in individual mice, bold line average for group. Experiment performed once. *** = P < 0.001, ns = not significant by two-way ANOVA. **C.** Growth curve of B16.F10 cells in NRP1^{Flox/+} and NRP1^{Flox/Flox} mice pre-treated with either anti-CD8 antibody or isotype control (n=5-7 mice per group). Dashed lines indicate growth in individual mice, bold line average for group. Data combined from two independent experiments. *** = P < 0.001, ns = not significant by two-way ANOVA. **D.** Enumeration of CD4+ and CD8+ T cells infiltrated into B16.F10 tumors in NRP1^{+/+}, NRP1^{Flox/+} and NRP1^{Flox/Flox} mice (n=6 per group). Data indicate mean ± SD. ** = P < 0.001, ns = not significant by two-way ANOVA. **E.** Experimental setup of mixed bone-marrow chimeras in C57BL/6 mice (left) and subsequent enumeration of CD8+ T cells in mice (middle graph). Ratio of CD8+ T cells from NRP1^{Flox/Flox} to NRP1^{Flox/+} bone-marrow derived cells (right graph) (n=6 mice per group). Experiment performed once. Data indicate mean ± SD. * = P < 0.05 by one-way ANOVA. **F.** Experimental setup using B16.F10 Sema3A KO or Sema3A OE cells (left) and growth curve of cells in untreated mice (right) (n=8 mice). Experiment performed once. ns = not significant by two-way ANOVA. **G.** Growth curve of B16.F10 Sema3A KO or Sema3A OE cells using similar experimental setup as in (F), but with OT-I treatment at day 7 post-injection (n=6). Experiment performed once. ** = P < 0.001 by two-way ANOVA. **H.** Enumeration of OT-I T cells in tumors (left graph) and their ratio of cells, normalized to the number in the B16.F10 Sema3A KO tumor (right) from same experiment as in (G). * = P < 0.05 by two-way ANOVA.

317

318

319 NRP1 is expressed on tumor-infiltrating CD8+ T cells in clear cell renal cell 320 carcinoma patients

321 We wished to explore if our findings were relevant to human cancer. Analysis of publicly
322 available TCGA data revealed that high Sema3A expression was associated with poorer

323 survival in clear cell renal cell carcinoma (ccRCC) (**Figure 6A**). We hence turned to a cohort
324 of ccRCC patients that had undergone nephrectomy (**Supplementary Table 1**) to explore
325 the role of the Sema3A/NRP1 pathway in cancer immunity (**Figure 6B**). We first quantified
326 NRP1 expression on CD8⁺ T cells from peripheral blood (PBMCs), and CD8⁺ TILs within
327 tumor and tumor-adjacent tissue. Significantly more CD8⁺ T cells in both tumor and tumor-
328 adjacent tissue expressed NRP1 (**Figure 6C-D, Supplementary Figure 6A**), suggesting
329 that these cells would be sensitive to Sema3A. In our murine model, NRP1 expression
330 correlated with antigen exposure (**Figure 1C**), and we therefore speculated that NRP1-
331 positive CD8⁺ TILs might be tumor-specific. Indeed, most NRP1⁺ TILs were also PD1-
332 positive (**Figure 6E-F**), demonstrating that they had either recently been activated or
333 experienced chronic exposure to antigen (41). To investigate this, we single-cell sorted
334 NRP1-negative and positive CD8⁺ T cells from four ccRCC patients and examined their
335 TCR repertoire. TCR diversity, as calculated by either Shannon (SA) and Simpson (SI)
336 diversity indices of CDR3 β (**Figure 6G**) and TRBV usage (**Figure 6H**), showed that NRP1⁺
337 CD8⁺ TILs were more clonal than NRP1⁻ T cells, further supporting the hypothesis that such
338 T cells had undergone clonal expansion following recognition of their cognate antigens (42)
339 (**Figure 6E-F**). Various cancer-testis (CT) antigens can be expressed by cancerous tissue
340 in ccRCC (43). We took advantage of this fact and screened four HLA-A2-positive patients
341 for the presence of HLA-A2-restricted CT-antigen-specific CD8⁺ T cells using a panel of 21
342 HLA-A2 tetramers loaded with CT epitopes (44). In the three patients, who had CT tetramer
343 positive TILs, we found that a larger proportion of NRP1⁺ CD8⁺ TILs were specific for CT-
344 antigens (**Figure 6I-J, Supplementary Figure 6B**). Taken together these data show that
345 NRP1⁺ CD8⁺ TILs were found in ccRCC patients, were activated, and were likely specific
346 for tumor-associated antigens.

347
348 We next wished to explore the spatial distribution of Sema3A and CD8⁺ T cells within the
349 TME. For this purpose, we stained ccRCC tissue sections from 12 patients from our ccRCC
350 cohort for Sema3A by immunohistochemistry (IHC). We observed widespread expression
351 of Sema3A both within the tumor as well as in adjacent non-neoplastic kidney tissue. In the
352 tumor, Sema3A was predominantly expressed by smooth muscle cells within the tunica
353 media of tumor vasculature but also in areas of fibromuscular stroma. In the adjacent tissue,

354 glomerular mesangial cells and smooth muscle cells within peritubular capillaries stained
355 positive for Sema3A (**Supplementary Figure 6C**). Next, strict serial sections from the same
356 formalin-fixed paraffin embedded tissue blocks were stained for CD31 and CD8 and
357 computationally aligned to the Sema3A sections. Pathological review confirmed that
358 expression of Sema3A co-localized with that of the blood vessel marker CD31. Furthermore,
359 CD8+ cells were often located within regions of high Sema3A expression (**Supplementary**
360 **Figure 6D**); indeed dual-staining of CD31 and CD8 in ccRCC clearly showed that CD8+
361 cells were restricted to the immediate area surrounding blood vessels (**Figure 6K**). To
362 further explore the effect of Sema3A on CD8+ cell infiltration and localization, we compared
363 regions within each tumor that were either Sema3A-rich or Sema3A-poor, allowing us to
364 control for variability in CD8+ cell infiltration between patients (**Figure 6L**). This analysis
365 confirmed that our selected Sema3A-rich regions expressed more CD31 than the Sema3A-
366 poor regions, underscoring the close association of Sema3A with the vasculature
367 (**Supplementary Figure 6E-F**). In 11 out of 12 examined patients, there were significantly
368 more CD8+ TILs in the Sema3A-rich areas than in Sema3A-poor areas, corresponding to
369 46% fewer CD8+ cells in Sema3A-poor regions (**Figure 6M**). Additionally, the CD8+ cells
370 that were present in Sema3A-poor regions were often found clustered near sources of
371 Sema3A (**Figure 6L, arrows**). The data presented here are consistent with a role for
372 Sema3A in modulating T cell infiltration and restricting CD8+ cells to perivascular areas
373 within the tumor.

374

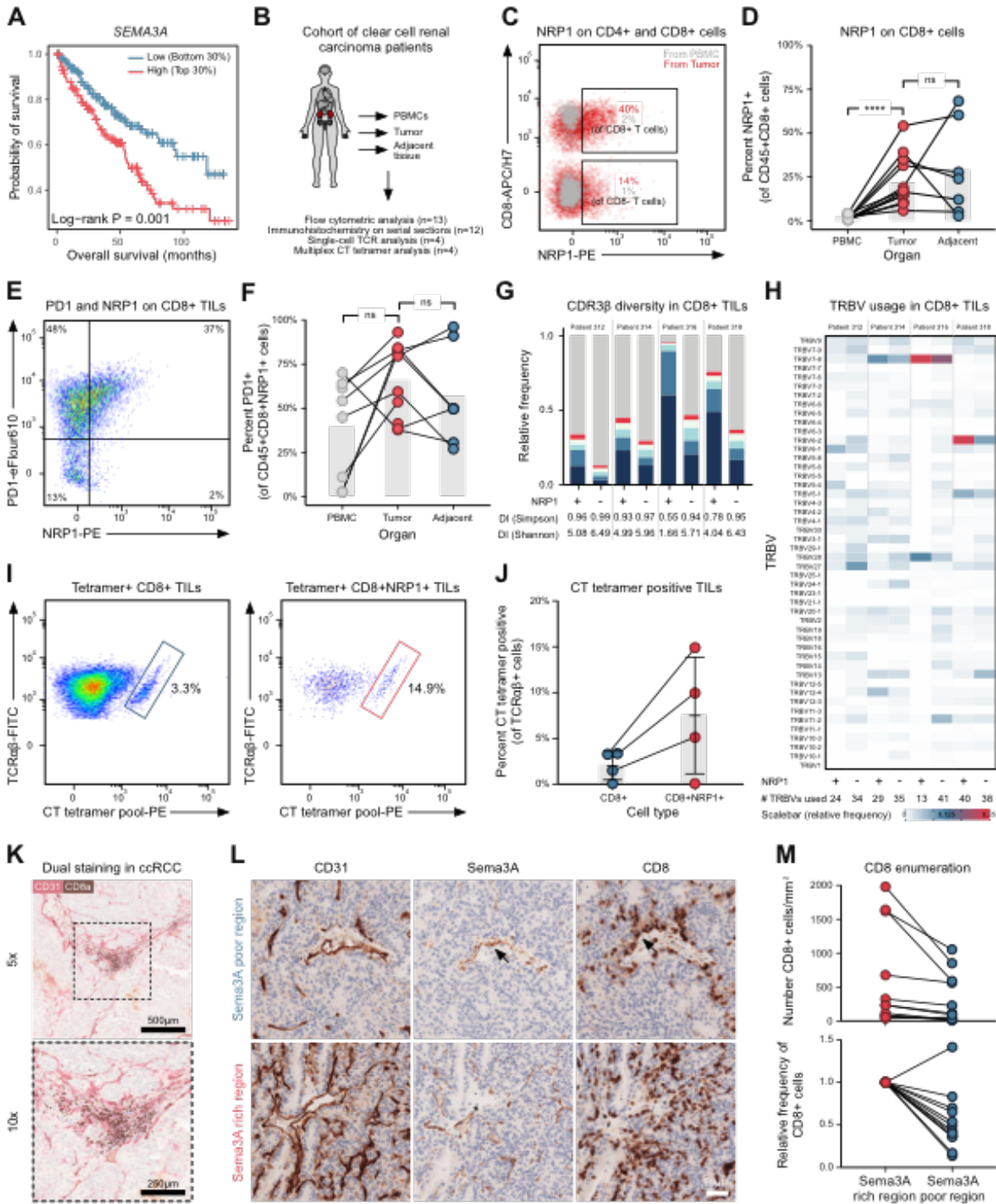


Figure 6: CD8+ TILs express NRP1 and are captured in Sema3A rich areas in ccRCC tumors.

A. Correlation of SEMA3A mRNA level with survival of ccRCC patients. Data from TCGA, using TIMER (71). **B.** Schematic representing ccRCC cohort of patient utilized in (C-M). **C.** Representative flow cytometric analysis of CD8 and NRP1 expression in PBMC and TILs in ccRCC patient. **D.** Analysis of NRP1 expression on CD8+ T cells in PBMC, tumor and tumor-adjacent tissue in ccRCC cohort (n=7-13). Bars indicate mean. **** = P < 0.0001, ns = not significant by two-way ANOVA. **E.** Representative flow cytometric analysis of PD1 and NRP1 on CD8+ TIL in ccRCC patient. **F.** Analysis of PD1 expression on NRP1 positive CD8+ T cells in PBMC, tumor and tumor-adjacent tissue in ccRCC cohort (n=7-13). Bars indicate mean. ns = not significant by two-way ANOVA. **G.** Analysis of CDR3β diversity in NRP1 positive (+) and negative (-) CD8+ TILs (n=4). Colored bars represent the five most abundant clonotypes. Grey bar represents remaining sequences. SI and SA diversity indices (DI) show that in all four patients, NRP1+ TILs are less diverse. **H.** Heatmap of TRBV usage in NRP1 positive (+) and negative (-) CD8+ TILs (n=4). Color indicates relative usage within all of individual patients, as indicated by scalebar. **I.** Representative flow cytometric analysis of TCRβ and CT tetramer positive CD8+ TILs (left) and NRP1+ CD8+ TILs (right). Error bars indicate mean ± SD. **J.** Graph of percentage CT tetramer positive NRP1+ (red) and NRP1- (blue) CD8+ TILs in four ccRCC patients. **K.** Representative CD8 (brown) and CD31 (red) staining in ccRCC tumor. Dashed area in top image indicates zoom area in bottom image. Scalebar, 500µm and 250µm. **L.** Representative CD31, Sema3A and CD8 staining in Sema3A poor region (top row) and Sema3A rich region (bottom row). Arrows indicate association between Sema3A and CD8 staining. Scalebar, 50µm. **M.** Enumeration of CD8+ TILs in Sema3A rich (red dots) and poor (blue dots) in patients (n=12). Abbreviations: CT, cancer testis. DI, diversity indices. SA, Shannon index. SI, Simpson index. TIL, tumor-infiltrating lymphocytes. TCGA, The Cancer Genome Atlas. TIMER, Tumor Immune Estimation Resource. TRBV, TCR beta chain variable.

376

377 **DISCUSSION**

378 In this study we showed that the secreted protein Sema3A has a previously
379 underappreciated role in controlling tumor-specific CD8+ T cells and highlight several
380 important conclusions concerning its function.

381

382 We established several lines of evidence that reveal a strong inhibitory effect of Sema3A on
383 CD8+ T cell migration in tumors. First, *in vitro* experiments provided functional insights into
384 how Sema3A inhibited key steps in T cell extravasation, including adhesion, transmigration
385 and mobility. Notably, these effects could be reversed using a blocking antibody against
386 NRP1, confirming that NRP1 is an important regulator of Sema3A signaling on CD8+ T cells.
387 Second, conditional knockout of NRP1 on T cells corroborated these findings *in vivo*,
388 resulting in higher CD8+ T cell infiltration into the TME. Conversely, significantly fewer
389 tumor-specific T cells homed to and infiltrated Sema3A-overexpressing tumors. Third, in
390 ccRCC patients CD8+ TILs were preferentially found in Sema3A-rich regions and beside
391 Sema3A-rich blood vessels, reminiscent of how tumor-associated macrophages can be
392 entrapped within Sema3A-rich hypoxic regions (22).

393

394 We explored the effect of Sema3A on IS formation. Previous studies have characterized
395 Sema3A as an inhibitor of T cell signaling and proliferation using *in vitro* assays (8, 12). We
396 extended these results and confirmed that key steps in synapse formation are affected,
397 including cell-cell binding, formation of close contact zones and organization of distinct
398 supramolecular activation clusters. These findings are in line with work by Ueda et al who
399 found that Sema3E inhibited IS formation in thymocytes (27). We further show that the F-
400 actin cytoskeleton becomes activated following Sema3A exposure. Although further
401 experiments are warranted to draw firm conclusions, this effect is ostensibly dependent on
402 myosin-IIA activity, since we could rescue T cell undulation using the drug Blebbistatin which
403 specifically prevents intra-cellular force generation by myosin-II (45). High resolution 3D
404 imaging has shown that myosin-IIA forms bona fide arcs above the pSMAC (46, 47) but then
405 moves inwards and contracts, thereby pinching the T cell away during termination of the IS

406 (24). This isotropic contraction of the actomyosin arc appears similar to myosin's role during
407 cytokinesis (48). Our data suggest that Sema3A leads to hyperactivation of myosin-II, thus
408 enforcing IS termination. Data does exist to provide a link between Sema3A binding and
409 myosin-II. Biochemical and crystallographic studies have shown that Sema3A signaling
410 converts the small GTPase Rap1A from its active GTP-bound state, to its inactive GDP-
411 bound state following binding to Plexin-A (15, 32). In epithelial and endothelial cells, active
412 Rap1-GTP can act as a negative regulator of myosin-II (33). It is therefore tempting to
413 speculate that Sema3A, by inhibiting Rap1-GTP activity, leads to hyperactivation of myosin-
414 II. Indeed, in both neurons (7, 49) and DCs (23), Sema3A has been shown to increase
415 myosin-II activity in line with this interpretation, however much of this pathway needs to be
416 further elucidated in T cells. We propose that Sema3A induces a cellular "paralysis" based
417 on integrin-actinomyosin contraction leading to motility paralysis and immunological
418 synapse preemption.

419

420 There is a growing interest in NRP1 in the context of T cell anti-tumor immunity. Much
421 research has focused on regulatory T cells (Treg), as NRP1 can be used to identify thymus-
422 derived regulatory T cells (50) and has been shown to play an important role in controlling
423 Treg function and survival (51). It has become clear that NRP1 is expressed on dysfunctional
424 tumor-specific CD8+ T cells (9–11), indicating that the protein might play an important role
425 in regulating CD8+ T cells as well. We show that initial NRP1 expression is controlled by
426 the level of TCR-engagement, that the protein remains expressed on tumor-specific CD8+
427 T cells *in vivo* and that NRP1 is found on a subset of tumor-specific CD8+ T cells in human
428 ccRCC patients. These latter results are in line with reports by Jackson et al. (52) and
429 Leclerc et al. (11) who found that approximately 10% of CD8+ TILs from melanoma patients
430 and 14% of non-small-cell lung cancer patients, respectively, were NRP1 positive. Unlike
431 us, Jackson et al. did not find any role for NRP1 in regulating CD8+ T cells when mice were
432 challenged with a leukemia cell line. An explanation for this discrepancy could be a lack of
433 Sema3A expression in this model. Indeed, we did not find any functional differences
434 between NRP1 knockout and wild-type T cells when challenging mice with H7N1 S-Flu, a
435 pathogen that did not lead to any meaningful upregulation of Sema3A in the lung.
436 Conversely, Sema3A knockout or overexpression in B16.F10.Ova cells was shown to have

437 significant effects on T cell migration and control of tumor growth when treated with tumor-
438 specific CD8+ T cells. An even stronger effect was seen in the lack of tumor growth in our
439 *Nrp1^{Flox/Flox}* mice. These results are in line with Delgoffe et al. (51) and Leclerc et al. (11)
440 who show similar control of tumor growth when treating mice with a blocking anti-NRP1
441 antibody. Hansen et al. also found strong anti-tumor effects in a comparable conditional
442 NRP1 knockout model but ascribed this to decreased Treg infiltration into the TME (53).
443 More likely, the remarkable control of tumor growth seen by us and others is due to
444 synergistic effects, as our data would suggest that ablation of NRP1 enhances CD8+ T cell
445 migration and effector functions as well. Research by Vignali and colleagues has shown that
446 NRP1 plays a key role in Treg survival and suppressive capabilities within the TME through
447 ligation with Sema4A (51, 54). Why does NRP1 enhance Treg function, but inhibit CD8+ T
448 cells? While not exploring this question in detail, we did find that Tregs to a larger extent
449 expressed other NRP1 co-receptors, including TGF β RI and II. Indeed, NRP1 has been
450 shown to enhance TGF β binding in Tregs (18). As Tregs are dependent on TGF β for their
451 function (55), one intriguing possibility is that NRP1 preferentially partners with these TGF β -
452 receptors on Tregs, while the only co-receptors available on CD8+ T cells are the proteins
453 of the Plexin-A family, which could provide a molecular basis for distinct signaling in each
454 cell type.

455
456 Our study highlights an underappreciated tumor-escape mechanism, namely inhibition of
457 tumor-specific T cells through cytoskeletal paralysis. We find that the effects of Sema3A on
458 CD8+ T cells are mainly mediated through the co-receptor NRP1, suggesting new
459 therapeutic avenues, for example by using antagonistic NRP1 antibodies. Enhancing
460 migration of tumor-specific T cells into tumors is critical for improving the efficacy of
461 checkpoint blockade (56) and adoptive T cell transfer therapies (57), making this an exciting
462 prospect. However, since the Sema3A-Plexin-A-NRP1 pathway also regulates the
463 maturation of endothelial cells (15) emphasis on timing and drug-target will be critical.

464 **METHODS**

465

466 **Cell lines and media**

467 Cell culture was performed using antiseptic techniques in HEPA filtered culture cabinets.
468 Cell lines were grown at 37c in a 5% CO₂ atmosphere. As indicated in text, for some
469 experiments, cells were cultured for 24 hours in a 1% O₂ chamber. All cell-lines were
470 screened for Mycoplasma. Adherent cells were split by Trypsin-EDTA detachment and
471 serially passaged and their viability regularly checked.

472 B16.F10 and B16.F10.Ova cell-lines were provided by Uzi Gileadi. The latter was generated
473 by transducing B16.F10 with a modified Ovalbumin construct, containing a start codon and
474 amino-acid 47 to 386 of the full-length ovalbumin, which ensures that Ovalbumin is not
475 secreted by the cell-line. LL/2 cells were a gift from Christopher W Pugh (Nuffield
476 Department of Medicine, University of Oxford).

477 B16.F10.Ova Sema3A knockout cells were generated using CRISPR/Cas9 genome-editing
478 (see below). B16.F10.Ova Sema3A overexpressing cells were generated by transducing
479 cells with a lentivirus encoding EFS-Sema3A cDNA (NCBI sequence NM_001243072.1)-
480 mCherry, cloned by VectorBuilder (see below). HEK293T cells were a gift from Tudor A.
481 Fulga (Radcliffe Department of Medicine, University of Oxford).

482 Adherent cells were kept in DMEM, 10% FCS, 2 mM Glutamine, 1 mM Sodium Pyruvate,
483 100 U/ml penicillin + 100 µg/ml streptomycin. For some experiments, 10 ng/mL murine IFN γ
484 (cat. no 315-05, PeproTech) or murine TNF α (cat. no. 315-01A, PeproTech) was added to
485 medium. T cells were kept in IMDM, 10% FCS, 2 mM Glutamine, 1 mM Sodium Pyruvate,
486 1x Non-essential amino acids, 100 U/ml penicillin + 100 µg/ml streptomycin, 10 mM HEPES,
487 50 µM β -mercaptoethanol. 10 IU IL-2 (cat. no AF-212-12, PeproTech) was added from
488 frozen stock just before use.

489

490 **Mouse strains and injection of tumor cells, T cells, antibodies and S-Flu H7N1**

491 All experiments were performed in mice on a C56BL/6 background. Mice were sex-matched
492 and aged between 6 and 12 weeks at the time of the first experimental procedure. All studies
493 were carried out in accordance with Animals (Scientific Procedures) Act 1986, and the
494 University of Oxford Animal Welfare and Ethical review Body (AWERB) under project licence

495 40/3636. CD4-Cre mice were a gift from Katja Simon (NDM, University of Oxford). LifeAct
496 mice were a gift from Shankar Srinivas (DPAG, University of Oxford). C57BL/6, OT-I and
497 CD45.1 mice were purchased from Biomedical services, University of Oxford. NRP1-floxed
498 mice were purchased from Jackson Laboratories (Stock No: 005247).

499 Cancer cell lines were split at 1:3 ratio 24 hours before injection into mice in order to keep
500 cells in log-phase. On the day of injection, cells were trypsinized and washed 3 times in
501 PBS to remove residual FBS. Suspensions of 1.5×10^5 cells in 100 μ L PBS were prepared
502 and kept on ice until injection. Mice were anesthetized using isoflurane and cells injected
503 intradermally.

504 For adoptive transfer of T cells into mice, OT-I splenocytes were stimulated for 48 hours
505 using SIINFEKL peptide and sorted as described below, washed 2 times in PBS and injected
506 i.v. via the tail vein.

507 For infection with S-Flu H7N1, mice were infected intranasally with 10 infectious units S-Flu
508 H7N1 in 50 μ L viral growth medium (DMEM with 2 mM Glutamine, 10 mM HEPES, 100 U/ml
509 penicillin + 100
510 μ g/ml streptomycin and 0.1% BSA) under anesthesia.

511 For CD8-depletion experiments, anti-CD8a (cat. no. BE0061, clone 2.43, BioXcell) or IgG2b
512 isotype control (cat. no. BE0090, clone LTF-2, BioXcell) were resuspended in PBS and
513 injected intraperitoneally at day -4, -1, 4 and 7 post injection of cancer cells.

514

515 **Mixed bone-marrow chimeras**

516 To generate mixed bone marrow chimeric mice, male C57BL/6 host mice were lethally
517 irradiated at 4.5 Gy for 300 seconds, followed by a 3 hour rest, and a subsequent 4.5 Gy
518 dose for 300 seconds. Mice were injected i.v. with a 1:1 mixture of CD45.1+ NRP1^{Flox/+} and
519 CD45.1+CD45.2+ NRP1^{Flox/Flox} bone marrow cells. Recipient mice received drinking water
520 containing antibiotics (0.16mg/mL Enrofloxacin (Baytril), Bayer Corporation). Mice were
521 rested for 10 weeks before experimental use.

522

523 **Analysis of publicly available transcriptional data**

524 For analysis of Sema3A co-receptors, we downloaded raw expression data collected from
525 mice from the “Immunological Genome Project data Phase 1” via the Gene Expression

526 Omnibus (series accession: GSE15907). We specifically focused on naïve CD8+ T cells
527 (accessions: GSM605909, GSM605910, GSM605911), CD8+ effector T cells (accessions:
528 GSM538386, GSM538387, GSM538388, GSM538392, GSM538393, GSM538394), and
529 CD8+ memory T cells (accessions: GSM538403, GSM538404, GSM538405). The raw
530 expression array files were processed using the affy package (58) and differential
531 expression of selected genes (CD72, NRP1, NRP2, PLXNA1, PLXNA2, PLXNA3, PLXNA4,
532 PLXNB1, PLXNB2, PLXNB3, PLXNC1, PLXND1, SEMA3A, SEMA3B, SEMA3C, SEMA3D,
533 SEMA3E, SEMA3F, SEMA3G, SEMA4A, SEMA4B, SEMA3C, SEMA4D, SEMA4F,
534 SEMA4G, SEMA5A, SEMA5B, SEMA6A, SEMA6B, SEMA6C, SEMA6D, SEMA7A, TIMD2,
535 HPRT, OAZ1, RPS18, NFATC2, TBX21, EOMES, CD28, PDCD1, CTLA4, LAG3, BTLA,
536 TIM3, ICOS, TNFRSF14, TNFSF14, CD160, CD80, LAIR1, CD244, CXCR1, CXCR2,
537 CXCR3, CXCR4, CXCR5, CCR1, CCR2, CCR3, CCR4, CCR5, CCR5, CCR6, CCR7,
538 CCR8, CCR9, CCR10) between naïve and effector and memory cells was examined using
539 the limma package (59) in R (60).

540 Analysis of TCGA data was conducted using TIMER (61).

541

542 **Harvesting and activating splenocytes**

543 Mice were euthanized using CO₂, and spleens were harvested and stored in T cell media
544 on ice. The spleen was strained through a 70 µm nylon mesh using the blunt end of a syringe
545 to make a single cell suspension. Cells were washed off the mesh by applying 5 mL of T cell
546 medium, followed by mixing of the solution by aspiration. Cells were then washed and
547 resuspended in 3 mL red blood-cell (RBC) lysis buffer for 5 minutes on ice. Cells were
548 washed again in T cell medium, counted and resuspended at 2×10⁶ cells per mL in T cell
549 medium.

550 10 IU/ml IL-2 (Cat. 212-12, PeproTech) and 25nM SIINFEKL (N4) peptide (Cambridge
551 Peptides) were added to the single cell solution. Approximately 200 µL cells were then
552 plated onto a 96-well U-bottom plate and allowed to expand for 48 hours. For TCR affinity
553 assays, SIINFEKL (N4), SIITFEKL (T4) or SIIQFEKL (Q4) peptide (Cambridge Peptides)
554 was used at indicated concentrations.

555

556 **Sorting CD8+ T cells using magnetic beads**

557 CD8a+ Negative T Cell Isolation Kit (Order no. 130-104-075, Miltenyi Biotec) was used to
558 sort T cells and was performed according to the manufacturer's protocol. Briefly, cells were
559 washed in MACS buffer (0.5% bovine serum albumin and 2 mM EDTA in PBS), incubated
560 with antibody cocktail, followed by magnetic beads for 10 minutes each on ice. Cells were
561 then loaded into a prewetted LS column (Order no. 130-042-401, Miltenyi Biotec) inserted
562 into a magnet in approximately 3-5 mL MACS buffer.

563

564 **Preparation of tissue from mice for flow cytometry**

565 When staining cells in B16.F10 and LL/2 tumors, or from lymph nodes, frontal cortex, lungs
566 or thymus, mice were euthanized using CO₂, and tumors were harvested and stored in T
567 cell media on ice. Organs were cut into smaller pieces with a scalpel and incubated for 30
568 minutes with reagents from a tumour dissociation kit (Order no. 130-096-730, Miltenyi
569 Biotec). Cells were strained through a 70 µm nylon mesh using the blunt end of a syringe to
570 make a single cell suspension. Cells were washed off the mesh by applying 5 mL T cell
571 media, followed by mixing of the solution by aspiration. After a wash, the cells were
572 resuspended in approximately 2 mL of 100% Percoll solution (Cat. no. 17-0891-01, GE
573 Healthcare), and layered carefully on top of 3 mL of 80% and 40% Percoll solution, and spun
574 for 30 minutes at 2000g. Cells at the 80-40% interphase were washed, and stained using
575 protocols outlined below.

576

577 **Flow cytometry**

578 For washing and staining cells for flow cytometry PBS with 2% BSA, 0.1% NaN₃ sodium
579 azide was used. Single colour controls were either cells or OneComp Compensation Beads
580 (Cat. No 01-1111-41, Thermo Fisher).

581 For surface staining, cells were washed with 200 µl FACS Buffer and blocked in 100 µl Fc
582 block (cat. no. 101319, TruStain FcX, clone 93, BioLegend, diluted 1:100) in FACS Buffer
583 for 10 min on ice and washed. Antibody cocktail was added and cells were stained on ice
584 for at least 20 min, in the dark and washed twice. When applicable, cells were fixed in 2%
585 PFA for at least 10 min at RT before acquisition. For quantification of number of cells in
586 tumors, lymph nodes and lungs in certain experiments, quantification beads (CountBright,
587 cat. no. C36950, Thermo Fisher) were used.

588 For intracellular staining, cells were fixed in 100 μ l/well of FoxP3 IC Perm/fix Buffer (Cat. no
589 00-8222-49, Thermo Fisher) for 20 min at RT. Cells were pelleted, the fixative removed, and
590 200 μ l/well of 1x Perm Buffer added for 20 min at RT and washed. Antibody mix was added
591 and cells stained for at least 20 min on ice in Perm buffer and washed twice.
592 Cells were analyzed on either Attune NxT (Life Technologies) or an LSR Fortessa X20 or
593 X50 (BD Biosciences) flow cytometers in the WIMM Flow Cytometry Facility and data was
594 analysed using FlowJo v10 (FlowJo) and R (60).

595

596 The following antibodies and tetramers were used for flow cytometry:

597

Specific for	Fluorophore(s)	Company	Clone
CD3e	BV650	BioLegend	17A2
CD4	APC710	Tonbo Bioscience	GK1.5
CD4	BUV810	BD	GK1.5
CD8a	BV711	BioLegend	53-6.7
CD11a	PE	BioLegend	M17/4
CD11b	APC	BioLegend	M1/70
CD19	BV425	BioLegend	6D5
CD24	APC710	BD	M1/69
CD25	PerCP/Cy5.5	BioLegend	3C7
CD31	BV510	BD	MEC13.3
CD44	APC/Cy7, PE/Cy7	BioLegend	IM7
CD45.1	FITC	eBioscience	A20
CD45.2	PerCP/Cy5.5	BioLegend	104
CD49d	BUV395	BD	9C10
CD62L	BV610,FITC	BioLegend	Mel-14
CD69	BUV737	BD	H1.2F3
CD103	PE/Cy7	BioLegend	2E7

CD105	PE-CF594	BD	MJ7/18
CD162	BV421	BD	2PH1
EpCAM	APC/Cy7	BioLegend	G8.8
F4/80	BV610	BioLegend	BM8
FoxP3	BV421	BioLegend	MF-14
IFN γ	PE	BioLegend	XMG1.2
Granzyme B	FITC	BioLegend	GB11
Ly6C	BV780	BioLegend	HK1.4
MHC-II	PerCP/Cy5.5	BioLegend	AF6-120.1
NRP1	BV421	BioLegend	3E12
NRP1	PE	BioLegend	3E12
H-2D ^B -NP tetramer	PE-Cy7	Made in-house	
PlexinA1	PE	R&D Systems	FAB4309P
PlexinA2	APC	R&D Systems	FAB5486A
PlexinA4	Conjugated to PE	Abcam	ab39350
Podoplanin	APC	BioLegend	8.1.1
TCR β	APC/Cy7	BioLegend	H57-597
TCR β	PE-CF594	BD	H57-597
TGF β RI	PE	R&D Systems	FAB5871P
TGF β RII	PE	R&D Systems	FAB532P
TNF α	PerCP/Cy5.5	BioLegend	MP6-XT22
Sema3A	FITC, PE	R&D Systems	IC1250P
VEGFR2	PE	BioLegend	Avas12
Zombie	Violet Dye	BV421	BioLegend
Zombie	Aqua Dye	BV525	BioLegend
Zombie	Near-infrared	APC-780	BioLegend

598

599 **RT-qPCR**

600 RNA was extracted from cells using RNeasy kit (QIAGEN), followed by quantification on
601 Nanodrop (Thermo Scientific). RNA was reverse transcribed using QuantiTect Reverse
602 Transcription Kit (QIAGEN). Both controls without RNA or reverse transcription were
603 included, and all experiments were performed in minimum technical triplicates and biological
604 duplicates. cDNA was diluted to 10-20 ng in 5 ul/well and added to qRT-PCR plates. Taqman
605 probes were combined with 2x Fast Taqman Master Mix and 5 ul/well added to the cDNA.
606 qRT-PCRs were run on a QuantStudio7 qRT-PCR machine (Life Technologies). Expression
607 was normalized to the house keeping gene HPRT.
608 The following TagMan probes were used: BNIP3 (Mm01275600-g1), HPRT (Mm03024075-
609 m1), PDK1 (Mm00554300-m1), PDL1 (Mm00452054-m1), SEMA3A (Mm00436469-m1)
610 and VEGFA (Mm00437306-m1).

611

612 **Western blot**

613 Cells were washed in PBS and pelleted, before being resuspended in lysis buffer with a
614 EDTA protease inhibitor for at least 30 minutes on ice to extract protein. Cell-debris was
615 removed by centrifugation at 4°C. Supernatant containing protein was collected and
616 quantified using Pierce BCA protein assay using diluted albumin as a standard.
617 Samples were mixed with Loading Buffer (Life Technologies) and Reducing Agent (Life
618 Technologies) and heated to 95°C for at least 5 minutes. 4-12% Bis-Tris gels and MES SDS
619 Buffer (Life Technologies) were used for proteins with a molecular weight below 200 kDa,
620 while proteins above 200 kDa were blotted on a 3-8% Tris-Acetate gels in MOPS Buffer (Life
621 Technologies). Proteins were separated at 200V for approximately one hour and transferred
622 onto either PVDF or nitrocellulose membrane using the TransBlot Turbo Transfer (BioRad)
623 system. Gels were blocked in 5% BSA/PBS solution (blocking buffer) for at least 30 minutes
624 at RT. Membranes were stained with primary antibody in fresh blocking buffer and incubated
625 at 4C overnight on a shaker, washed five times in PBS with Triton-X (0.1% Tween-20 in
626 PBS) followed by incubation with fluorescent secondary antibodies (LICOR) diluted 1:20000
627 in blocking buffer for 1 hour on a shaker. Membranes were dried and imaged using the
628 Odyssey Near-Infrared imaging system (LI-COR).

629 The following antibodies were used: Anti-Neuropilin 1 antibody (Abcam, ab184783), anti-
630 PlexinA1 (R&D Systems, AF4309), anti-PlexinA2 (R&D Systems, AF5486), anti-GAPDH
631 (Santa Cruz, sc-32233), anti- β -Actin (Cell Signaling Technology, 13E5).

632

633 **CRISPR/Cas9 editing and verification of B16.F10.Ova cells**

634 A sgRNA targeting Sema3a was cloned into Cas9-2A-EGFP expression vector pX458. This
635 vector was electroporated into 1x10 B16.F10.Ova cells suspended in Solution V (Lonza)
636 using the Amaxa 2B nucleofector (Lonza) with settings P-020. After 48 hours, single cells
637 were sorted using the SH800 cell sorter (SONY) and expanded. Clones were genotyped by
638 high-throughput sequencing. Briefly, the targeted locus was PCR amplified from each clone
639 and subsequently indexed with a unique combination of i5 and i7 adaptor sequences.
640 Indexed amplicons were sequenced on the MiSeqV2 (Illumina) and demultiplexed reads
641 from each clone were compared to the wildtype Sema3a reference sequence using the
642 CRISPResso webtool (62).

643

644 **Lentiviral transduction of cells**

645 A lentiviral Sema3A overexpression vector was purchased from VectorBuilder. To generate
646 viral particles, this transfer vector was co-transfected into HEK-293T cells along with the
647 packaging and envelope plasmids pCMV-dR8.91 and pMD2.G using polyethylenimine.
648 Crude viral supernatant was filtered and used to transduced B16.F10.Ova cells. mCherry
649 positive transduced cells were selected by FACS using the SH800 cell sorter (SONY) to
650 create a Sema3A overexpression cell line.

651

652 **Protein production of Sema3A and mutant Sema3A**

653 Recombinant mouse Sema3A_{S-P} (residues 21–568), Sema3A_{S-P-I} (residues 21–675, without
654 a HIS tag) along with the Nrp1-binding deficient mutant Sema3A (residues 21–568, L353N-
655 P355S), here called mutant Sema3A, were cloned into a pHLsec vector optimized for large
656 scale protein production as described before (63). The L353N P355S mutation in mutant
657 Sema3A introduces an N-linked glycan to the Sema3A-Nrp1 interaction site, which is
658 sufficient to block the formation of Sema3A-Nrp1-PlxnA2 signaling complex as described
659 previously (5). Additionally, in all proteins, furin sites (R551A and R555A) were removed to

660 prevent Sema3A proteolytic processing and enable more controlled purification and sample
661 homogeneity. Proteins were expressed in HEK293T cells. Proteins were purified from buffer-
662 exchanged medium by immobilized metal-affinity followed by size-exclusion
663 chromatography using a Superdex 200 column (GE).

664 For some experiments, purified Sema3A protein was labelled with AF647 using Alexa Fluor
665 647 Antibody Labeling Kit according to protocol (Invitrogen, cat. no A20186) at a F/P rate at
666 1-2.

667

668 **Live-cell imaging of cells for migration studies, LifeAct and IRM quantification**

669 u-Slide I 0.4 Luer (Cat. no. 80172, Ibidi), u-Slide 8 well (Cat. no. 80826, Ibidi) and u-Slide
670 Angiogenesis (Cat. no. 81506, Ibidi) were used for live-cell imaging of T cell motility and
671 adhesion.

672 Proteins were immobilized on the plates by resuspension in PBS and allowing them to
673 adhere for 2 hours at room-temperature, followed by three washes in PBS with 1-2% BSA.

674 The following proteins and concentrations were used: ICAM-1-Fc (Cat. 553006, BioLegend,
675 at 10 µg/mL), CD3 (clone 145-2C11, BioLegend, at 10ug/mL), CXCL12 (Cat. 578702,
676 BioLegend, at 0.4 µg/mL, BioLegend). Sema3A_{S-P} and mutant Sema3A (described above)
677 were either ligated to surfaces in a similar manner or added to the medium while imaging at
678 5 µM. Plates were used immediately after preparation.

679 For experiments, T cells were activated for 48 hours using peptide stimulation, sorted and
680 washed. In cases where αNRP1 or isotype control treatment was applied, T cells were
681 incubated with these antibodies for 15 minutes at 37 °C, before an additional wash. T cell
682 medium without phenol red and IL-2 was prewarmed and used to resuspend cells. Cells
683 were added to plates placed on a stage in environmental chamber set at 37C directly at the
684 microscope. DeltaVision Elite Live cell imaging microscope, Zeiss LSM 780 or 880 confocal
685 microscopes were used with a Zeiss Plan-Neofluar 10x (0.3 NA), 40x (0.6 NA) or 63x (1.3
686 NA) lens.

687 In cases where T cells were tested under shear stress, cells were loaded into Hamilton
688 syringes, and installed on a Harvard Apparatus PHD 2000 pump, and connected to
689 chambered slides through 0.4 luer tubes. Flow rates and control of directionality of flow (ie,

690 that it was laminar) were measured using fluorescent beads over set distances to calibrate
691 instrument.

692 In cases where T cells were treated with drugs while imaging, drugs were first resuspended
693 in T cell medium and carefully added on top of solution while images were being acquired.
694 Care was taken always to take into consideration the correct concentration under growing
695 levels of media. The following drugs were used: Jasplakinolide (Cat. J4580, Sigma, used at
696 5 μ M) and Blebbistatin (Cat. 203390, EMD Millipore, used at 100 μ M), as well as Sema3A_{S-P}
697 (used at 1-5 μ M).

698 Data was acquired at 1 sec to 1 minute per frame as indicated, and analyzed in Fiji/ImageJ
699 (64). For cell tracking and visualization of spiderplots the Trackmate package was used (65).
700 Subsequent tracks were analyzed in R (60) and visualized using the ggplot2 package (66).
701 For cell contours, IRM and LifeAct, data was thresholded and collected in Fiji/ImageJ, then
702 exported to R for analysis and visualization. For quantification of IRM and LifeAct area while
703 adding drugs, movies were edited such that the analyzed frames were equal to the timing
704 indicated in figures (ie to start 3-5 minutes before adding the first drug). In videos with a
705 frame-rate of 1 frame per second, 3 frames around the frame in which drugs was added was
706 removed to avoid blurry or distorted images.

707

708 **Transwell chemotaxis assay**

709 Trans-migration was assessed in 24-well transwell plates with 3- μ m pore size (Corning Life
710 Sciences). The lower chamber was loaded with 500 μ l T cell medium with 10 IU IL-2 and
711 with or without 50ng/mL CXCL10. 10⁵ CD8⁺ OT-I CD8⁺ cells were stimulated and sorted as
712 described above and added in a volume of 100 μ l of T cell medium to the upper chamber,
713 in either the presence of a blocking α NRP1 antibody or an isotype control, and 5 μ M
714 Sema3A_{S-P}. As a positive control, effector cells were placed directly into the lower chamber.
715 As a negative control, migration medium alone was placed in the upper chamber. Plates
716 were incubated for 3 hours at 37C in a 5% CO₂ atmosphere. Thereafter, the Transwell
717 inserts were removed and the contents of the lower compartment were recovered. Cells
718 from the lower chamber were stained and the cells were quantified by flow cytometry.

719

720 **Immunological synapses analysis on supported lipid bilayers (SLB)**

721 The concentrations of the ligands used were as follows: 5 $\mu\text{g/ml}$ to achieve 10
722 molecules/ μm^2 of biotinylated H2Kb-SIINFEKL, 68 ng/ml to achieve 100 molecules/ μm^2 of
723 12x-HIS-tagged CD80 (AF488-labelled), and 122 ng/ml to achieve 200 molecules/ μm^2 of
724 12x-His tagged ICAM-1 (AF405-labelled). These concentration were determined based on
725 titrations on bead supported bilayers analyzed by flow cytometry. Sema3A_{S-P-I} (described
726 above) was used, as this protein had no HIS-tag and so could not interfere with binding of
727 other tagged proteins in bilayer.

728 For live cell imaging, supported lipid bilayer presenting H2Kb-SIINFEKL, CD80, and ICAM-
729 1 were assembled in sticky-Slide VI 0.4 luer (Ibidi) channels. The entire channel was filled
730 with a liposome suspension to form bilayer all along the channel. Live cells on SLBs were
731 imaged using the Olympus FluoView FV1200 confocal microscope that was enclosed in an
732 environment chamber at 37 °C and operated under standard settings. 60x oil immersion
733 objective (1.4 NA) was used with 2x digital zoom for time-lapse imaging at 20 second
734 intervals.

735 For fixed cell imaging, SLBs presenting H2Kb-SIINFEKL, CD80, and ICAM-1 were
736 assembled in 96-well glass-bottom plates (MGB096-1-2-LG-L, Brooks). 50,000 cells were
737 introduced into the wells at 37C and fixed 10 minutes later by adding 8% PFA in 2x PHEM
738 buffer. After 3 washes with 0.1% BSA in HBS the fixed cells were imaged on the InCell 6000
739 wide-field fluorescence high-throughput imaging station using a 40x air objective (0.75 NA).
740 The imaging station was programmed to visit specific equivalent locations in each of the
741 desired wells in the 96-well plate.

742 Analysis of fixed cell images was carried out by the MATALB based TIAM HT package (26).

743 The source-code is available on the github repository: <https://github.com/uvmayya/TIAM HT>.

744

745 **Acquisition of tissue from ccRCC patients**

746 Acquisition and analysis of ccRCC samples were approved by Oxfordshire Research Ethics
747 Committee C. After informed written consent was obtained, samples were collected, and
748 store until use by Oxford Radcliffe Biobank (project reference 17/A100 and 16/A075).

749

750 **Analysis of CDR3 and TRBV usage in CD8+ ccRCC TILs**

751 cDNA from single cells was obtained using a modified version of the SmartSeq2 protocol
752 (67). Briefly, single cells were sorted into plates containing lysis buffer. cDNA was generated
753 by template switch reverse transcription using SMARTScribe Reverse Transcriptase
754 (Clontech), a template-switch oligo and primers designed for the constant regions of Trac
755 and Trbc genes. TCR amplification was achieved by performing two rounds of nested PCR
756 using Phusion High-Fidelity PCR Master Mix (New England Biolabs). During the first PCR
757 priming, indexes were included, to identify each cell. A final PCR was performed to add the
758 Illumina adaptors. TCR libraries were sequenced on Illumina MiSeq using MiSeq Reagent
759 Kit V2 300-cycle (Illumina). FASTQ files were demultiplexed for each cell. Sequences from
760 clones were analysed using MiXCR (68). Post analysis was performed using VDJtools (69).

761

762 **CT-tetramer staining**

763 HLA-A2 monomers were made in-house, and loaded with CT-antigens through UV-directed
764 ligand exchange using published protocols (70). Following ligand exchange, all monomers
765 were tetramerized through binding to PE-Streptavidin, washed, and combined to allow for
766 "cocktail" staining of cells. Frozen vials of tumor tissue were thawed, dissociated, and CD45-
767 positive sorted, followed by staining with 0.5 µg of each tetramer in 50 ul for one hour at RT.
768 Otherwise staining proceeded like described previously.

769

770 The following CT-antigens were loaded into HLA-A2 monomers:

Ligand	Antigen
MLMAQEALAFI	LAGE-1
SLLMWITQC	LAGE-1
SLLMWITQA	NY-ESO-1
KVLEYVIK	MAGE-A1
GLYDGMEHL	MAGE-A10
YLQLVFGIEV	MAGE-A2
FLWGPRALV	MAGE-A3
KVAELVHFL	MAGE-A3
FLWGPRALV	MAGE-A3

ALKDVEERV	MAGE-C2
KVLEFLAKL	MAGE-C2
LLFGLALIEV	MAGE-C2
ELAGIGILTV	MART1
TLNDECWPA	Meloe-1
AMAPIKVRL	PRDX5 (OMT3-12)
LKLSGVVRL	RAGE-1
KASEKIFYV	SSX2
SVYDFFVWL	TRP2
YMDGTMSQV	Tyrosinase
CQWGRLWQL	BING4
VLPDVFIRCV	GnT-V

771

772 **Immunohistochemistry and image acquisition and analysis**

773 The diagnostic hematoxylin and eosin (H&E) stained slides for 12 cases of clear cell renal
774 cell carcinoma were reviewed to identify corresponding formalin-fixed paraffin embedded
775 tissue blocks that contained both tumor and adjacent non-tumor tissue. Strictly serial 4 µm
776 sections were then cut from the most appropriate block from each case. These sections
777 underwent immunohistochemistry staining on a Leica BOND-MAX automated staining
778 machine (Leica Biosystems). Briefly, sections were deparaffinized, underwent epitope
779 retrieval and endogenous peroxidase activity was blocked with 3% hydrogen peroxide (5
780 minutes). Subsequently, sections were incubated with the primary antibody (30 minutes)
781 followed by post-primary and polymer reagents (8 minutes each). Next, 3,3'-
782 Diaminobenzidine (DAB) chromogen was applied (10 minutes) (all reagents contained
783 within the BOND Polymer Refine Detection kit, Leica Biosystems, catalogue no. DS9800).
784 For double immunohistochemistry staining, the above cycle was repeated twice with the first
785 cycle using Fast red chromogen labelling (all reagents contained within the BOND Polymer
786 Refine Red Detection kit, Leica Biosystems, catalogue no. DS9390) and the second cycle
787 DAB chromogen labelling. At the end of both the single and double immunohistochemistry
788 protocols, the sections were counterstained with hematoxylin (5 minutes), mounted with a
789 glass coverslip and left to dry overnight. The following primary antibodies were used during

790 staining: CD31 (Agilent Technologies, JC70A, 1:800), CD8 (Agilent Technologies, C8/144B,
791 1:100) and Sema3A (Abcam, EPR19367, cat. no. ab199475, 1:4000).
792 Stained slides were scanned at x400 magnification using the NanoZoomer S210 digital slide
793 scanner (Hamamatsu). Sema3A-stained digital images were reviewed by a trained
794 pathologist (PSM) and the extent of staining was quantified in the regions where expression
795 was deemed to be highest ('Sema3A-rich') and lowest (Sema3A-poor) within the same
796 tumor, using custom-made Matlab (MathWorks) scripts (% staining = DAB+ pixels/total
797 pixels x 100; raw data, image analysis, and data processing scripts are available upon
798 request). This analysis was repeated in the same regions on adjacent serial sections for
799 CD31 (as for Sema3A) and CD8 (for which discrete cell counts were calculated from stained
800 regions using a water shedding process).

801

802 **Statistical analysis**

803 Statistical analysis was performed in Prism software (GraphPad) or R (60). Data was tested
804 for Gaussian distribution. For multiple comparisons, either one-way or two-way analysis of
805 variance (ANOVA) was used with Tukey's test to correct for multiple comparisons. For
806 comparison between two groups, Students t test, Student's paired t test, or one-tailed or
807 two-tailed Mann–Whitney test were used.

808

809 **AUTHORS CONTRIBUTIONS**

810 *Conceptualization:* M.B.B., V.C., E.Y.J., M.L.D., T.A.F., M.F.; *Formal analysis:* M.B.B.,
811 Y.S.M., V.A., M.R., P.S.M., V.M., M.F.; *Funding acquisition:* V.C., E.Y.J., M.L.D., T.A.F.,
812 M.F., C.W.P.; *Investigation:* M.B.B., Y.S.M., V.A., P.S.M., U.G., S.V., M.R., C.K., J.C.,
813 A.V.H., V.M., P.R., L.R.O., M.F., H.C.Y.; *Methodology:* V.C., U.G., M.B.B., M.L.D., M.F.,
814 H.C.Y., Y.S.M., A.R.T.; *Project administration:* V.C.; *Resources:* U.G., S.V., V.J., M.R., V.W.,
815 Y.K., V.M., A.R.T.; *Software:* M.R., J.A.B., M.B.B.; *Supervision:* V.C.; *Visualization:* M.B.B.,
816 P.S.M., V.M., M.F.; *Writing – original draft:* M.B.B., V.C., E.Y.J., M.L.D., M.F.; *Writing –*
817 *review & editing:* M.B.B., V.C., E.Y.J., M.L.D., M.F., Y.S.M., C.K., P.S.M., V.J.

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845

846 **COMPETING INTERESTS**

847 Authors declare no competing interests.

848

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1092 **MAIN FIGURE LEGENDS**

1093

1094 **Figure 1. Tumor-specific CD8+ T cells up-regulate NRP1 and Plexin-A1 allowing for**
1095 **Sema3A binding.**

1096 **A-B.** Representative histogram of flow cytometric analysis of surface NRP1 expression on
1097 human NY-ESO-1-specific HLA-A2 restricted CD8+ T cells and murine OT-I CD8+ T cells
1098 following 48 hours stimulation with cognate peptides. Cells are gated on CD45, CD8 and
1099 TCR β . Experiment repeated three times.

1100 **C.** Analysis of NRP1 up-regulation using peptides with varying TCR affinities. Cells are gated
1101 on CD45.1, CD8 and TCR β . Cells from 3 mice per group, experiment was performed once.
1102 Data indicate mean \pm SD.

1103 **D.** Quantification of surface binding of Sema3A_{S-P} on naïve and 48 hour stimulated OT-I T
1104 cells. Cells are gated on CD8 and CD3. Experiment was repeated three times. Data indicate
1105 mean \pm SD of representative experiment.

1106 **E.** Confocal imaging of 48 hour stimulated OT-I T cells stained with AF647-labelled
1107 Sema3A_{S-P} shows that the protein can bind to the cell membrane (white arrow) and within
1108 the cell (black arrow).

1109 **F.** Flow cytometric analysis of PD-1 and NRP1 expression on OT-I T cells 11 days after
1110 adoptive transfer in spleen, non-antigen expressing tumor (B16.F10) and antigen-
1111 expressing tumor (B16.F10.Ova) (n=6). Data representative of two independent
1112 experiments and indicate mean \pm SD of six mice per group.

1113 **G.** Schematic of NRP1 interactions partners (left). Flow cytometric analysis of expression of
1114 selected NRP1 interactions partners on OT-I T cells 11 days after adoptive transfer (n=5)
1115 (right). Experiment was performed once. Data indicate mean \pm SD.

1116

1117 Abbreviations: gMFI, geometric mean fluorescence intensity. N4, SIINFEKL. Q4, SIIQFEKL.
1118 T4, SIITFEKL.

1119

1120 **Figure 2. Sema3A negatively regulates CD8+ T cell adhesion, motility and migration**
1121 **through NRP1.**

- 1122 **A.** Representative brightfield and IRM images of 48 hour stimulated OT-I T cells adhering to
1123 ICAM-1 and CXCL12 coated plates with either Sema3A_{S-P} or IgG immobilized.
- 1124 **B.** Quantification of contact area per single cell using live-cell microscopy for 10 minutes
1125 after OT-I T cells were added to plate (n=60 cells). Data representative of three independent
1126 experiments and indicate mean \pm SEM. **** = $P < 0.0001$ by Student's t-test.
- 1127 **C.** Relative frequency of cell polarity from brightfield images. A polarity of 1 indicates a shape
1128 of a perfect circle, 0 a rectangular shape. Representative images of OT-I T cells illustrated
1129 above graph.
- 1130 **D.** Representative spider plots showing the migration paths of individual T cells pre-treated
1131 with either a NRP1 blocking antibody or isotype control antibody on similar plates as in (A).
- 1132 **E.** Graph of single cell distance (left) and single cell velocity (right) in same experiment as
1133 (D). (n=314-744 cells per group). Data combined from five independent experiments indicate
1134 mean \pm SD. *** = $P < 0.001$, ns = not significant by Kruskal-Wallis test.
- 1135 **F.** Representative spider plots showing the migration path of individual OT-I T cells on similar
1136 plates as in (A), with flow rates at 0 or 80 $\mu\text{m}/\text{sec}$. Arrows indicate flow direction.
- 1137 **G.** Quantification of percent of OT-I cells that detach in same experiment as (F) (n=20-73
1138 cells per condition). Data representative of two independent experiments and indicate mean
1139 \pm SEM. * = $P < 0.05$, *** = $P < 0.001$, ns = not significant by two-way ANOVA.
- 1140 **H.** Representative graph of number of stimulated OT-I T cells able to transmigrate through
1141 3 μm Boyden chamber with CXCL12 in bottom chamber, with or without Sema3A_{S-P} in top-
1142 chamber. OT-I T cells were pre-treated with either a blocking NRP1 antibody or isotype
1143 control antibody. Data representative of two independent experiments and indicate mean \pm
1144 SD. *** = $P < 0.001$, ns = not significant, by two-way ANOVA.

1145

1146 Abbreviations: IRM, interference reflection microscopy. Sec, second.

1147

1148 **Figure 3. Sema3A negatively regulates CD8+ T cells' immunological synapse**
1149 **formation.**

- 1150 **A.** Live-cell imaging visualizing surface interface using IRM of stimulated CD8+ T cells
1151 dropped on an activating surface with immobilized ICAM-1 and CD3 and Sema3A_{S-P} or IgG

1152 present in medium (left). Cell contour of representative cells from either condition (right).
1153 Color of contour indicates time from 0 to 200 sec as denoted on scalebar.

1154 **B.** Quantification of maximum size of cell contact area (top) and spreading speed from initial
1155 contact to maximum contact area (bottom) (n=25 cells per group) in same experiment as
1156 (A). Data combined from three independent experiments and indicate mean \pm SD. ** = $P <$
1157 0.01, *** = $P <$ 0.001, by Mann-Whitney test.

1158 **C.** Live-cell imaging of activated T cells pre-treated with Sema3A_{S-P-I}-AF647 and allowed to
1159 form synapses on supported lipid bilayers with ICAM-1, CD80 and H-2Kb-SIINFEKL. Arrows
1160 in merged image indicate cells that have bound Sema3A and do not form immunological
1161 synapses.

1162 **D.** Representative image from high-throughput analysis of immunological synapses on
1163 supported lipid bilayers as in (C) with OT-I T cells pre-treated with Sema3A or not.

1164 **E.** Quantification of immunological synapses with or without Sema3A_{S-P-I} pre-treated OT-I T
1165 cells. Data from six independent experiments (n=90-1100 cells per mouse per group). ** =
1166 $P <$ 0.01, by paired t-test.

1167

1168 Abbreviations: IRM, interference reflection microscopy. Sec, seconds.

1169

1170 **Figure 4. Sema3A affects T cell actin dynamics through actomyosin II activity.**

1171 **A.** Representative flow cytometric analysis of F-actin content with no or varying exposure to
1172 Sema3A_{S-P} treatment in 48 hour stimulated OT-I T cells as measured by Phalloidin-staining.
1173 Percentage indicate positive cells in each condition. Data representative of two independent
1174 experiments.

1175 **B.** Representative brightfield images of 48 hour stimulated LifeAct OT-I T cells adhering to
1176 ICAM-1 and CD3 coated plates before and after Sema3A_{S-P} added to medium.

1177 **C.** Representative confocal images of LifeAct in OT-I T cells (left) and their contour plots
1178 (right) from same experiment as in (B). Image taken at cell-surface interface. Dashed white
1179 line indicate area used for (D). Color of contour indicates time from 0 to 300 sec as denoted
1180 on scalebar.

1181 **D.** Kymograph before (top) and after (bottom) Sema3A_{S-P} added to medium on area
1182 indicated with white dashed line in (C). Dotted line along edge of cell denoted example of
1183 data used for calculating data in (E).

1184 **E.** Quantification of F-actin velocity at cell edge before and after treatment with either
1185 Sema3A_{S-P}, Jasplakinolide or mutant Sema3A (n=25-33 cells per group) using same
1186 experimental setup as in (B). Data combined from three independent experiments and
1187 indicate mean \pm SD. **** = $P < 0.0001$, ns = not significant, by paired t-test.

1188 **F.** Intensity plot of LifeAct signal before and after Sema3A_{S-P} treatment of a single OT-I T
1189 cell (left) or quantified on multiple cells exposed to Sema3A_{S-P} (right) using same
1190 experimental setup as in (B). Arrows indicate measured cell width. ** = $P < 0.01$, by paired
1191 t-test.

1192 **G.** Cell width dynamics measured like (F) over time before (white background) or after (grey
1193 background) Sema3A_{S-P}, Jasplakinolide or mutant Sema3A addition to medium.

1194 **H.** Quantification of IRM area of individual OT-I T cells (grey lines) or average for group (red
1195 line) over time, with no treatment (leftmost white background), under treatment with Sema3A
1196 (grey background) and then Blebbistatin (rightmost white background). Above
1197 representative contour plots of single cell under different treatments, with color denoting time
1198 (150 sec total). Cells were allowed to settle, and form contact for 3-5 min before data
1199 acquisition. Area normalized to cell area at $t = 0$ sec. Data combined from three independent
1200 experiments (n=27 cells). *** = $P < 0.001$, **** = $P < 0.0001$, ns = not significant by two-way
1201 ANOVA at time-points 90, 270 and 450 sec.

1202 **I.** Quantification of IRM area of individual OT-I T cells and representative contour plots as
1203 in (H), but with treatment with Blebbistatin (grey background) before Sema3A_{S-P} (rightmost
1204 white background). Data combined from three independent experiments (n=37 cells). * = P
1205 < 0.05 , ns = not significant by two-way ANOVA at time-points 90, 270 and 450 sec.

1206

1207 Abbreviations: Min, minutes. Sec, seconds. t, time.

1208

1209 **Figure 5: Nrp1-deficiency enhances anti-tumor migration and activity of CD8+ T cells.**

1210 **A.** Growth curve of B16.F10 cells in NRP1^{+/+}, NRP1^{Flox/+} and NRP1^{Flox/Flox} mice (left) and
1211 Kaplan-Meier survival curve (right). Dashed lines indicate growth in individual mice, bold line

1212 average for group. Combined data from 4 independent experiments with 3-6 mice per group.
1213 *** = $P < 0.001$, ns = not significant by two-way ANOVA.

1214 **B.** Growth curve of LL/2 cells in NRP1^{+/+}, NRP1^{Flox/+} and NRP1^{Flox/Flox} mice (left) and Kaplan-
1215 Meier survival curve (right) (n=3-6 mice per group). Dashed lines indicate growth in
1216 individual mice, bold line average for group. Experiment performed once. *** = $P < 0.001$,
1217 ns = not significant by two-way ANOVA.

1218 **C.** Growth curve of B16.F10 cells in NRP1^{Flox/+} and NRP1^{Flox/Flox} mice pre-treated with either
1219 anti-CD8 antibody or isotype control (n=5-7 mice per group). Dashed lines indicate growth
1220 in individual mice, bold line average for group. Data combined from two independent
1221 experiments. *** = $P < 0.001$, ns = not significant by two-way ANOVA.

1222 **D.** Enumeration of CD4⁺ and CD8⁺ T cells infiltrated into B16.F10 tumors in NRP1^{+/+},
1223 NRP1^{Flox/+} and NRP1^{Flox/Flox} mice (n=6 per group). Data indicate mean \pm SD. ** = $P < 0.001$,
1224 ns = not significant by two-way ANOVA.

1225 **E.** Experimental setup of mixed bone-marrow chimeras in C57BL/6 mice (left) and
1226 subsequent enumeration of CD8⁺ T cells in mice (middle graph). Ratio of CD8⁺ T cells from
1227 NRP1^{Flox/Flox} to NRP1^{Flox/+} bone-marrow derived cells (right graph) (n=6 mice per group).
1228 Experiment performed once. Data indicate mean \pm SD. * = $P < 0.05$ by one-way ANOVA.

1229 **F.** Experimental setup using B16.F10 Sema3A KO or Sema3A OE cells (left) and growth
1230 curve of cells in untreated mice (right) (n=8 mice). Experiment performed once. ns = not
1231 significant by two-way ANOVA

1232 **G.** Growth curve of B16.F10 Sema3A KO or Sema3A OE cells using similar experimental
1233 setup as in (F), but with OT-I treatment at day 7 post-injection (n=6). Experiment performed
1234 once. ** = $P < 0.001$ by two-way ANOVA.

1235 **H.** Enumeration of OT-I T cells in tumors (left graph) and their ratio of cells, normalized to
1236 the number in the B16.F10 Sema3A KO tumor (right) from same experiment as in (G). * =
1237 $P < 0.05$ by two-way ANOVA.

1238

1239 **Figure 6: CD8⁺ TILs express NRP1 and are captured in Sema3A rich areas in ccRCC**
1240 **tumors.**

1241 **A.** Correlation of SEMA3A mRNA level with survival of ccRCC patients. Data from TCGA,
1242 using TIMER (71).

- 1243 **B.** Schematic representing ccRCC cohort of patient utilized in (C-M).
- 1244 **C.** Representative flow cytometric analysis of CD8 and NRP1 expression in PBMC and TILs
1245 in ccRCC patient.
- 1246 **D.** Analysis of NRP1 expression on CD8+ T cells in PBMC, tumor and tumor-adjacent tissue
1247 in ccRCC cohort (n=7-13). Bars indicate mean. **** = $P < 0.0001$, ns = not significant by
1248 two-way ANOVA.
- 1249 **E.** Representative flow cytometric analysis of PD1 and NRP1 on CD8+ TIL in ccRCC patient.
- 1250 **F.** Analysis of PD1 expression on NRP1 positive CD8+ T cells in PBMC, tumor and tumor-
1251 adjacent tissue in ccRCC cohort (n=7-13). Bars indicate mean. ns = not significant by two-
1252 way ANOVA.
- 1253 **G.** Analysis of CDR3 β diversity in NRP1 positive (+) and negative (-) CD8+ TILs (n=4).
1254 Colored bars represent the five most abundant clonotypes. Grey bar represents remaining
1255 sequences. SI and SA diversity indices (DI) show that in all four patients, NRP1+ TILs are
1256 less diverse.
- 1257 **H.** Heatmap of TRBV usage in NRP1 positive (+) and negative (-) CD8+ TILs (n=4). Color
1258 indicates relative usage within all of individual patients, as indicated by scalebar.
- 1259 **I.** Representative flow cytometric analysis of TCR $\alpha\beta$ and CT tetramer positive CD8+ TILs
1260 (left) and NRP1+ CD8+ TILs (right). Error bars indicate mean \pm SD.
- 1261 **J.** Graph of percentage CT tetramer positive NRP1+ (red) and NRP1- (blue) CD8+ TILs in
1262 four ccRCC patients.
- 1263 **K.** Representative CD8 (brown) and CD31 (red) staining in ccRCC tumor. Dashed area in
1264 top image indicates zoom area in bottom image. Scalebar, 500 μ m and 250 μ m.
- 1265 **L.** Representative CD31, Sema3A and CD8 staining in Sema3A poor region (top row) and
1266 Sema3A rich region (bottom row). Arrows indicate association between Sema3A and CD8
1267 staining. Scalebar, 50 μ m.
- 1268 **M.** Enumeration of CD8+ TILs in Sema3A rich (red dots) and poor (blue dots) in patients
1269 (n=12).
- 1270
- 1271 Abbreviations: CT, cancer testis. DI, diversity indices. SA, Shannon index. SI, Simpson
1272 index. TIL, tumor-infiltrating lymphocytes. TCGA, The Cancer Genome Atlas. TIMER, Tumor
1273 Immune Estimation Resource. TRBV, TCR beta chain variable.

1274

1275 **SUPPLEMENTARY DATA**

1276

1277 **Supplementary Table 1**

Baseline characteristics of patient cohort.		
Characteristics	Number (range)	Percent
Age (years)		
Mean	64.4 (42-86)	
Gender		
Male	13	56.5%
Female	10	43.5%
Tumour grade (ISUP)		
1	0	0
2	2	8,7%
3	12	52.2%
4	6	26.1%
N/A	3	13%
Tumour location		
Right	14	60.9%
Left	9	39.1%
Type of surgery		
Radical nephrectomy	17	73.9%
Partial nephrectomy	6	26.1%
Tumour stage		
pT1a	1	4,5%
pT1b	5	22.7%
pT2a	1	4.5%
pT2b	0	0

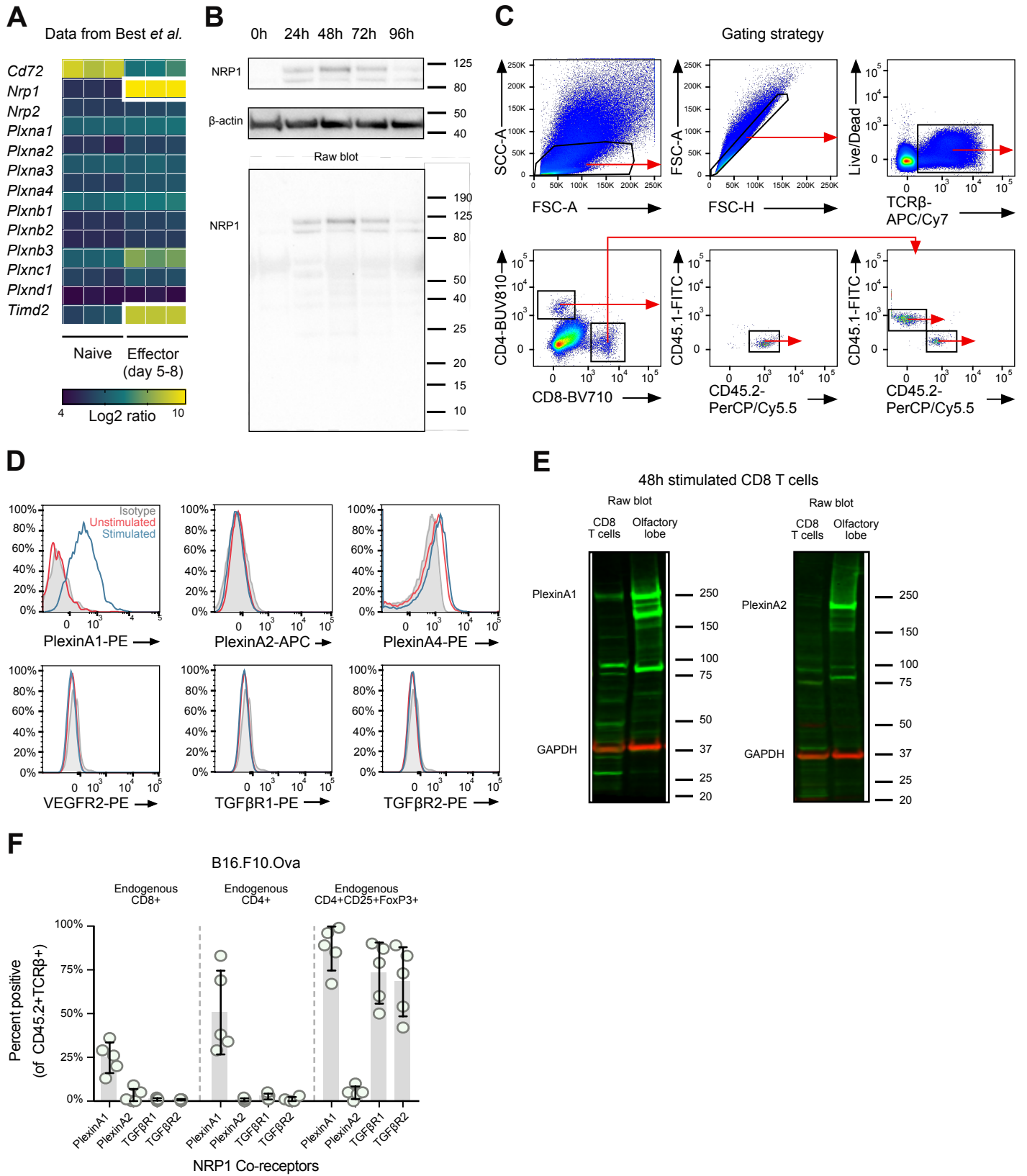
pT3a	14	63.6%
pT3b	0	0
pT3c	1	4.5%
pT4	0	0
N/A	1	4.5%

1278

1279

1280

Supplementary Figure 1



1281 **Supplementary Figure 1 (relates to Figure 1).**

1282 **A.** Heatmap of transcript levels of known semaphorin receptors on naïve and effector OT-I
1283 T cells following infection with vaccinia-OVA. Data from Best et al. 2013 (16).

1284 **B.** Western blot showing NRP1 up-regulation in OT-I T cells following stimulation with
1285 SIINFEKL. Experiment was performed once.

1286 **C.** Gating strategy for Figure 1F-G.

1287 **D.** Flow cytometric analysis of Plexin-A1, Plexin-A2, Plexin-A4, VEGFR2, TGF β R1 and
1288 TGF β R2 on unstimulated and 48 hour SIINFEKL stimulated OT-I T cells. Experiment
1289 representative of three.

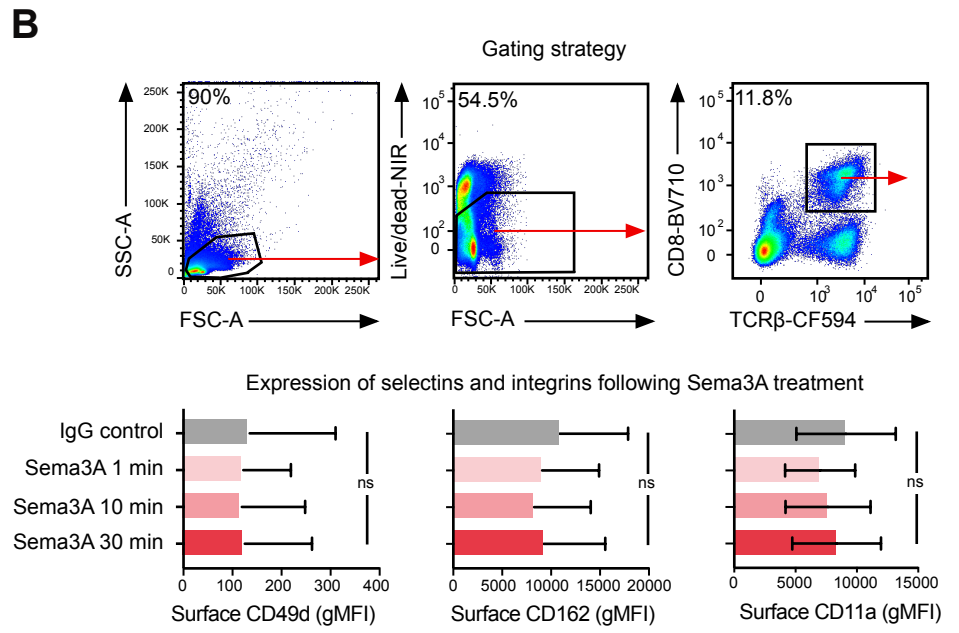
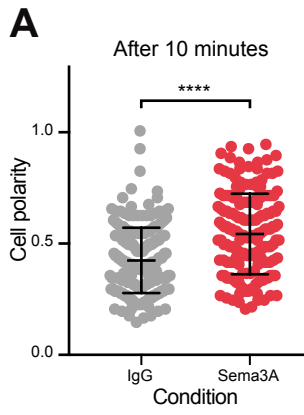
1290 **E.** Western blots showing expression of Plexin-A1 (left, green) and Plexin-A2 (right, green)
1291 in 48 hour stimulated OT-I T cells and olfactory lobe (positive control). Experiment performed
1292 once. Loading control GAPDH shown in red.

1293 **F.** Flow cytometric analysis of Plexin-A1, Plexin-A2, TGF β R1 and TGF β R2 expression on
1294 OT-I T cells and endogenous CD8+ TILs, 11 days after adoptive transfer of OT-I T cells in
1295 antigen-expressing tumor (B16.F10.Ova) (n=5). Error bars indicate SD.

1296

1297

Supplementary Figure 2



1298 **Supplementary Figure 2 (relates to Figure 2).**

1299 **A.** Relative frequency of cell polarity of 48 hour stimulated OT-I T cells treated with IgG or
1300 Sema3A_{S-P}. A polarity of 1 indicates a shape of a perfect circle, 0 a rectangular shape.
1301 Experiment repeated three times. **** = $P < 0.0001$ by Student's t-test.

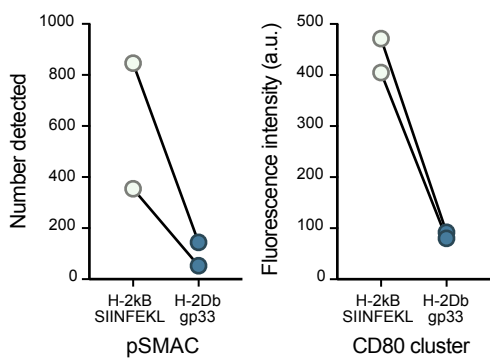
1302 **B.** Gating strategy for analyzing 48 hour stimulated OT-I splenocytes treated with Sema3A_{S-P}
1303 _P (top). Bar graphs of gMFI of CD49d, CD162 and CD11a following Sema3A_{S-P} treatment at
1304 indicated times. ns = not significant, by Kruskal-Wallis test.

1305

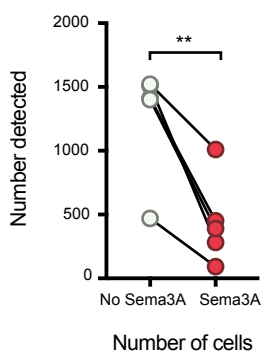
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Supplementary Figure 3

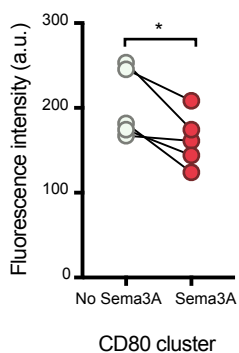
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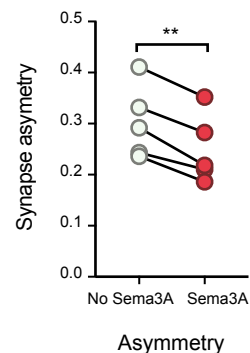
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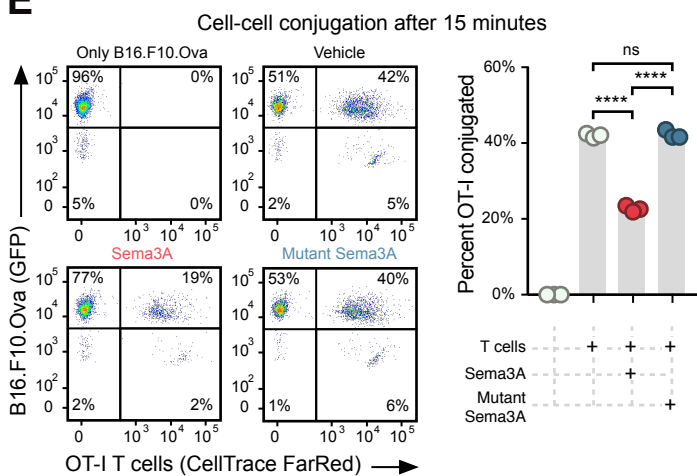
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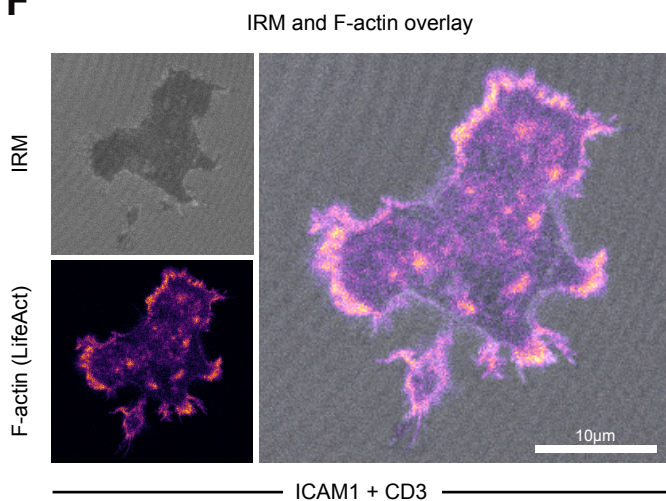
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E



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1307 **Supplementary Figure 3 (relates to Figure 3-4).**

1308 **A.** Quantification of pSMAC (left) and CD80 clustering (right) in immunological synapses of
1309 48 hour stimulated OT-I T cells when presented with a relevant (H-2Kb-SIINFEKL) or
1310 irrelevant (H-2D-gp33) MHC monomer loaded onto the bilayer.

1311 **B.** Quantification of 48 hour stimulated OT-I T cells detected in high-throughput assay with
1312 or without Sema3A_{S-P-I} pre-treatment. ** = $P < 0.01$, by paired t-test.

1313 **C.** Fluorescence intensity of CD80 signal introduced by 48 hour stimulated OT-I T cells in
1314 high-throughput assay with or without Sema3A_{S-P-I} pre-treatment. * = $P < 0.05$, by paired t-
1315 test.

1316 **D.** Analysis of radial symmetry of synapses in OT-I T cells in high-throughput assay with or
1317 without Sema3A_{S-P-I} pre-treatment. Asymmetry of the synapse is quantified as the distance
1318 between the centroids of the CD80 cluster and that of the pSMAC relative to the diameter
1319 of the cell. ** = $P < 0.01$, by paired t-test.

1320 **E.** Gating strategy and representative images showing number of B16.F10.Ova cells and T
1321 cells as either singlets or doublets under four different conditions: cancer cells alone, with
1322 normal media, media with Sema3A_{S-P} or with mutant Sema3A (left). Quantification of 3
1323 biological replicates, showing approximately 50% reduction in cell-cell conjugation when
1324 Sema3A is present (right). Data representative of three independent experiments. **** = P
1325 < 0.0001 , ns = not significant, by two-way ANOVA. Gray bars indicate mean.

1326 **F.** Images from live-cell imaging of OT-I \times LifeAct T cells showing concordance between
1327 IRM shadow and F-actin signal. Scalebar 10 μm .

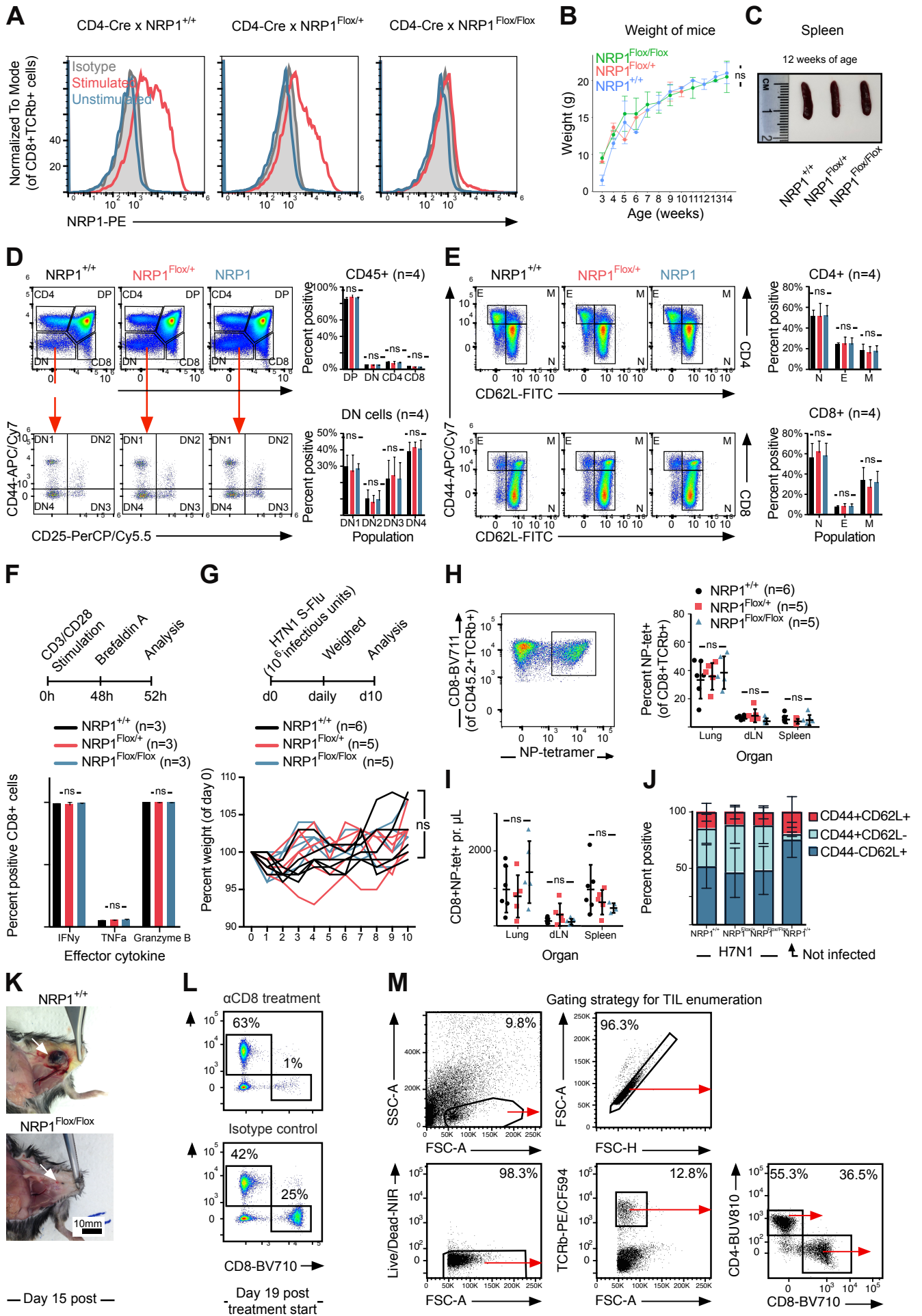
1328

1329 Abbreviations: a.u., arbitrary unit.

1330

1331

Supplementary Figure 4



1332 **Supplementary Figure 4 (relates to Figure 5).**

1333 **A.** Flow cytometric analysis of naïve or CD3/CD28 stimulated splenocytes from either
1334 $Nrp1^{+/+}$, $Nrp1^{Flox/+}$ or $Nrp1^{Flox/Flox}$ mice. Cells are gated on CD8 and TCR β . Experiment
1335 performed three times.

1336 **B.** The weight of female littermates (n=17) were followed for 12 weeks and revealed no
1337 difference between genotypes. Data indicate mean \pm SD. ns = not significant by two-way
1338 ANOVA.

1339 **C.** The size of spleens from female littermate mice of different genotypes at 12 weeks of
1340 age.

1341 **D.** Representative plots showing the distribution of double negative, double positive, CD4
1342 and CD8 positive thymocytes (upper panel, left) and DN1, DN2, DN3 and DN4 populations
1343 (lower panel, left) in $Nrp1^{+/+}$, $Nrp1^{Flox/+}$ or $Nrp1^{Flox/Flox}$ mice (n=4 per genotype) as analyzed
1344 by flow cytometry. Cells are gated on CD45.2. Quantification of cell populations in different
1345 genotypes (upper and lower histograms, right). Experiment performed once. ns = not
1346 significant by two-way ANOVA.

1347 **E.** Representative plots showing T cell effector populations in splenic CD4+ (top) and CD8+
1348 (bottom) T cells. Cells are gated on CD45.2 and TCR β (n=4 per genotype). Data combined
1349 from two independent experiments. ns = not significant by two-way ANOVA.

1350 **F.** Cytokine production following ex vivo stimulation by CD3/CD28. Experimental design
1351 (upper panel). Quantification of IFN γ , TNF α and Granzyme B by intracellular staining (lower
1352 panel). Cells are gated on TCR β and CD8 (n=3 mice per genotype). Experiment repeated
1353 twice. ns = not significant by two-way ANOVA.

1354 **G.** Weight of mice following H7N1 S-Flu infection. Experimental design (upper panel).
1355 Weight of mice, normalized to day 0 of individual mouse weight (lower panel). Experiment
1356 performed once (n=5-6 mice per genotype). ns = not significant by two-way ANOVA.

1357 **H.** Analysis of H7N1 S-Flu-specific T cells 10 days post-infection. Example H-2D^B-NP
1358 tetramer staining in lung of infected mouse (left figure). Quantification of H-2D^B-NP tetramer
1359 positive CD8+ T cells in lung, dLN and spleen (right figure). Cells are gated on CD45.2,
1360 TCR β and CD8 (n=5-6 per genotype). Experiment performed once. ns = not significant by
1361 two-way ANOVA.

1362 **I.** Quantification of total number of infiltrating H-2D^B-NP tetramer positive CD8⁺ in lung, dLN
1363 and spleen 10 days post-infection. Experiment performed once. ns = not significant by two-
1364 way ANOVA.

1365 **J.** Analysis of effector subpopulations in lung 10 days post-infection in different genotypes
1366 of mice (n=5-6 mice per genotype).

1367 **K.** Representative image of B16.F10 tumors 15 days post-injection in Nrp1^{+/+} (upper image)
1368 and Nrp1^{Flox/Flox} (lower image) mice. Arrows indicates tumors. Scalebar 10 mm.

1369 **L.** Representative flow cytometric analysis of peripheral blood in mice treated with either
1370 aCD8 antibodies (upper scatterplot) or isotype control (lower scatterplot).

1371 **M.** Gating strategy used for flow cytometric analysis of TIL enumeration in mice.

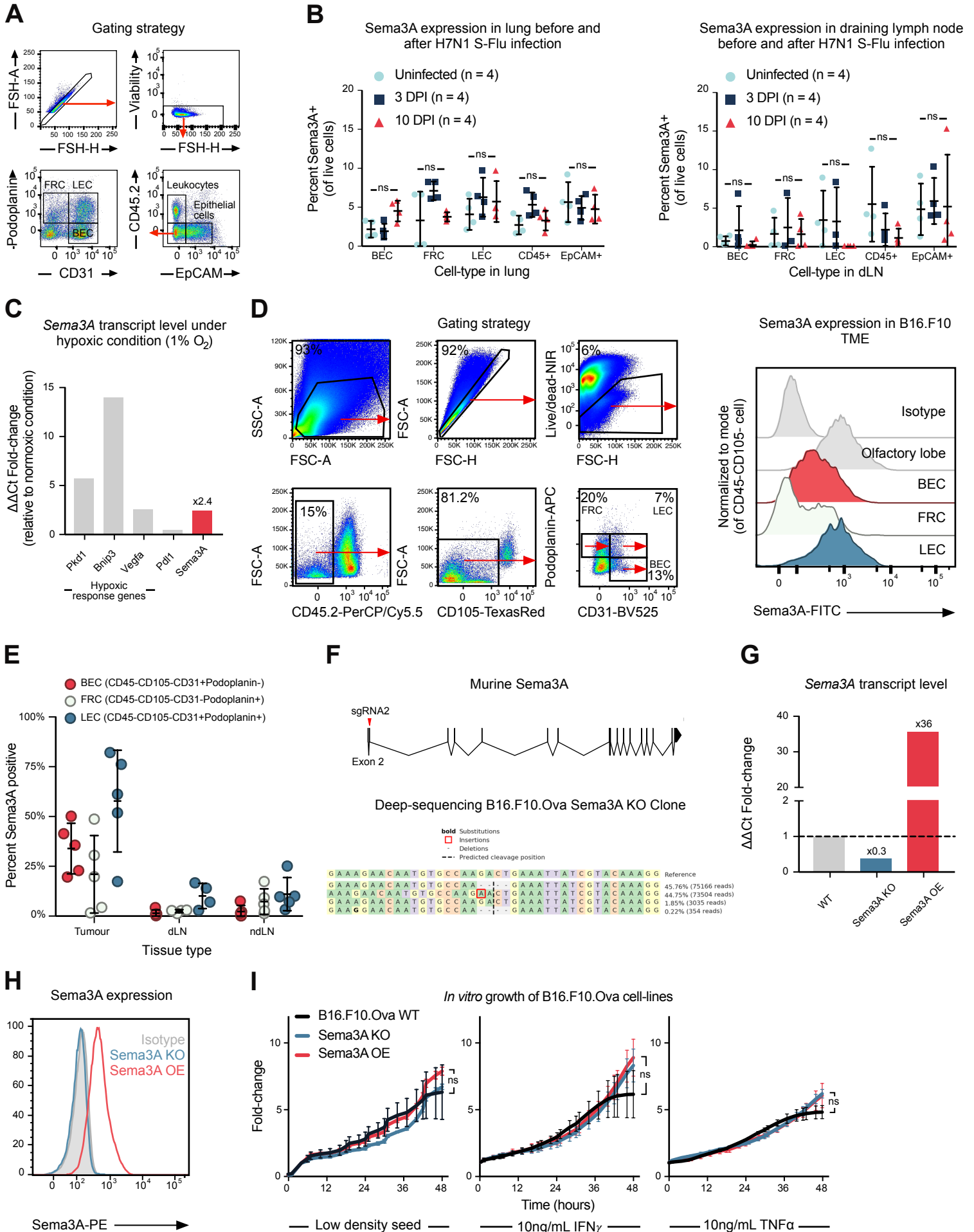
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1373 Abbreviations: dLN, draining lymph node. DN, double negative. E, effector T cells. N, naïve
1374 T cells. M, memory T cells. TIL, tumor-infiltrating leukocytes.

1375

1376

Supplementary Figure 5



1377 **Supplementary Figure 5 (relates to Figure 5).**

1378 **A.** Gating strategy for analyzing Sema3A expression among leukocytes, epithelial and
1379 endothelial cells in lung and dLN.

1380 **B.** Quantification of Sema3A positive cells in different cell populations in uninfected (n=4),
1381 and infected mice, at 3 days DPI (n=4) and 10 DPI (n=4) in lung (left panel) or dLN (right
1382 panel). *Nrp1*^{Flox/Flox} mice used. Data combined from two independent experiments. ns = not
1383 significant by two-way ANOVA.

1384 **C.** Quantification of *Pkd1*, *Bnip3*, *Vegfa*, *Pdl1* and Sema3A mRNA level following 24 hour
1385 culture in 1% O₂ chamber.

1386 **D.** Gating strategy (left) and representative histograms (right) analyzing Sema3A positive
1387 cell populations in B16.F10 tumors 11 days post-injection. Olfactory lobe is used as a
1388 positive control for Sema3A expression.

1389 **E.** Quantification of Sema3A positive cells in same experiment as in (D) in tumor, dLN and
1390 ndLN.

1391 **F.** Genomic organization of murine Sema3a gene, indicating where CRISPR guide RNA
1392 targets with red arrow (upper figure). MiSEQ sequence results for chosen Sema3A KO clone
1393 showing 4 base deletion in two alleles (46% of all reads), a frameshift in one allele (45% of
1394 all reads) and WT reads in 2% of all reads.

1395 **G.** RT-qPCR analysis show down- and up-regulation of Sema3A in Sema3A KO and OE
1396 cell lines, respectively. Normalized to *Hprt*. Experiment performed once.

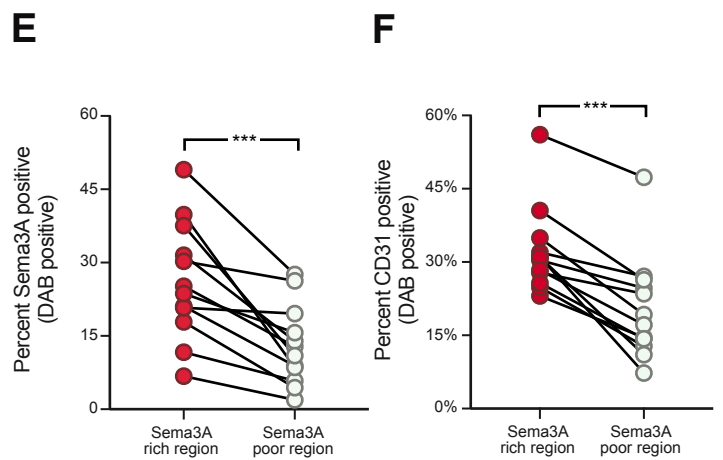
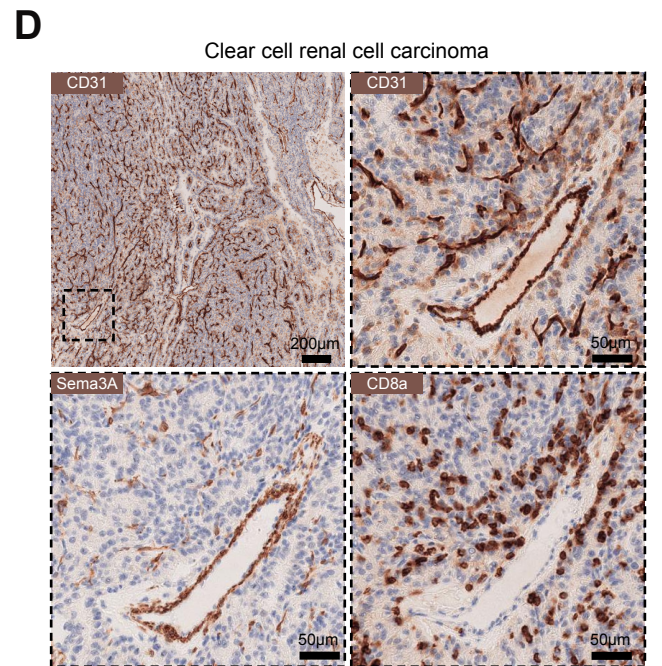
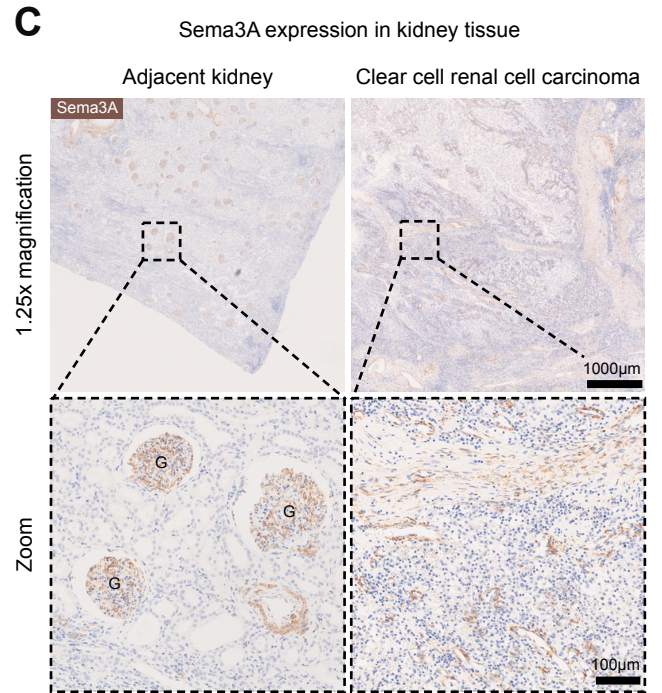
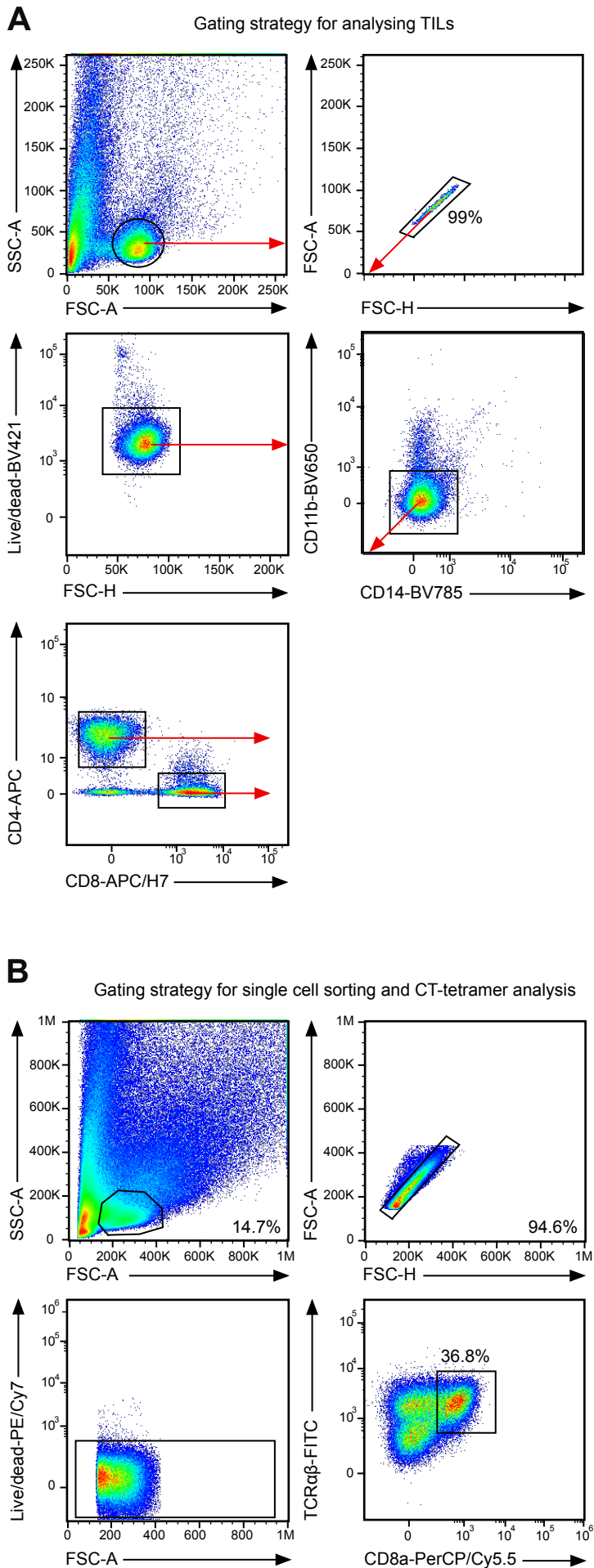
1397 **H.** Intracellular staining shows no detectable expression of Sema3A in Sema3A KO cells,
1398 and expression in Sema3A OE cells, as expected. Experiment performed once, at low
1399 seeding density.

1400 **I.** Growth of WT, Sema3A KO and Sema3A OE B16.F10.Ova cell lines in normal, IFN γ or
1401 TNF α -rich media. Experiment performed once. Data indicate mean \pm SD. ns = not significant
1402 by two-way ANOVA.

1403

1404 Abbreviations: BEC, blood endothelial cells. dLN, draining lymph node. DPI, days post-
1405 infection. FRC, fibroblastic reticular cells. KO, knockout. LEC, lymphatic endothelial cells.
1406 ndLN, non-draining lymph node. OE, overexpressing. TME, tumor microenvironment. WT,
1407 wild-type.

Supplementary Figure 6



1408 **Supplementary Figure 6 (relates to Figure 6).**

1409 **A.** Gating strategy for analyzing TILs from ccRCC patients.

1410 **B.** Gating strategy for single cell sorting and CT-tetramer analysis.

1411 **C.** Sema3A expression in tumor-adjacent and tumor tissue of ccRCC patient. G indicates
1412 kidney glomeruli. Scalebars indicate 1000 μm (upper row) and 100 μm (lower row).

1413 **D.** Serial sections from ccRCC tumor stained for CD31, Sema3A and CD8a. Dashed box in
1414 upper left image indicates the region depicted at higher magnification in the three other
1415 images. Scalebar indicates 200 μm (upper left) and 50 μm (other images).

1416 **E.** Expression of CD31 in Sema3A rich and poor regions. **** = $P < 0.0001$ by paired t-test.

1417 **F.** Expression of Sema3A in selected Sema3A rich and poor regions. **** = $P < 0.0001$ by
1418 paired t-test.

1419

1420 Abbreviations: ccRCC, clear cell renal cell carcinoma. DAB, 3,3'-Diaminobenzidine.

1421