

1 **Salivary gland macrophages assist tissue-resident CD8<sup>+</sup> T cell immune surveillance**

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33 **One sentence summary**

34 Combined *in vitro* and *in vivo* imaging of salivary gland-resident tissue memory CD8<sup>+</sup> T cells (T<sub>RM</sub>) uncovers  
35 their unique migratory behavior and describes a novel accessory function of tissue macrophages to assist T<sub>RM</sub>  
36 surveillance.

37 **Abstract**

38 Tissue macrophages and tissue resident memory CD8<sup>+</sup> T cells (T<sub>RM</sub>) play important roles for pathogen sensing  
39 and rapid protection of barrier tissues. To date, it is incompletely understood how these two cell types  
40 cooperate for efficient organ surveillance during homeostasis. Here, we used intravital imaging to show that  
41 T<sub>RM</sub> dynamically crawled along tissue macrophages in murine submandibular salivary glands (SMG) during  
42 the memory phase following a viral infection. *Ex vivo* confined SMG T<sub>RM</sub> integrated an unexpectedly wide  
43 range of migration modes: in addition to chemokine- and adhesion receptor-driven motility, SMG T<sub>RM</sub>  
44 displayed a remarkable capacity of autonomous motility in the absence of chemoattractants and adhesive  
45 ligands. This unique intrinsic SMG T<sub>RM</sub> motility was transmitted by friction and adaptation to  
46 microenvironmental topography through protrusion insertion into permissive gaps. Analysis of extracellular  
47 space in SMG using super-resolution shadow imaging showed discontinuous attachment of tissue  
48 macrophages to neighboring epithelial cells, offering paths of least resistance for patrolling T<sub>RM</sub>. Upon tissue  
49 macrophage depletion, intraepithelial SMG T<sub>RM</sub> showed decreased motility and reduced epithelial crossing  
50 events, and failed to cluster in response to local inflammatory chemokine stimuli. In sum, our data uncover  
51 a continuum of SMG T<sub>RM</sub> migration modes and identify a new accessory function of tissue macrophages to  
52 facilitate T<sub>RM</sub> patrolling of the complex exocrine gland architecture.

53

54 **Keywords**

55 Tissue-resident CD8<sup>+</sup> T cells; non-lymphoid tissue; tissue macrophages; intravital imaging; super-resolution  
56 shadow imaging; exocrine glands; chemokines; adhesion

## 57 **Introduction**

58 Naïve CD8<sup>+</sup> T cells (T<sub>N</sub>) continuously traffic through lymphoid tissue such as peripheral lymph nodes (PLN)  
59 and spleen, where they screen antigen presenting dendritic cells (DCs) for the presence of cognate peptide-  
60 MHC (pMHC) complexes. Intravital two-photon microscopy (2PM) of peripheral lymph nodes (PLN)  
61 uncovered a high amoeboid-like T<sub>N</sub> motility of 12-15 μm/min (1-4), facilitating their search for rare cognate  
62 pMHC-presenting DCs interspersed on a 3D stromal scaffold of fibroblastic reticular cells (FRC) (5-10).  
63 Intranodal motility is mediated by the CCR7 ligands CCL19 and CCL21 that drive F-actin polymerization at the  
64 leading edge in a Gαi-dependent manner to generate a retrograde cortical actin flow. Cortical actin flow is  
65 conveyed by the integrin LFA-1 into forward movement without generating substantial substrate adhesion  
66 (11-15).

67 During viral infections, effector CD8<sup>+</sup> T cells (T<sub>EFF</sub>) generated in reactive lymphoid tissue disseminate into non-  
68 lymphoid tissues (NLT) including gut, lung, genitourinary tract, and skin to eliminate infected cells. After  
69 clearance of viral antigens, part of T<sub>EFF</sub> differentiate into central memory T cells (T<sub>CM</sub>) and continue to patrol  
70 lymphoid organs, while others stably reside in NLT and to a minor extent in lymphoid tissue as non-  
71 recirculating, self-renewing tissue-resident memory T cells (T<sub>RM</sub>). T<sub>RM</sub> in gut, skin, and genitourinary tract act  
72 as “first line” sentinels that eliminate infected cells and trigger an organ-wide alert status through cytokine  
73 secretion upon pathogen re-encounter (16-21). The scanning behavior of epidermal T<sub>RM</sub> has been extensively  
74 studied. These cells display characteristically elongated, dendritic shapes and move in a Gαi-dependent  
75 manner with speeds of 1-2 μm/min in proximity to the extracellular matrix (ECM)-rich basement membrane  
76 (BM) separating epidermis from dermis, i.e. in plane with the bottom keratinocyte layer (22, 23). Upon  
77 pathogen reencounter, epidermal CD8<sup>+</sup> T cells follow local chemokine signals to accumulate around infected  
78 cells (24). CD8<sup>+</sup> T cell accumulation is considered critical for cooperative elimination of infected stromal cells  
79 through repeated cytotoxic attacks (25). Similarly, CD8<sup>+</sup> T<sub>RM</sub> of the small intestine continuously patrol the  
80 absorptive epithelial layer (26).

81 T<sub>RM</sub> are also present in exocrine glands of the head and neck region, including submandibular salivary glands  
82 (SMG). Salivary glands are targeted by several bacteria and viruses including human beta- and gamma-  
83 herpesviruses, which can cause disease, mostly in immunocompromised individuals (27, 28). Similar to skin



84 and gut, SMG contains epithelial tissue basally anchored onto connective tissue. However, while skin and gut  
85 3D geometry is evenly layered, the SMG epithelium has an arborized structure, with acini secreting saliva  
86 into intermediate and collecting ducts. The glandular epithelium is separated by a BM from the supporting  
87 interstitium containing blood and lymphatic vasculature, fibroblasts and tissue macrophages (29). In tissue  
88 sections, most CD8<sup>+</sup> T<sub>RM</sub> in SMG are localized within the abundant acini and ducts, implying a mechanism that  
89 allows T<sub>EFF</sub> arriving in interstitial venules to cross the BM below the epithelial compartment and develop into  
90 memory T cells (30, 31). During acute inflammation of NLT, CD8<sup>+</sup> T cell recruitment is driven by chemokines  
91 and adhesion receptors (32). In contrast, the cellular dynamics of homeostatic T<sub>RM</sub> surveillance in SMG after  
92 viral infection and the involvement of tissue macrophages in this process have not been explored to date.  
93 Here, we used intravital microscopy of mouse SMG in the memory phase following a systemic viral infection  
94 to uncover a high baseline motility of T<sub>RM</sub>, which often followed tissue macrophage topology. *Ex vivo*,  
95 confinement alone in the absence of chemoattractants and adhesion receptors was sufficient to induce SMG  
96 T<sub>RM</sub> migration through friction- and protrusion-insertion-driven motility, which was further tuned by  
97 chemokines and adhesion molecules. Using super-resolution microscopy to explore extracellular space  
98 distribution in SMG, we observed discontinuous attachment of tissue macrophages to surrounding  
99 epithelium, offering paths of least resistance to migrating T<sub>RM</sub>. Accordingly, tissue macrophage depletion  
100 resulted in a significant disruption of T<sub>RM</sub> patrolling behavior. Taken together, our data uncover a new  
101 accessory role for tissue macrophages to enable T<sub>RM</sub> surveillance of salivary glands. Our observations suggest  
102 a continuum of chemokine- and adhesion receptor-dependent and -independent migration modes and  
103 topographic features facilitating this task.

104 **Results**

105 *Systemic viral infection leads to the establishment of  $T_{RM}$  in salivary glands*

106 We used a viral infection model for a comparative analysis of CD8<sup>+</sup> T cell populations in lymphoid tissue and  
107 SMG (**Fig. 1A and Fig. S1A**). Systemic infection with lymphocytic choriomeningitis virus (LCMV)-OVA, a  
108 replication-competent, attenuated LCMV mutant expressing ovalbumin (OVA) as model antigen (33), led to  
109 transient and low viral titers in spleen on day 3 p.i. that remained below the detection limit in PLN and SMG  
110 (**Fig. S1B**). Adoptively transferred GFP<sup>+</sup> OT-I CD8<sup>+</sup> TCR tg T cells (which recognize the OVA<sub>257-264</sub> peptide in the  
111 context of H2-K<sup>b</sup>) (34) underwent a prototypic expansion – contraction kinetic in spleen and PLN over the  
112 course of 30 days (**Fig. S1, C and D**). Despite the lack of detectable viral titers, OT-I T cells accumulated in  
113 SMG from day 6 p.i. onwards, with a stable population maintained until at least day 30 p.i. (**Fig. S1, C and D**).  
114 By day 30 p.i., OT-I T cells isolated from SMG but not PLN or spleen showed increased expression of CD103  
115 and CD69, while losing the KLRG-1<sup>+</sup> population present on day 6 p.i. (**Fig. S1, E and F**). SMG OT-I T cells also  
116 upregulated PD-1 and CD44 surface levels (not shown), supporting the observation that most SMG CD8<sup>+</sup> T  
117 cells had developed into *bona fide*  $T_{RM}$  at day 30 p.i. (35). Memory OT-I CD8<sup>+</sup> T cells isolated from PLN were  
118 approximately 65% CD62L<sup>+</sup> CD44<sup>high</sup>  $T_{CM}$ , with the remaining population being CD62L<sup>-</sup> CD44<sup>high</sup> memory T cells.  
119 To take this heterogeneity into account, we refer to memory T cells isolated from PLN as  $T_{PLN-M}$ .  
120  $T_{RM}$  establishment in SMG was also observed at  $\geq 30$  day after systemic VSV-OVA infection (not shown). In  
121 addition, we detected memory P14 CD8<sup>+</sup> TCR tg T cells (which recognize the LCMV epitope gp<sub>33-41</sub> in the  
122 context of H2-D<sup>b</sup>) (36) in SMG after infection with the LCMV Armstrong strain (**Fig. S2, A and B**). In sum,  
123 systemic viral infection led to the recruitment and retention of CD8<sup>+</sup> T cell populations in SMG, even in the  
124 absence of detectable viral titers in this organ.

125

126 *SMG  $T_{RM}$  migration is characterized by dynamic cell shape changes*

127 We next determined the localization of  $T_{PLN-M}$  and  $T_{RM}$  in their target organs during the memory phase. Tissue  
128 sections showed that most GFP<sup>+</sup> OT-I T cells in PLN and SMG were dispersed evenly in the tissue at day 30  
129 p.i. (**Fig. 1B**). In PLN, OT-I  $T_{PLN-M}$  cells localized mainly with smooth muscle actin (SMA)<sup>+</sup> FRCs, whereas most  
130 OT-I  $T_{RM}$  cells in SMG were within or adjacent to EpCAM<sup>+</sup> acini and ducts (**Fig. 1B**). We developed a sequential

131 surgery method to visualize homeostatic tissue surveillance of  $T_{\text{PLN-M}}$  and  $T_{\text{RM}}$  in PLN and SMG, respectively,  
132 in the same host by 2PM (37).  $T_{\text{PLN-M}}$  displayed characteristic amoeboid shapes and moved with high speeds  
133 comparable to  $T_{\text{N}}$  ( $11.8 \pm 4.0 \mu\text{m}/\text{min}$ , median  $\pm$  SD) (**Fig. 1, C and F to H; movie S1**). Compared to  $T_{\text{PLN-M}}$ , SMG  
134  $T_{\text{RM}}$  displayed more pronounced shape changes, with several protrusions probing the microenvironment  
135 during migration, at times with thin and elongated cell bodies (**Fig. 1, D to G; movie S2**). While  $T_{\text{PLN-M}}$  and  $T_{\text{RM}}$   
136 covered large distances throughout the observation period of intravital imaging sequences (20-60 min), both  
137 populations differed in their speed and arrest coefficients, i.e. percentage of track segments with speeds  $<$   
138  $2.5 \mu\text{m}/\text{min}$ . Thus, SMG  $T_{\text{RM}}$  were significantly slower than  $T_{\text{PLN-M}}$  (**Fig. 1H**) and had higher arrest coefficients  
139 (**Fig. 1I**). Nonetheless, SMG  $T_{\text{RM}}$  retained a relatively high motility coefficient, which is a proxy of a cell's ability  
140 to scan the environment during random migration, of  $> 15 \mu\text{m}^2/\text{min}$  (**Fig. 1J**). Accordingly, their median speed  
141 of  $6.8 \pm 3.4 \mu\text{m}/\text{min}$  was notably higher than values reported for epidermal  $T_{\text{RM}}$  ( $1-2 \mu\text{m}/\text{min}$ ) (22), with some  
142 cells achieving speeds of  $> 12 \mu\text{m}/\text{min}$ . Both  $T_{\text{PLN-M}}$  and SMG  $T_{\text{RM}}$  retained a fast response to antigenic  
143 stimulation, as systemic administration of cognate peptide resulted in immediate arrest and secretion of IFN-  
144  $\gamma$  (**Fig. 1, J and K; Fig. S2, C and D**).

145 We measured similar speeds for GFP<sup>+</sup> P14  $T_{\text{RM}}$  in SMG before and after cognate peptide administration (**Fig.**  
146 **S2, A to D**). Furthermore, GFP<sup>+</sup> OT-I T cells patrolled the structurally comparable lacrimal gland (LG) in the  
147 same speed range ( $7.6 \pm 4.3 \mu\text{m}/\text{min}$ ; median  $\pm$  SD;  $n = 255$  tracks), suggesting that migration parameters of  
148  $T_{\text{RM}}$  patrolling exocrine glands during homeostasis are independent of TCR specificity and reflect tissue  
149 properties. In sum, our data uncover a remarkably fast motility of exocrine gland-resident CD8<sup>+</sup> T cells, which  
150 was characterized by dynamic shape changes.

151

#### 152 *$T_{\text{RM}}$ migrate along tissue macrophages during SMG surveillance*

153 To explore the microenvironmental context of exocrine gland  $T_{\text{RM}}$  migration, we used a CD11c-YFP reporter  
154 strain that labels SMG CD64<sup>+</sup> F4/80<sup>+</sup> tissue macrophages (29). CD11c-YFP<sup>+</sup> cells were also positive for the  
155 macrophage marker Iba-1, whereas some Iba-1<sup>+</sup> cells were CD11c-YFP<sup>low/negative</sup>, indicating that most but not  
156 all tissue macrophages were labeled in CD11c-YFP mice (**Fig. S3, A and B**). Confocal analysis of thick tissue  
157 sections showed that CD11c-YFP<sup>+</sup> tissue macrophages extended numerous protrusions from their cell bodies

158 throughout the SMG tissue and were located within EpCAM<sup>+</sup> ducts and acini, as well as SMA<sup>+</sup> perivascular  
159 structures of the interstitium (**Fig. S3C**). Most macrophage protrusions were phosphotyrosine-positive (**Fig.**  
160 **S3, D and E**) and enriched in F-actin (not shown), suggesting the presence of podosomes or focal adhesions  
161 at these sites.

162 To assess the spatial relationship between tissue macrophages and T<sub>RM</sub>, we transferred GFP<sup>+</sup> OT-I T cells into  
163 CD11c-YFP recipients one day prior to infection with LCMV-OVA and analyzed tissue sections by confocal  
164 microscopy in memory phase ( $\geq$  day 30 p.i.). We observed a striking spatial proximity of T<sub>RM</sub> and tissue  
165 macrophages in SMG, with approximately 70% of OT-I T cells directly in contact with CD11c-YFP<sup>+</sup> cells (**Fig. 2,**  
166 **A and B; movie S3**). The close spatial association between tissue macrophages and T<sub>RM</sub> was confirmed by  
167 correlative light and electron microscopy imaging, with both cell membranes adjacent to each other (**Fig. 2C**).  
168 Electron microscopy images also highlighted the compact tissue structure of SMG, with tight junctions of  
169 acinar and ductal epithelium surrounded by a dense ECM (**Fig. 2D**).

170 Their spatial proximity to SMG macrophages in tissue sections raised the question whether patrolling T<sub>RM</sub>  
171 migrate alongside macrophages. 2PM imaging of GFP<sup>+</sup> T<sub>RM</sub> in LCMV-OVA memory phase CD11c-YFP recipients  
172 indeed confirmed that T<sub>RM</sub> crawled along CD11c-YFP<sup>+</sup> macrophages during most of the observation period,  
173 with T<sub>RM</sub> shapes often closely matching the underlying macrophage topology. This was particularly evident  
174 along thin macrophage protrusions, which T<sub>RM</sub> often followed (**Fig. 2E; movie S4**). At the same time, T<sub>RM</sub>  
175 protrusions occasionally detached from macrophages, apparently scanning the surrounding environment.  
176 Accordingly, we identified occasional T<sub>RM</sub> track segments which were not associated with tissue  
177 macrophages. T<sub>RM</sub> speeds were slightly elevated when in contact with tissue macrophages than when not  
178 ( $7.0 \pm 5.3$  versus  $6.1 \pm 4.8$   $\mu\text{m}/\text{min}$ ;  $p < 0.001$ ). Occasionally, we observed small T<sub>RM</sub> clusters around tissue  
179 macrophages. Adoptively transferred CXCR3<sup>-/-</sup> T<sub>RM</sub> failed to accumulate at tissue macrophage clusters,  
180 suggesting the existence of local CXCL9/CXCL10 “hotspots” at these sites (**Fig. 2F**).

181 We examined whether the noticeable proximity between tissue macrophages and T<sub>RM</sub> also occurred in other  
182 exocrine glands and species. A comparable association of T<sub>RM</sub> and tissue macrophages was observed in LG  
183 after LCMV-OVA infection (**Fig. S3F**). Furthermore, CD3<sup>+</sup> T cells colocalized with CD68<sup>+</sup> macrophages in human  
184 parotid gland sections, both as dispersed individual cells and in clusters (**Fig. S3, G and H**). Taken together,

185 SMG  $T_{RM}$  colocalized with and moved alongside tissue macrophages during homeostatic tissue patrolling of  
186 several exocrine glands.

187

188 *SMG  $T_{RM}$  motility is induced by confinement and can be tuned by external factors*

189 The close proximity of  $T_{RM}$  to tissue macrophages *in vivo* prompted us to examine the molecular factors  
190 involved in this interaction. We performed quantitative PCR analysis of cytokine and chemokine expression  
191 by CD11c-YFP<sup>+</sup> cells sorted from SMG in steady-state, acute (day 6 p.i.) and memory phase (day 30 p.i.) of  
192 LCMV-OVA infection. We observed detectable mRNA levels of the cytokines IL-1 and TNF, as well as the  
193 chemokines CCL3, CCL4, CXCL2, CXCL9, CXCL10 and CXCL16 (not shown). Expression levels were similar at all  
194 time points analyzed, reflecting the lack of detectable viral spread to this organ (**Fig. S1B**). Given the  
195 expression of promigratory chemokines and adhesion receptors including ICAM-1 on tissue macrophages  
196 (38), we examined their influence on  $T_{RM}$  migration parameters. To this end, we employed under agarose  
197 assays that allow to precisely control environmental factors and provide the confinement required for T cell  
198 motility (**Fig. 3A**) (15). To benchmark our system, we transferred  $T_N$  on CCL21 - and ICAM-1-coated plates as  
199 surrogate lymphoid tissue microenvironment. We observed high chemokinetic  $T_N$  motility with similar speeds  
200 as measured *in vivo* ( $13.3 \pm 5.9 \mu\text{m}/\text{min}$ ) (**Fig. 3, B and C; movie S5**) (14, 15).  $T_{RM}$  showed a high motility ( $11.4$   
201  $\pm 3.0 \mu\text{m}/\text{min}$ ) when migrating on CXCL10 + CXCL12- and ICAM-1-coated plates, which was only slightly lower  
202 than that of  $T_N$  (**Fig. 3C; movie S6**). These observations show that SMG  $T_{RM}$  respond to presence of  
203 chemokines and adhesion molecules with high speeds.

204 We then examined T cell displacement on plates coated with fatty acid-free human serum albumin (HSA) and  
205 thus free of chemoattractants and specific adhesion ligands. In line with previous findings (15),  $T_N$  and  $T_{PLN-M}$   
206 remained essentially immobile throughout the observation period (**Fig. 3, D to F; movies S7 and S8**). Under  
207 these conditions, only 16% of  $T_N$  and 31% of  $T_{PLN-M}$  migrated faster than  $3 \mu\text{m}/\text{min}$ , and showed low  
208 directionality (**Fig. 3, G and H**). In contrast, most SMG  $T_{RM}$  showed robust intrinsic motility on HSA-coated  
209 plates despite the absence of chemoattractants and adhesion molecules (**Fig. 3, D to F; movies S7 and S8**).  
210 Almost 70% of SMG  $T_{RM}$  migrated faster than  $3 \mu\text{m}/\text{min}$  with high directionality, with their median speed of  
211  $5.5 \mu\text{m}/\text{min}$  approaching values observed *in vivo* (**Fig. 3, G and H**). High temporal resolution imaging revealed

212 that migratory  $T_{RM}$  often formed several protrusions along the leading edge that appeared to probe the  
213 environment, followed by rapid displacement of the cell body along one of the protrusions (**movie S9**). Thus,  
214 unexpectedly, confinement alone was sufficient to induce spontaneous SMG  $T_{RM}$  migration, representing to  
215 the best of our knowledge the first observation of such a motility mode in resting T cells. Their speeds were  
216 increased in presence of chemokines and adhesion molecules, suggesting that external promigratory factors  
217 tune intrinsic cell motility.

218

#### 219 *Friction mediates $T_{RM}$ migration in the absence of chemokines and ICAM-1*

220 We set out to characterize the requirements for autonomous  $T_{RM}$  motility in under agarose assays. Reflecting  
221 the absence of chemoattractants and integrin ligands, pertussis toxin (PTx) treatment or addition of the  $\beta$ 1-  
222 blocking peptide RGD did not affect  $T_{RM}$  speeds in this setting (**Fig. 3I**). Although Mac-1 binds weakly to serum  
223 albumin (39), addition of anti-Mac-1 mAb did not cause a significant reduction in  $T_{RM}$  speeds (**Fig. 3I**). These  
224 observations suggested a friction-based migration mechanism (40). Friction is the resisting force when two  
225 elements slide against each other and may be composed of a number of fundamental forces. While the  
226 nature of the weak interactions between  $T_{RM}$  and migratory surface causing friction are not defined, we  
227 hypothesized that these might in part involve bivalent cations. Indeed, chelation of bivalent cations by EDTA  
228 caused a strong decrease of  $T_{RM}$  speeds under agarose (**Fig. 3I**). High temporal resolution imaging showed  
229 that despite the lack of translocation in presence of EDTA,  $T_{RM}$  continued to probe the environment via  
230 transient protrusion formation, essentially “running on the spot” (**Fig. 3, J and K, movie S10**). This behavior  
231 precipitated a loss in the motility coefficient (**Fig. 3L**). In sum, our data suggest that bivalent cation-dependent  
232 friction between  $T_{RM}$  and the confining 2D surfaces generated sufficient traction for translocation in the  
233 absence of considerable surface binding.

234

#### 235 *SMG $T_{RM}$ insert protrusions between adjacent structures for translocation*

236 In addition to friction-based migration, protrusion insertion has emerged in recent years as a complementary  
237 mechanism to allow cell migration without specific adhesions (40). The continuous probing of  $T_{RM}$  in presence  
238 of EDTA (**Fig. 3J**) provided an opportunity to test whether topographic features of the environment such as

239 narrow intercellular spaces may rescue cell motility by permitting insertion of pseudopods as mechanical  
240 “footholds” (41, 42). As a surrogate approach to re-introduce a “2.5D” environmental geometry in under  
241 agarose assays, we co-transferred a surplus of  $T_N$  together with  $T_{RM}$  and performed time-lapse imaging in the  
242 presence of EDTA and in the absence of chemoattractants and adhesion molecules (**Fig. 4A**). Remarkably,  
243  $T_{RM}$  localized within  $T_N$  clusters frequently showed lateral displacement despite the presence of EDTA (**movies**  
244 **S10 and S11**). Under these conditions,  $T_{RM}$  displacement occurred through insertion of protrusions between  
245 adjacent  $T_N$  and subsequent translocation of the cell body accompanied by dynamic cell shape changes (**Fig.**  
246 **4B**).  $T_{RM}$  within  $T_N$  clusters were significantly faster than isolated  $T_{RM}$  ( $5.8 \pm 3.0$  and  $2.3 \pm 1.7$   $\mu\text{m}/\text{min}$ ,  
247 respectively), displayed higher directionality, and resembled in cell shape and speeds  $T_{RM}$  migrating *in vivo*  
248 (**Fig. 4C to E**). Once  $T_{RM}$  had traversed  $T_N$  clusters, they returned to their probing behavior without efficient  
249 translocation, indicating a close interdependence on physical contact and motility (**movie S10**).

250 We then examined whether potential residual molecular interactions between naive T cells and  $T_{RM}$  might  
251 act as drivers of migration. We therefore transferred uncoated polystyrene beads with  $T_{RM}$  in under agarose  
252 assays. These beads replaced  $T_N$  as surrogate 2.5D structures and allowed to examine protrusion insertion in  
253 the absence of potential adhesive interactions. In this setting,  $T_{RM}$  recapitulated the behavior observed within  
254  $T_N$  clusters, showing effective cell displacement only when in contact with clusters of beads for protrusion  
255 insertion (**Fig. 4F; movie S12**).  $T_{RM}$  speeds increased to  $6.4 \pm 1.9$   $\mu\text{m}/\text{min}$  and became more directional when  
256 in contact with beads, whereas isolated  $T_{RM}$  showed no displacement (**Fig. 4, G and H**). In this setting, we  
257 further observed that  $T_{RM}$  moved around dense bead areas, in line with a search for permissive gaps for  
258 locomotion (**movie S12**). In sum, SMG  $T_{RM}$  displayed a unique ability to migrate by adapting to topographic  
259 features of the environment through protrusion insertion and shape deformation, even in the absence of  
260 considerable friction, chemoattractants and adhesion receptors.

261

#### 262 *Residual in vivo motility of SMG $T_{RM}$ in presence of integrin and chemokine receptor blockade*

263 Our *in vitro* experiments raised the question to which extent external cues govern  $T_{RM}$  motility *in vivo*. We  
264 explored the molecular mechanisms underlying  $T_{RM}$  scanning of SMG, focusing on well-described canonical  
265 chemoattractant- and integrin-signaling pathways. Integrins provide traction and force transmission through

266 engagement of their ligands expressed by many cell types including macrophages, such as ICAM-1. SMG  $T_{RM}$   
267 express  $\alpha 1$ ,  $\alpha 4$ ,  $\alpha E$ ,  $\alpha L$ ,  $\beta 1$ ,  $\beta 2$  and  $\beta 7$  integrins, and low levels of  $\alpha V$  (**Fig. S1 and 4A**). To assess their  
268 involvement in SMG  $T_{RM}$  immune surveillance, we administered a mix of integrin-blocking mAbs against the  
269 major lymphocyte integrin  $\alpha L$  (CD11a/CD18, LFA-1), the E-cadherin ligand  $\alpha E$  (CD103), and  $\alpha 4$  (VLA-4 and  
270  $\alpha 4\beta 7$ ) to LCMV-OVA memory phase mice containing  $T_{PLN-M}$  and  $T_{RM}$  (**Fig. 5A**). We confirmed that mAbs were  
271 saturating surface integrins at the time point analyzed (**Fig. S4B**). We then followed OT-I T cell motility in PLN  
272 and SMG on  $\geq$  day 30 p.i., using dual surgery 2PM as described above. Integrin blockade significantly lowered  
273  $T_{PLN-M}$  speeds from 11.7 to 8.8  $\mu\text{m}/\text{min}$  (**Fig. S4C**), similar to the decreased cell speeds of CD18-deficient  $T_N$  in  
274 lymphoid stroma (14). In contrast,  $T_{RM}$  speeds and crawling along tissue macrophages remained unaltered by  
275 this treatment (**Fig. 5, B and C; movie S13**). We did not detect Mac-1 (CD11b/CD18) expression on SMG  $T_{RM}$   
276 by flow cytometry, and addition of anti-Mac1 mAb to the integrin blocking mix did not decrease  $T_{RM}$  speeds  
277 or guidance by tissue macrophages (not shown). Similarly, inclusion of blocking mAbs against  $\alpha 1$  and  $\alpha V$   
278 together with  $\alpha L$ ,  $\alpha 4$  and  $\alpha E$  had no impact on  $T_{RM}$  cells speeds or association with macrophages ( $6.5 \pm 2.7$   
279  $\mu\text{m}/\text{min}$ ;  $n = 68$  tracks).

280 Poor surface saturation of blocking anti- $\beta 1$  mAbs on OT-I T cells preempted us to assess the role of  $\beta 1$   
281 integrins for  $T_{RM}$  motility by this approach (not shown). As alternative, we directly administered the  $\beta 1$ -  
282 blocking peptide RGD or the control peptide RAD through the Wharton's duct (WD) into SMG and followed  
283 its impact on  $T_{RM}$  motility parameters by 2PM. The WD channels saliva from SMG into the oral cavity and can  
284 be used to administer reagents or pathogens through retrograde duct cannulation (43). Control experiments  
285 using WD administration of OVA<sub>257-264</sub> peptide led to instantaneous arrest of  $T_{RM}$  similar to systemic injection,  
286 suggesting efficient peptide permeation of SMG by this route (not shown). While WD injection of either  
287 peptide slightly lowered  $T_{RM}$  speeds, we did not observe any impact on RGD administration on  $T_{RM}$  motility  
288 parameters as compared to control peptide (**Fig. 5D**). This reflected low  $\beta 1$  integrin levels in the interface  
289 between macrophages and  $T_{RM}$  (**Fig. S4D**). Furthermore, E-cadherin levels on macrophages and  $T_{RM}$  were  
290 barely detectable in SMG tissue sections, arguing against a role for this cadherin in mediating close spatial  
291 association with tissue macrophages (**Fig. S4E**).



292 Cytokine-driven chemoattractant production plays a key role for T cell trafficking. Since the CXCR3 ligands  
293 CXCL9 and CXCL10 play a role in T<sub>RM</sub> clustering in SMG (**Fig. 2F**), we co-transferred WT and CXCR3<sup>-/-</sup> OT-I T  
294 cells one day prior to LCMV-OVA infection. Consistent with recent reports (44), we found that absence of  
295 CXCR3 did not impair T<sub>RM</sub> formation in SMG after viral infection. Non-clustered CXCR3<sup>-/-</sup> OT-I T<sub>RM</sub> showed no  
296 significant differences in speeds as compared to WT T<sub>RM</sub> (**Fig. 5E**), and lack of CXCR3 did not prevent T<sub>RM</sub>  
297 patrolling along tissue macrophages (**Fig. 2F**).

298 To comprehensively assess a function for potential chemoattractants, we inhibited G $\alpha$ i signaling by systemic  
299 PTx treatment (45) and performed 2PM analysis of OT-I T cell motility parameters on  $\geq$  day 30 after LCMV-  
300 OVA infection. To control for inhibitor efficacy, we took advantage of the dual surgery of PLN and SMG in the  
301 same recipient. Systemic PTx administration significantly slowed T<sub>PLN-M</sub> down from 11.3  $\mu$ m/min in control  
302 versus 8.6  $\mu$ m/min in PTx-treated recipients (**Fig. S4F**), resembling observations made with PTx-treated T<sub>N</sub> in  
303 PLN (11, 13). Speeds were also decreased in SMG T<sub>RM</sub> (from 6.6 to 5.5  $\mu$ m/min) by PTx treatment (**Fig. 5F**),  
304 suggesting a role for chemoattractants in mediating high T<sub>RM</sub> speeds. Nonetheless, we observed a robust  
305 residual motility and continued T<sub>RM</sub> crawling along tissue macrophages in presence of PTx (**Fig. 5G, movie**  
306 **S14**). These data suggest that while G $\alpha$ i-coupled receptors contribute to SMG T<sub>RM</sub> motility, they are not  
307 required for T<sub>RM</sub> association with tissue macrophages. Finally, since matrix metalloproteinases (MMP) play  
308 roles in cancer cell invasion, we interfered with MMP activity using the broad MMP-9, MMP-1, MMP-2, MMP-  
309 14 and MMP-7 inhibitor marimastat as described (46). Yet, MMP inhibition did not reduce T<sub>RM</sub> migration  
310 compared to vehicle and rather resulted in a minor increase in speeds (not shown). Taken together, with  
311 exception of a minor effect by PTx, the *in vivo* inhibitor treatment examined here did not alter T<sub>RM</sub> motility  
312 and close spatial proximity to tissue macrophages.

313 To directly assess intercellular adhesion between tissue macrophages and T<sub>RM</sub> *ex vivo*, we co-incubated  
314 freshly isolated cells for 20 min and analyzed cluster formation by flow cytometry (**Fig. 5H**). As positive control  
315 for T cell association with tissue macrophages, we pre-incubated macrophages with cognate OVA<sub>257-264</sub>  
316 peptide. While addition of OVA<sub>257-264</sub> to tissue macrophages induced detectable association with T<sub>RM</sub>, baseline  
317 association between both populations remained low (**Fig. 5, H and I**). Finally, we performed under agarose  
318 assays in presence of tissue macrophages. On the few occasions when motile T<sub>RM</sub> contacted co-plated tissue

319 macrophages, these contacts were mostly transient (**Fig. 5, J and K; movie S15**). Furthermore,  $T_{RM}$  did not  
320 crawl along macrophage protrusions as observed *in vivo* (**Fig. 5J**). These data suggested that  $T_{RM}$  association  
321 to macrophages occurred preferentially in the SMG microenvironment. Thus, within the technical limitations  
322 of our experimental approach, our *in vivo* and *in vitro* observations did not identify specific molecules that  
323 provide strong adhesion of SMG  $T_{RM}$  to tissue macrophages. Importantly, our data do not exclude the  
324 presence of unidentified adhesion receptors mediating  $T_{RM}$  association to tissue macrophages *in vivo*.

325

#### 326 *Discontinuous macrophage attachment within SMG*

327 We considered that tissue microanatomy may contribute to the close spatial association between  $T_{RM}$  and  
328 tissue macrophages observed *in vivo*. Based on our observation that *ex vivo* SMG  $T_{RM}$  are able to insert  
329 protrusions into narrow spaces between adjacent structures lacking adhesion between them (**Fig. 4**), we  
330 decided to examine macrophage attachment within SMG applying the super-resolution shadow imaging  
331 microscopy (SUSHI) technique. SUSHI was originally developed to visualize the complex topology of the  
332 extracellular space (ECS) in living brain slices (47). It was used to study dynamic changes in ECS in response  
333 to a hyperosmotic challenge, which leads to cell shrinkage and ECS widening in brain tissue. Here, we adapted  
334 SUSHI imaging to acutely sliced SMG sections, which were superfused with the cell-impermeable fluorescent  
335 dye Calcein (**Fig. 6A**). Steady-state imaging revealed that the interstitium contained more ECS as compared  
336 to the tightly packed epithelium (**Fig. 6, B and C**). We reasoned that SUSHI in combination with hyperosmotic  
337 challenge could be applied to explore attachment between neighboring cells. Performing time-lapse ECS  
338 imaging, we acutely increased the osmolarity to induce cell shrinkage, which led to a strong increase of ECS  
339 in the interstitium (**movie S16**). In turn, interepithelial junctions remained relatively stable and only mildly  
340 increased their spacing under osmotic challenge, reflecting the presence of adherens and tight junctions  
341 known to link epithelial cells (**Fig. 6D**). In contrast, hyperosmolarity induced intraepithelial CD11c-YFP<sup>+</sup>  
342 macrophages detachment from the adjacent epithelium (**Fig. 6, E and F**). This observation confirms previous  
343 reports that tissue macrophages do not form continuous adhesive contacts with the epithelium, unlike the  
344 extensive cell-to-cell contacts between acinar epithelial cells (48).

345 We then investigated the spatial arrangement of macrophage protrusions with regard to epithelial BM  
346 markers. The laminin ligand CD49f ( $\alpha 6$ ) was prominent on the basal side of acini and to a lesser extent on  
347 ducts, which were identified by the presence of the tight junction protein ZO-1 on the luminal side. In some  
348 cases, tissue macrophages appeared to cross adjacent acini and ducts via their protrusions (**Fig. 6G**). For a  
349 detailed examination, we analyzed laminin-stained tissue sections from immunized CD11c-YFP mice. We  
350 observed in some cases macrophage protrusions penetrating between adjacent acini, or between epithelium  
351 and connective tissue, thus bridging adjacent compartments separated by BM (**Fig. 6H; movie S17**). Using  
352 correlative confocal and transmission electron microscopy, we validated that some macrophage protrusions  
353 transversed BM (**Fig. 6I**). Taken together, our data support the notion of discontinuous attachment of tissue  
354 macrophages to neighboring cells and occasional penetration of macrophage protrusions across the  
355 epithelial BM.

356

### 357 *Depletion of tissue macrophages disrupts $T_{RM}$ patrolling*

358 Our observations prompted us to examine  $T_{RM}$  motility in the absence of tissue macrophages. To this end,  
359 we generated bone marrow chimera by reconstituting C57BL/6 or Ubi-GFP mice with control CD11c-YFP or  
360 CD11c-DTR bone marrow. At 6 weeks of reconstitution, we adoptively transferred GFP<sup>+</sup> or DsRed<sup>+</sup> OT-I T cells,  
361 followed by LCMV-OVA infection. In some experiments, we directly transferred OT-I T cells into CD11c-DTR  
362 mice and infected mice with LCMV-OVA. Both approaches allowed us to deplete CD11c<sup>+</sup> macrophages by  
363 diphtheria toxin (DTx) treatment in the memory phase without affecting the unfolding of the adaptive  
364 immune response. Macrophage depletion in the memory phase had no impact on CD45<sup>+</sup> and OT-I T cell  
365 numbers recovered from spleens and SMG up to one week after DTx treatment (not shown).

366 2PM imaging in DTx-treated mice revealed that SMG  $T_{RM}$  patrolling behavior was disrupted when  
367 macrophages were depleted (**Fig. 7A; movie S18**).  $T_{RM}$  motility was decreased, reflected by less displacement  
368 (**Fig. 7B**) and slower speeds (**Fig. 7C**). Also, we occasionally observed cells that returned and migrated back  
369 the same path within acini and ducts after macrophage depletion (**Fig. 7A**). To quantify this behavior, we  
370 developed a method to specifically retrieve U-turns from track parameters (**Fig. 7D**). This analysis confirmed  
371 that the percent of T cell tracks showing U-turns was doubled in DTx-treated CD11c-DTR SMG from 8.1 to

372 16.7 % of tracks (**Fig. 7E**). For comparison, PTx treatment had essentially no impact on U-turn frequency (1.15  
373 fold increase as compared to PTx<sub>mut</sub>). We observed a similar impact of macrophage depletion on T<sub>RM</sub> speeds  
374 in LG (from  $7.6 \pm 4.3$  to  $5.5 \pm 3.2$   $\mu\text{m}/\text{min}$ ), with a 2.5 fold increase in U-turns (**Fig. S5**).

375 We next asked how impaired motility impacts organ surveillance. We generated tracks *in silico* from the data  
376 sets obtained by 2PM imaging of DTx- and control-treated SMG and assessed the average T<sub>RM</sub> dwell time in  
377 a sphere of 80  $\mu\text{m}$  diameter as surrogate epithelial structure (**Fig. 7D**). This analysis uncovered a nearly  
378 threefold increased sphere dwell time from  $24 \pm 1.8$  min for control SMG to  $69 \pm 6.5$  min (median  $\pm$  SEM) for  
379 DTx-treated CD11c-DTR SMG (**Fig. 7F**). For comparison, sphere dwell time was increased from  $31 \pm 1.8$  min  
380 in PTx<sub>mut</sub>-treated to  $46 \pm 2.45$  min for PTx-treated SMG. Taken together, macrophage depletion disrupted  
381 motility parameters and increased the propensity of T<sub>RM</sub> to make U-turns.

382 We investigated whether lack of macrophages may also affect T<sub>RM</sub> transitions into and out of epithelium as  
383 part of the impaired motility pattern. To address this point, we developed an approach to optically separate  
384 epithelial from connective tissue. We reconstituted irradiated Ubi-GFP mice expressing GFP in all cells with  
385 CD11c-YFP bone marrow before transfer of DsRed<sup>+</sup> OT-I T cells and systemic LCMV-OVA infection. We found  
386 that in these chimera, acini and ducts of surgically prepared SMG were GFP<sup>bright</sup> and readily identifiable by  
387 their glandular shapes, whereas connective tissue was GFP<sup>low</sup>. Using case-by-case 3D rendering of 2PM image  
388 sequences in memory phase ( $\geq 30$  days p.i. with LCMV-OVA), we observed that DsRed<sup>+</sup> T<sub>RM</sub> were not  
389 restricted to individual epithelial structures but occasionally crossed between adjacent acini or between  
390 epithelial and connective tissue compartments in a bidirectional manner along macrophage protrusions (**Fig.**  
391 **7G top; movie S19**). We confirmed this observation in a mouse model expressing membrane tomato and  
392 CD11c-YFP (**Fig. 7G bottom**). In total, 75% of T<sub>RM</sub> transits (n = 42) into and out of epithelial structures occurred  
393 along macrophage protrusions (**Fig. 7H**). Given that not all tissue macrophages are YFP<sup>+</sup> (**Fig. S3B**), the actual  
394 percentage of macrophage-assisted transitions may still be higher. DTx treatment of CD11c-DTR SMG  
395 reduced, but did not abolish, T<sub>RM</sub> transit into or out of acini and ducts. In total, we observed 55 T<sub>RM</sub> crossing  
396 events into or out of acini in CD11c-YFP versus 12 events in CD11c-DTR chimera SMG. These data  
397 corresponded to a 77% fewer crossing events per h track duration and a 71% fewer transitions per 1000  $\mu\text{m}$

398 track length in macrophage-depleted SMG (**Fig. 7, I and J; movie S18**). Reduced  $T_{RM}$  crossing into and out of  
399 epithelial structures was also observed when we prolonged DTx treatment for 5 days (**movie S20**).

400

#### 401 *Impaired intraorgan accumulation of SMG $T_{RM}$ after macrophage depletion*

402 Tissue macrophages are best characterized for their core functions of maintenance or restoration of tissue  
403 homeostasis by engulfing apoptotic cells (efferocytosis), clearing debris and initiation of repair (49-52).

404 Accordingly, we observed massively increased numbers of infected cell foci in macrophage-depleted SMG  
405 after WD infection with murine cytomegalovirus expressing OVA and mCherry (53), as compared to SMG

406 containing tissue macrophages (**Fig. S6, A and B**). The efferocytic function of tissue macrophages was  
407 independent of the presence of  $T_{RM}$  (**Fig. S6C**), although the latter partially suppressed viral replication as

408 assessed by decreased mCherry intensity in viral foci (**Fig. S6D**). In support of this, we observed CD11c-YFP<sup>+</sup>  
409 cells engulfing MCMV-infected cells after SMG infection (**Fig. S6, E and F**). These observations preempted the

410 use of a viral rechallenge model to assess a function for tissue macrophages in facilitating  $T_{RM}$  patrolling.

411 We therefore designed an alternative experiment to assess the support of SMG macrophages for  $T_{RM}$   
412 surveillance and local cluster formation. We treated LCMV-OVA-immunized CD11c-YFP and CD11c-DTR BM

413 chimera mice with DTx, followed one day later by local injection of the CXCR3 ligand CXCL10 into SMG (**Fig.**  
414 **7K**). We also administered anti- $\alpha 4$  and LFA-1 blocking mAbs that block recruitment of circulating T cells to

415 SMG (44) but do not affect  $T_{RM}$  motility in this organ (**Fig. 5B**). At 4 h after CXCL10 administration, we isolated  
416 SMG and quantified  $T_{RM}$  enrichment in thick confocal SMG sections according to the area marked by the co-

417 injected fluorescent marker. CXCR3<sup>-/-</sup> OT-I T cells did not show accumulation in CXCL10 injection sites,  
418 supporting the specificity of chemokine-triggered clustering (49 - 63 > 500  $\mu\text{m}$  versus 60 - 68 cells/cm<sup>2</sup> cells

419 < 500  $\mu\text{m}$  from injection site; range from two SMG each). WT OT-I  $T_{RM}$  were twofold enriched at CXCL10  
420 injection sites, suggesting that these cells had followed a CXCL10 gradient or became retained during their

421 surveillance path (**Fig. 7L**). In contrast, local accumulation of  $T_{RM}$  was lost when macrophages had been  
422 depleted, although  $T_{RM}$  numbers outside the site of chemokine injection remained comparable to

423 macrophage-containing SMG (**Fig. 7L**). These data suggest a key role for SMG macrophages to assist  $T_{RM}$   
424 patrolling within and between epithelial structures and to cluster at local inflammatory sites (**Fig. S7**).

425 **Discussion**

426 After clearing of pathogens,  $T_{RM}$  display a remarkable capacity to patrol heterogeneous tissues without  
427 impairing vital organ functions (16-20). Their scanning behavior evolved because T cells are MHC-restricted  
428 and hence need to physically probe membrane surfaces of immotile stromal cells. The key point of this study  
429 was to examine how these cells achieve this feat in the complex arborized epithelial structure of SMG during  
430 homeostatic immune surveillance. Our main finding is that  $T_{RM}$  mostly moved along tissue macrophages, and  
431 that depletion of macrophages impaired  $T_{RM}$  patrolling. These observations assign a new accessory role to  
432 tissue macrophages in addition to their core functions for tissue homeostasis and sentinels of infection. Our  
433 data suggest two non-exclusive options to explain macrophage guidance of  $T_{RM}$ : first, through unidentified  
434 specific adhesive interaction(s) independent of ICAM-1 and other adhesion molecules; and second, by  
435 offering paths of least resistance within the exocrine gland microenvironment for protrusion insertion by  
436 autonomously moving T cells. Our data provide evidence for the second option without discarding the first  
437 one. Reductionist *in vitro* experiments revealed that SMG  $T_{RM}$  respond to exogenous cues from  
438 chemoattractant and adhesion molecules. Remarkably, confinement alone suffices to trigger friction- and  
439 protrusion insertion-based motility without exogenous chemoattractants or adhesion molecule. We  
440 speculate that the continuum of intrinsic motility and integration of external factors permits  $T_{RM}$  to patrol  
441 these exocrine glands in homeostasis and rapidly respond to inflammatory stimuli.

442 Macrophages and T cells closely cooperate during the onset of inflammation, the effector phase and  
443 contraction through antigen presentation, cytokine secretion and effector functions such as phagocytosis.  
444 Yet, little is known whether and how these two cell types collaborate for surveillance of NLT during  
445 homeostasis. Tissue macrophages are best characterized for their core function of maintenance or  
446 restoration of tissue homeostasis by engulfing apoptotic cells, clearing debris and initiation of repair (49-52,  
447 54). A recent study has identified a role for tissue macrophages for cloaking of microlesions (55), a behavior  
448 we also observed in SMG after local laser injury (not shown). Tissue macrophages also serve as sentinels of  
449 infection, leading to cytokine secretion and leukocyte recruitment (16, 56, 57). In recent years, several non-  
450 phagocytic and non-sentinel functions were assigned to macrophages, as core functions of parenchymal  
451 parts of organs are outsourced to accessory cells. Accessory macrophage functions include blood vessel and

452 mammary duct morphogenesis, hematopoietic stem cell maintenance, pancreatic cell specification, lipid  
453 metabolism, relay of long-distance signals during zebrafish patterning and electric conduction in the heart  
454 (58, 59). Our data suggest a novel accessory function, which is to facilitate T<sub>RM</sub> patrolling within and between  
455 acini and ducts of arborized secretory epithelium.

456 Our initial assumption was that specific adhesion receptors drive T cell association with tissue macrophages,  
457 while chemoattractants fuel their high baseline motility. Tissue macrophage express ICAM-1 and other  
458 adhesion molecules that can serve as ligands for T cell adhesion receptors, as well as chemoattractants (38).  
459 It was therefore startling that - against our initial expectations - we were unable to find evidence for strong  
460 adhesive contacts between salivary gland macrophages and T<sub>RM</sub>. The experimental systems we have used to  
461 address this point encompass *in vivo* inhibition of adhesion receptors in combination with reductionist *in*  
462 *vitro* adhesion assays. Such assays have previously been employed to identify intercellular adhesion through  
463 specific molecular interactions, such as ICAM-1-driven binding between T cells and DCs (60). It is important  
464 to note our data do not rule out the presence of specific adhesive and/or promigratory interactions between  
465 T<sub>RM</sub> and tissue macrophages *in situ*. For instance, low T<sub>RM</sub> binding to tissue macrophages *in vitro* may be owing  
466 to altered gene expression patterns after macrophage isolation (61). Along the same line, we have not  
467 examined talin-deficient T cells that lack functional integrins, and poor surface mAb saturation preempted  
468 an analysis of CD44 for SMG T<sub>RM</sub> motility (62). Of note, PTx treatment induced a minor but significant  
469 reduction in T<sub>RM</sub> speeds *in vivo*. Yet, PTx treatment had essentially no impact on U-turn frequency and  
470 movement along tissue macrophages. In line with this, recent observations suggest that guidance and  
471 adhesion do not necessarily correlate, as T<sub>N</sub> migrate along the FRC network even in the absence of LFA-1 and  
472 CCR7 (15). The influence of the physical properties of the microenvironment is increasingly recognized to  
473 play a central role for decision-taking by migrating leukocytes (63, 64). Yet, technical limitations in recreating  
474 the complex tissue microenvironment of exocrine glands under controlled *in vitro* conditions limit the  
475 experimental scope to address this issue in a definite manner.

476 The canonical model of leukocyte migration postulates chemoattractant-stimulated F-actin polymerization  
477 at the leading edge (4). The resulting retrograde F-actin flow in turn generates traction and cell body  
478 translocation via an integrin “clutch” that binds to adhesion receptors of the ECM or on the surface of



479 neighboring cells. Although integrin-independent migration in 3D matrices has become a widely accepted  
480 concept in cell biology based on studies with cell lines and DCs (40), several studies uncovered integrin  
481 involvement during immune surveillance of skin T cells (65, 66). Thus, it remained unclear to which extent  
482 integrin-free motility occurs in primary lymphocytes, which contain less cytoplasm and surface area as  
483 compared to DCs and cell lines. Another open question was whether memory T cells from distinct anatomical  
484 locations would employ similar or tissue-specific mechanisms of host surveillance. Work by Zaid et al. has  
485 identified a critical role of G-protein-coupled receptor signaling during scanning by epidermal T<sub>RM</sub> (66). Our  
486 own observations confirm that similar to T<sub>N</sub> and T<sub>PLN-M</sub>, *ex vivo* confined epidermal T<sub>RM</sub> do not migrate in the  
487 absence of integrin ligands or chemoattractants (not shown). Spontaneous motility under 2D confinement  
488 appears to constitute therefore a distinctive hallmark of SMG T<sub>RM</sub> not shared by other resting T cells. Isolated  
489 T<sub>RM</sub> showed high intrinsic protrusive activity *in vitro*, which may reflect high F-actin turnover and/or increased  
490 Rho-ROCK-mediated actomyosin contractility. In fact, low adhesiveness under confinement induces  
491 spontaneous amoeboid motility via cortical contractility in adherent mesenchymal cell lines (67, 68),  
492 suggesting that T<sub>RM</sub> may use a similar mechanism for autonomous migration *in vitro* and *in vivo*. Yet, it  
493 remains currently unknown how this unique motility program is imprinted in SNG T<sub>RM</sub> and whether it is shared  
494 by tissue-resident cells from other exocrine glands.

495 Adhesion-free motility in 2D conditions has been proposed for large, blebbing carcinoma cells, based on  
496 friction mediated by a large interface between migrating cells and substrates (69). We show that 2D  
497 confinement suffices to induce T<sub>RM</sub> motility through cation-dependent friction, since these cells become  
498 unable to translocate their cell bodies in presence of EDTA. Friction is composed of multiple nanoscale forces  
499 between two interfaces. For instance, electrostatic and van der Waals forces have been implicated in cell  
500 migration and non-specific adherence to substrate (63, 70, 71). As chelation of bivalent cations reduced  
501 friction below a threshold for cell translocation in our setting, electrostatic forces are likely to be relevant. In  
502 principle, cells may compensate for a lower friction by increasing the contacting surface area (72). However,  
503 lymphocytes are likely too small to generate a sufficiently large interface under these conditions. In turn, T<sub>RM</sub>  
504 regained the capability to translocate in presence of EDTA when narrow spaces are created by immotile  
505 neighboring cells or beads that lack strong adhesion to each other. This motility mode correlated with



506 continuous changes in cell shapes owing to the intrinsic protrusion formation capacity of  $T_{RM}$ . Thus,  $T_{RM}$   
507 continuously formed multiple simultaneous protrusions that probed the environment, leading to their  
508 insertion into permissive gaps and subsequent cell body translocation. How  $T_{RM}$  protrusions generated  
509 tractive force for cell translocation under these conditions remains incompletely understood. One possibility  
510 is that protrusions insert into gaps of the 3D environment akin to cogs of a cogwheel and transmit the  
511 necessary force for translocation through retrograde actin flow along irregularly shaped surfaces, even in the  
512 absence of adhesion receptors. In fact, this translocation mode is reminiscent of the “squeezing and flowing”  
513 mechanism proposed for DCs (73), although SMG  $T_{RM}$  do not require a chemokine gradient for displacement.  
514 We also observed that  $T_{RM}$  avoided areas of high bead density, thus choosing the path of least resistance in  
515 this mode.

516 The efferocytic function of tissue macrophages conceivably requires physical contact with surrounding cells  
517 to detect and phagocytose senescent or infected cells. Since salivary gland macrophages do not form  
518 continuous tight and adherens junctions with neighboring cells (48), these cells may create a path of least  
519 resistance for patrolling  $T_{RM}$ . We speculate that the flexible anchorage of macrophage protrusions between  
520 epithelial cells may facilitate the insertion of F-actin-rich pseudopods by  $T_{RM}$  before squeezing of the nucleus  
521 as biggest organelle (40, 42, 74).  $T_{RM}$  migration along macrophages may be further assisted by unknown  
522 adhesion receptors or other molecular interactions between these cells. In any event, the non-proteolytic  
523 path finding is beneficial to preserve the integrity of the target tissue, as it does not require constant repair  
524 of newly generated discontinuities in the ECM (75). The scanning strategy adopted by  $T_{RM}$  resembles the  
525 migration pattern of T cell blasts in 3D collagen networks, where these cells routinely bypass dense collagen  
526 areas, while probing the environment for permissive gaps for cell body translocation (76). In fact, leukocytes  
527 have recently been shown to use the nucleus to identify the path of least resistance in complex 3D  
528 environments with different pore sizes (64). This migration mode preserves tissue integrity is energetically  
529 favorable by avoiding ECM degradation.

530 Reflecting the multiple functions of tissue macrophages, depletion studies make the interpretation of the  
531 physiological function of macrophage-assisted  $T_{RM}$  surveillance of SMG experimentally difficult to dissect. As  
532 example, when we locally infected macrophage-depleted SMG with MCMV, we observed massively increased

533 numbers of viral foci as compared to control SMG owing to a lack of efferocytosis. Our local CXCL10  
534 deposition experiment in combination with impaired recruitment of circulating T cells suggests that  
535 macrophages facilitate local  $T_{RM}$  accumulation at sites where inflammatory chemokines are produced. This  
536 resembles observations made in skin infection models where CXCR3 promotes  $CD8^+$  T cell accumulation at  
537 sites of viral replication necessary for efficient elimination of infected cells (21, 77). A recent study by Förster  
538 and colleagues has uncovered an unexpectedly low killing rate of cytotoxic T cells against viral-infected  
539 stromal cells (78). Thus, effective stromal cell elimination requires cooperativity through repeated cytotoxic  
540 attacks by multiple  $CD8^+$  T cells. Conceivably, the promigratory accessory function of tissue macrophages  
541 described here helps to cluster a quorum of  $T_{RM}$  for successful stromal cell killing. Furthermore, unlike the  
542 monoclonal  $T_{RM}$  population created in our experimental setting, not all  $T_{RM}$  recognize the same pathogen  
543 under physiological conditions. This might impose a requirement for T cells to scan local sites of pathogen  
544 re-emergence and to form clusters for timely elimination of fast-replicating microbes.

545 In sum, our data assign a previously unnoticed interplay between tissue-resident innate and adaptive  
546 immune cell populations. These findings further suggest a noticeable capacity of SMG  $T_{RM}$  to integrate a  
547 continuum of intrinsic and external signals, friction and 3D structures for efficient motility, providing these  
548 cells with maximal flexibility for NLT surveillance. We propose that such a mode of tissue patrolling is ideally  
549 adapted to the arborized epithelial architecture of exocrine glands by permitting homeostatic surveillance  
550 while maintaining responsiveness to local inflammatory cues.

551 **Materials and Methods**

552 *Mice*

553 OT-I TCR (34) and P14 TCR transgenic mice (36) were backcrossed to Tg(UBC-GFP)30Scha “Ubi-GFP” (79) or  
554 hCD2-dsRed (80) mice. Ubi-GFP (GFP<sup>+</sup>) OT-I mice backcrossed to CXCR3<sup>-/-</sup> mice have been described (81).  
555 Tg(Itgax-Venus)1Mnz CD11c-YFP (82) and Tg(Itgax-DTR/EGFP)57Lan CD11c-DTR mice were used as recipients  
556 or bone marrow donors for lethally irradiated C57BL/6 or Ubi-GFP mice. C57BL/6 mice were purchased from  
557 Janvier (AD Horst). All mice were maintained at the Department of Clinical Research animal facility of the  
558 University of Bern, at the Theodor Kocher Institute and the University of Fribourg. All animal work has been  
559 approved by the Cantonal Committees for Animal Experimentation and conducted according to federal  
560 guidelines.

561

562 *T cell transfer and viral infections*

563 CD8<sup>+</sup> T cells were negatively isolated from spleen, peripheral and mesenteric lymph nodes of GFP<sup>+</sup> or dsRed<sup>+</sup>  
564 OT-I or GFP<sup>+</sup> P14 mice, using the EasySep™ Mouse CD8<sup>+</sup> T cell Isolation Kit (Stem Cell Technologies). CD8<sup>+</sup> T  
565 cell purity was confirmed to be > 95% by flow cytometry prior to cell transfer. 10<sup>4</sup> OT-I T cells were i.v.  
566 transferred into recipient mice 24 h before i.p. infection with 10<sup>5</sup> pfu LCMV-OVA (33). Experimental read-  
567 outs for the acute, cleared and memory phase of viral infection were performed 6, 15 and ≥ 30 days p.i.,  
568 respectively.

569

570 *LCMV virus titer*

571 C57BL/6 mice were infected i.p. with 10<sup>5</sup> pfu LCMV-OVA and sacrificed 3 or 5 days later. PLN, spleens and  
572 SMG were harvested and organs were snap frozen in liquid nitrogen. Recombinant LCMV-OVA infectivity was  
573 measured by immunofocus assay on MC57 cells as previously described <sup>96</sup>.

574

575 *Antibodies and reagents*

576 Alexa633-conjugated anti-PNAd MECA79, αL-integrin FD441.8 and anti-α4-integrin PS/2 mAbs were from  
577 nanotools (Freiburg, Germany). Anti-α1-integrin Ha31/8 was from BD Bioscience, anti-α4 integrin PS/2 and

578 anti- $\alpha$ E integrin M290 were from BioXCell, anti- $\alpha$ V integrin RMV-7 was from BioLegend, and anti-Mac1 mAb  
579 M1/70 was purified from hybridoma supernatant. TexasRed-Dextran 70 kDa was from Molecular Probes.  
580 Cascade Blue (MW 10 kDa) was purchased from Invitrogen. TRITC-Dextran (MW 70 kDa) and Diphtheria Toxin  
581 whereas purchased from Sigma. Pertussis toxin (PTx) and enzymatically inactive mutant PTx (PTx<sub>mut</sub>) were  
582 obtained from List Biological Laboratories. Sodium Pyruvate (100 mM; #11360-039), HEPES buffer (1M;  
583 #15630-056), Minimum essential Medium Non-essential amino acids (MEM NEAA, #11140-035), L-Glutamine  
584 (200 mM; #25030-024), PenStrep (#15140-122) and RPMI-1640 (#21875-034) were purchased from Gibco  
585 and Fetal Bovine Serum (FCS, #SV30143.03) was purchased from HyClone.

586

### 587 *Flow cytometry analysis*

588 PLN and spleen were harvested at the indicated time points and single cell suspensions were obtained by  
589 passing organs through cell strainers (70  $\mu$ m; Bioswistec). Red blood cell lysis was performed on splenocytes  
590 in some experiments. For analysis of SMG and LG, organs were minced and treated with 2 U/ $\mu$ l collagenase  
591 II (Worthington Biochem), 2 U/ $\mu$ l bovine DNase I (Calbiochem) and - only for intracellular stainings of  
592 cytokines – 5  $\mu$ g/ml Brefeldin A (B6542, Sigma-Aldrich) in CMR (RPMI/10% FCS/1% HEPES/1% PenStrep/2  
593 mM L-Glutamine/1 mM Sodium Pyruvate) for 30 min at 37°C, passed through a 70  $\mu$ m cell strainer and  
594 washed with PBS/5 mM EDTA. We used following reagents for flow cytometry:

Antibody	clone	company	Order number
anti-CD3-APC	145-2C11	Biolegend	100312
anti-CD8a-PE	53-6.7	BD Biosciences	553033
anti-CD8a-PerCP	53-6.7	Biolegend	100732
anti-CD8a-APC/Fire750	53-6.7	Biolegend	100766
anti-CD11a-PE	M17/4	Biolegend	101107
anti-CD11b-PE	M1/70	BD Biosciences	553311
anti-CD11c-APC	HL3	BD Biosciences	550261
anti-CD18-PE	M18/2	Biolegend	101407

anti-CD29-PE	HMβ1-1	Biologend	102207
anti-CD44-PE	IM7	BD Biosciences	553134
anti-CD45-PerCP	30-F11	BD Bioscience	557235
anti-CD45-BV711	30-F11	Biologend	103147
anti-CD45.1-AF488	A20	Biologend	110718
anti-CD45R/B220-APC	RA3-6B2	Biologend	103212
anti-CD49a-PE	HMα1	Biologend	142603
anti-CD49b-Biotin	DX5	Biologend	108903
anti-CD49d-PE	PS/2	Southern Biotech	1520-09L
anti-CD51-PE	RMV-7	Biologend	104105
anti-CD64-AF647	X54-517	BD Bioscience	558539
anti-CD69-PE	H1.2F3	Biologend	104508
anti-CD103-APC	2E7	Biologend	121414
anti-CD103-Biotin	M290	BD Bioscience	557493
anti-β7-integrin-Biotin	FIB504	Biologend	321209
anti-F4/80-FITC	BM8	Biologend	123108
anti-F4/80-APC	BM8	Biologend	123116
anti-KLRG1-PE	2F1	BD Bioscience	561621
anti-KLRG-1-PE-Cy7	2F1	Biologend	138415
anti-KLRG1-APC	2F1	Biologend	138411
anti-NK1.1-APC	PK136	Biologend	108710
anti-Siglec-F-PE	E50-2440	BD Bioscience	562068
Streptavidin-APC	-	Biologend	405207
anti-rat IgG1 K-APC	-	Biologend	400412
armenian hamster IgG-PE	-	Biologend	400907

595 Single cell suspensions were stained for surface antigens on ice for 30 min with the indicated antibodies and  
 596 washed in FACS buffer (FB; PBS/2% FCS/1 mM EDTA) or FB with 5 µg/ml Brefeldin A for intracellular cytokine

597 stainings. All sample were washed in FB after staining, and for intracellular stainings, cells were permeabilized  
598 and fixed in Cytofix/Cytoperm (#51-2090KZ, BD Biosciences) for 20 min on ice. Fixative was removed by  
599 washing with Perm/Wash buffer (#51-2091KZ, BD Biosciences) and subsequent intracellular staining steps  
600 were performed in Perm/Wash buffer. Cells were washed again prior to acquisition and at least  $10^5$  cells in  
601 the lymphocyte FSC/SSC gate were acquired using a FACSCalibur (BD Bioscience), LSR II (BD Bioscience), LSR  
602 II SORP Upgrade (BD Bioscience) or Attune NxT Flow cytometer (ThermoFisher). Total cell counts were  
603 obtained by measuring single cell suspensions in PKH26 reference microbeads (Sigma) for 1 min at high  
604 speed. Gating for CD103<sup>+</sup> and KLRG1<sup>+</sup> was set according to isotype controls. For CD69 staining, positive and  
605 negative gates were set according to distinguishable populations and FMO was subtracted from the final %  
606 of CD69<sup>+</sup> cells as background.

607

#### 608 *Immunofluorescence*

609 Mice were anesthetized with i.p. injection of ketamine and xylazine and perfused with ice-cold 1% PFA.  
610 Organs were harvested and fixed overnight in 2% PFA prior to embedding in TissueTek O.C.T. compound  
611 (Sakura) for cryostat sectioning or 5% low-melting-point agarose (Sigma) for vibratome (Microslicer™ DTK-  
612 1000) sectioning. 6  $\mu$ m-thick frozen cryostat sections were permeabilized, blocked and stained with 0.05%  
613 Triton-X 100 in 5% skimmed milk or 0.05% Tween 20 and 3% BSA for 1h, washed 3 times with PBS/1%  
614 BSA/0.05% Tween (TBPBS) and stained with goat-anti-Iba1 1/200 (ab5076, Abcam) and anti-phosphotyrosine  
615 (pTyr) (ab179530, Abcam) for 2 h at RT prior to mounting with Fluoromount-G (Electron Microscopy  
616 Sciences).

617 For vibratome sections, 100  $\mu$ m-thick section were collected in a 48-well plate and blocked with TBPBS for 2  
618 h, then blocked with F<sub>c</sub>-block o.n. at 4°C (hybridoma supernatant; 2.4 mg/ml diluted 1/800 in TBPBS). After  
619 washing once with TBPBS for 1 h, sections were stained in TBPBS for 2-3 days at 4°C (in 100  $\mu$ l, 3 sections per  
620 well) with Alexa647-conjugated anti-EpCAM (1/160 dilution; clone G8.8, 118212, Biolegend), eFluor660-  
621 conjugated anti-E-cadherin (1/200 dilution; clone DECMA-1, 50-3249-1633, eBioscience), polyclonal rabbit  
622 anti-Laminin (1/1000 dilution; Z0097, Dako) or Cy3-conjugated anti- $\alpha$ -smooth muscle cell actin (clone 1A4,  
623 C6198, Sigma). Sections were washed 3 times for 1 h with TBPBS and incubated with secondary Cy3-

624 conjugated anti-rabbit Ig (1/400 in TBPBS; 111-165-144, Jackson Immune Research), then washed 3 times 1  
625 h with TBPBS and one time with PBS. Images were acquired with a Zeiss LSM510 or Leica SP5 confocal  
626 microscope and processed using Adobe Photoshop CS6 and Imaris 8.4.1. We used Imaris software for surface  
627 rendering and channel masking function to separate fluorophores with close emission spectra (i.e. GFP and  
628 YFP).

629

### 630 *2PM image acquisition and analysis*

631 2PM intravital imaging of the popliteal lymph node was performed as described (83). In brief, mice were  
632 anesthetized with ketamine/xylazine/acepromazine. The right popliteal lymph node was surgically exposed.  
633 Prior to recording, Alexa 633-conjugated MECA-79 (10 µg/mouse) was injected i.v. to label HEV. 2PM was  
634 performed with an Olympus BX50WI microscope equipped with a 20X Olympus (NA 0.95) or 25X Nikon (NA  
635 1.0) objective and a TrimScope 2PM system controlled by ImSpector software (LaVisionBiotec). Some of the  
636 image series were acquired using an automated system for real-time correction of tissue drift (84). For 2-  
637 photon excitation, a Ti:sapphire laser (Mai Tai HP) was tuned to 780 or 840 nm. For 4-dimensional analysis  
638 of cell migration, 11 to 20 x-y sections with z-spacing of 2-4 µm (22-64 µm depth) were acquired every 20 s  
639 for 20-60 min; the field of view was 150-350 x 150-350 µm. Emitted light and second harmonic signals were  
640 detected through 447/55-nm, 525/50-nm, 593/40-nm and 655/40-nm bandpass filters with non-descanned  
641 detectors in case of C57BL/6 recipient mice. For CD11c-YFP<sup>+</sup> recipient mice or bone marrow chimera, we used  
642 447/55-nm, 513/20-nm, 543/30-nm and 624/30-nm bandpass filters.

643 For imaging of the SMG, neck and thorax of the mouse were shaved, and residual hair removed with hair  
644 removal cream (Veet). Subsequently, the animal was fixed on its back onto a custom-built SMG imaging stage  
645 and stereotactic holders were attached to the head for stabilization. A 10 x 5 mm piece of skin on the right  
646 side of the neck was excised to expose the right SMG lobe, which was micro-surgically loosened from  
647 surrounding tissue. The right SMG lobe was flipped to the right and gently immobilized in between 2 cover  
648 glasses to minimize motion artifacts from heartbeat and breathing. During the whole operation and imaging  
649 procedure tissue was kept moist. During imaging, the temperature at the SMG was monitored and kept at  
650 37°C by a heating ring. In most experiments, mice were operated twice (for PLN and SMG) in alternating

651 order to directly compare behavior of cells in different organs of the same recipient. Prior to imaging, blood  
652 vessels were labeled by i.v. injection of 400 – 600  $\mu\text{g}$  of 10 kDa Cascade-blue dextran or 70 kDa TexasRed  
653 Dextran. Surgical exposure of the LG was essentially performed as for the SMG, with the mouse fixed on its  
654 left flank onto the custom-built SMG imaging stage and a 10 x 5 mm piece of skin excised between the right  
655 ear and eye of the mouse.

656 Sequences of image stacks were transformed into volume-rendered four-dimensional videos with Volocity  
657 6.0 or Imaris 6.00-9.00 (Bitplane), which was also used for semi-automated tracking of cell motility in three  
658 dimensions. Drift in image sequences was corrected using a MATLAB script recognizing 3D movement in a  
659 reference channel or by using the correct drift function of Imaris. Since our filter set up does not allow  
660 complete separation of GFP and YFP signals, we performed spectral unmixing of GFP and YFP using the Image  
661 J plugin “Spectral\_Unmixing” from Joachim Walter. Cellular motility parameters were calculated from x, y,  
662 and z coordinates of cell centroids using Volocity, Imaris and MATLAB protocols. The motility coefficient, a  
663 measure of the ability of a cell to move away from its starting position, was calculated from the gradient of  
664 a graph of mean displacement against the square root of time. We defined U-turns as the steepest turn over  
665 five steps of a track, if it is over more than 166 degrees and has a skew line distance between the first and  
666 last step smaller than one mean step of the respective track (**Fig. 7D**) to exclude continuous turns. The given  
667 binomial proportion 95% confidence intervals are Wilson Score intervals. We generated 100 synthetic tracks  
668 of 12 h duration for each condition using a sampling strategy, which was designed to preserve the correlation  
669 between velocity and turning angle and the autocorrelation of velocity and turning angle (10). We then took  
670 the first timestep further than 40  $\mu\text{m}$  away from the origin of each track as simulated dwelling time in an  
671 acinus of 80  $\mu\text{m}$  diameter. These analyses were performed using scientific computing packages for Python.  
672 For the image series depicted in the Figures, raw 2PM data was filtered with a fine median filter (3x3x1), and  
673 brightness and contrast were adjusted. Shape factors were determined by rendering and tracking cells in  
674 Imaris, and manually excluding all cells that did not move along a horizontal axis. The signal from the filter  
675 cells was projected into a single z-slice and the shape-factor of the 2D image calculated with Volocity.

676

677 *In vivo inhibitor treatment*



678 Gai signaling by chemokines was blocked as described previously (45). Briefly, mice were treated with 3 µg  
679 PTx or PTx<sub>mut</sub> by i.p. injection 3 h prior to imaging. For depletion of CD11c-positive cells in CD11c-DTR mice  
680 or BM chimera, 4 ng/g Diphtheria Toxin (DTx) was i.p. injected 24 h prior to imaging. Depletion efficiency of  
681 CD11c<sup>+</sup> cells was determined by flow cytometry and was above 98% in all organs analyzed. For the  
682 synchronous blocking of integrins, 100 µg each of the purified mAbs M290, FD441.8, M1/70 and PS/2 were  
683 injected i.v. 16 h prior to imaging. Surface saturation of blocking mAbs in PLN and SMG suspensions was  
684 determined at the end of the experiment by sample staining with or without the same mAb clones used for  
685 blocking, followed by a fluorescently labeled secondary mAb and flow cytometry. RGD peptide or as control  
686 GRADSP (RAD) peptide (SIGMA) was injected via Wharton's duct cannulation (approximately 600 nmol of  
687 either peptide in 30 µl per lobe in PBS), as described previously (43). For this procedure, mice were  
688 anesthetized with ketamine/xylazine and their upper incisors rested on a metal rod and the lower incisors  
689 pulled down with string, which kept the mouth open. With the aid of a stereomicroscope, we located the  
690 orifice of the Wharton's duct in the sublingual caruncle and inserted a pointed glass-capillary (Untreated  
691 Fused Silica Tubing - L × I.D. 3 m × 0.10 mm, #25715, Sigma). The glass capillary was connected to a Hamilton  
692 Micro-syringe (Hamilton) via fine bore polythene tubing (0.28 mm, #800/100/100, Smiths), which allowed  
693 the injection of small volumes. For inhibition of MMPs, Marimastat (#S7156) was obtained from Sellcheck  
694 and diluted in PBS/10% DMSO (0.2 mg/g) or the corresponding volume of PBS/10% DMSO was injected i.p.  
695 90 min before starting imaging (46). OVA<sub>257-264</sub> (#BAP-201) and gp<sub>33-41</sub> (#BAP-206) peptides were obtained  
696 from ECM microcollections and 200 µg/100 µl saline injected i.v. immediately prior to imaging or 6 - 12 h  
697 prior to organ harvest for FACS staining.

698

#### 699 *Viral infection via Wharton's duct cannulation*

700 Wharton's duct cannulation was prepared as described above. Approximately 12500 pfu MCMV-  
701 OVA<sub>mCherry</sub> (85) were injected into the Wharton's duct (WD) of DTx-treated CD11c-DTR or CD11c-YFP mice  
702 in memory phase of LCMV-OVA infection. Mice were euthanized 48 h post infection and SMG tissue fixed in  
703 4% PFA at 4°C for 12 h.

704

705 *Under agarose assays*

706  $T_N$  were isolated from spleen and PLN of a naive mouse using CD8<sup>+</sup> T cell isolation kit from Stemcell. SMG-  
707 derived macrophages were isolated from uninfected CD11c-YFP mice and sorted for CD11c-YFP<sup>+</sup> cells.  $T_{RM}$   
708 and  $T_{CM}$  were isolated from SMG and PLN respectively of > 30 d LCMV-OVA-infected C57BL/6 mice. Single  
709 cells suspension of SMG and PLN were stained with APC-conjugated anti-KLRG1 mAb and sorted for GFP<sup>+</sup> or  
710 DsRED<sup>+</sup> KLRG1<sup>-</sup>  $T_{RM}$  and  $T_{PLN-M}$ , respectively. A 17-mm diameter circle was cut into the center of 60-mm dishes.  
711 The hole was sealed from the bottom part of the dish using aquarium silicone (Marina) and a 24-mm glass  
712 coverslip. After the silicone dried, we overlaid a 5 mm-high ring cut from a 15-ml falcon tube and sealed the  
713 borders with low melting point paraffin. Coverslips were washed with PBS and coated with 3% human serum  
714 albumin (HSA; A1653, Sigma) o.n. at 4°C or for 3 h at 37°C. In some experiments, coverslips were coated with  
715 10 µg/ml fibronectin (11080938001, Roche315-02, PeproTech). Fresh medium was added every 2 days and  
716 macrophages were cultured for 6-7 days. For naïve T cell migration, coverslips were coated with 20 µg/ml  
717 Protein A (6500-10, BioVision) for 1 h at 37°C, washed 3 times with PBS and blocked with 1.5 % BSA for 2 h  
718 at 37°C or o.n. at 4°C. After washing once with PBS, cover glasses were coated for 2 h at 37°C with 100 nM  
719 recombinant ICAM1-Fc (796IC, R&D Systems) and washed 2 times with PBS. Five ml of 2 x HBSS and 10 ml of  
720 2 x RPMI containing 1% HSA for  $T_{RM}$  and  $T_{CM}$  and 20% FBS for macrophage and naïve T cell experiments, were  
721 mixed and heated in a water bath to 56°C. Golden agarose (100 mg; 50152, Lonza) was dissolved and heated  
722 in 5 ml distilled water before adding to the prewarmed medium to give a 1% agarose mix. After cooling to  
723 37°C, 500 µl of the agarose mix was added on top of the coverslip. In some cases, inhibitors were added (200  
724 µg/ml PTx, 5 µg/ml anti-Mac1 mAb, 10 µM RGD or GRADSP, 2.5 mM EDTA, or 5 µg/ml Hoechst (H21492,  
725 Invitrogen). After incubation for 30 min at 4°C, the dish was warmed up to 37°C before adding 1 ml of PBS  
726 outside the ring to prevent agarose drying. We punched a sink hole (diameter approximately 2 mm) at the  
727 side of the agarose. Sorted T cell populations were suspended in RPMI/0.5% HSA and in some cases treated  
728 with 5 µg anti-Mac1, 10 µM RGD or GRADSP, and pelleted in an Eppendorf tube. Cells were resuspended in  
729 the smallest possible achievable volume (ca. 5-10 µl) and 0.3 µl were injected in the opposite side from the  
730 sink hole using a 2.5-µl Eppendorf pipette. In some experiments, polystyrene beads (Sigma-Aldrich, LB30 or  
731 78462) were co-injected with the cells. From the sink hole surplus of medium was collected to confine cells

732 between the agarose and the glass slide. Time-lapse images were taken from the center of the dish using a  
733 Zeiss fluorescent microscope (AxioObserver, Zeiss).

734

#### 735 *Correlative Confocal and Transmission Electron Microscopy*

736 Correlative confocal and transmission electron microscopy (TEM) was carried out as described (86). Briefly,  
737 CD11c-YFP mice were perfused with PBS and SMG were fixed *in situ* by left ventricle injection of 1.5%  
738 glutaraldehyde/2% PFA in 0.1 M sodium cacodylate buffer (pH 7.4). SMG were harvested and immersed in  
739 the same solution for 16 h. Fixed samples were cryoprotected in 30% sucrose prior to embedding in OCT and  
740 freezing. Thirty  $\mu\text{m}$  sections were cut with a CM1520 cryostat (Leica) and collected on Superfrost Plus slides  
741 (Thermo Fisher Scientific). Sections were processed for confocal imaging using PBS as mounting medium to  
742 prevent dehydration. After confocal image acquisition, the coverslips were gently removed and sections  
743 adherent to the slide were processed for TEM as described (86). Briefly, sections were postfixed using the  
744 ferrocyanide-reduced osmium-thiocarbohydrazide-osmium (R-OTO) procedure, *en bloc* stained in 1% uranyl  
745 acetate and dehydrated through increasing concentration of ethanol. Finally, sections were embedded by  
746 overlaying a BEEM capsule filled with Epoxy resin. The BEEM capsules containing the embedded sections  
747 were detached by immersing the slides in liquid nitrogen, leaving the section facing up on the resin block.  
748 The specimens were mounted on a Leica Ultracut UCT and 70-90 nm thick serial sections were collected on  
749 formvar-coated copper slot grids and imaged with a ZEISS Leo912AB Omega fitted with a 2k  $\times$  2k bottom-  
750 mounted slow-scan Proscan camera controlled by the EsivisionPro 3.2 software. Using the florescent confocal  
751 and bright field images, the same areas were relocated in the electron microscope and several images were  
752 acquired through the different serial section. Acquired TEM images were then aligned and overlaid with the  
753 confocal images by means of the eC-CLEM Icy plugin (87).

754

#### 755 *Super-resolution shadow imaging (SUSHI)*

756 After euthanizing mice with CO<sub>2</sub>, submandibular salivary glands (SMG) were isolated from 8-11 weeks old  
757 C57BL6 or CD11c-YFP mice and submerged in ice-cold PBS. SMG were embedded in 4% low gelling agarose  
758 (Sigma), cut in 300  $\mu\text{m}$ -thick transversal slices and submerged in cold complete RPMI medium containing:

759 10% FCS (Hyclone). Slices were left to recover at room temperature for 15-30 min before entering the imaging  
760 chamber of a custom-built 3D-STED microscopy setup (47). First, the positively labelled (YFP) macrophages  
761 were identified at a depth of 20-30  $\mu\text{m}$  below the surface and imaged in STED mode (excitation 485 nm,  
762 depletion 597 nm, objective HC PL APO 63X/1.30 NA, Leica) with the following acquisition parameters: field  
763 of view: 200  $\mu\text{m}$  X 200  $\mu\text{m}$ ; pixel size: 48 nm X 48 nm; pixel dwell time: 30  $\mu\text{s}$ , frame acquisition time: 20 min.  
764 The medium was exchanged in the chamber with the complete RPMI containing 400  $\mu\text{M}$  Calcein dye, which  
765 was allowed for 20 - 30 min to disperse throughout the extracellular space of the tissue. Subsequently, we  
766 acquired a SUSHI image to identify a region of interest around the macrophages. We performed a  
767 hyperosmolar challenge by exchanging the chamber solution (300  $\mu\text{l}$ ) with high osmolar solution (350  
768 mOsm/L), and acquired time lapse images to track changes in ECS topology with a 20-min interval between  
769 the image frames. All image analysis including morphological measurements were done on raw images using  
770 the "Plot Line Profile" function in ImageJ on structures of interest. Brightness and contrast were adjusted  
771 using the "Brightness and Contrast" function in ImageJ. It was applied for illustration purposes only and did  
772 not affect the quantitative analysis. No filtering or any other image processing was applied, other than  
773 inverting the look-up-tables (LUT). The YFP signal was used only to identify macrophages and was not  
774 recorded during subsequent imaging. Only unambiguously recognizable macrophages and ECS were  
775 analyzed. Image analysis was performed on SMG slices from two mice in two independent experiments.

776

#### 777 *Confocal imaging of the p-Tyr signal and quantification*

778 Mice were perfused with PBS containing 4% paraformaldehyde, SMG were isolated and fixed in the same  
779 solution at 4°C for 18 h, followed by at least 5 h dehydration in 30% sucrose. Glands were embedded in OCT  
780 (Tissue-Tek) and cut at a thickness of 6 and 20  $\mu\text{m}$  at the cryostat, flash dried and fixed with 4% PFA for 10  
781 min at room temperature. Sections were permeabilized using 0.2% Triton X-100, blocked in 10% serum of  
782 the secondary antibody and 2% BSA containing PBS and stained with antibodies for 18 h at 4°C in the same  
783 solution, after being washed in PBS and mounted in Prolong Gold containing DAPI (Invitrogen, Carlsbad, CA).  
784 Fluorescence microscopy was performed using the LSM880 confocal microscopes with 40x oil (Plan-  
785 Apochromat 40x 1.3 Oil DIC M27) or 63x oil (Plan-Apochromat 63x 1.3 Oil DIC M27) objectives (Zeiss,

786 Oberkochen, Germany). All images were recorded using sequential excitation. The lack of spectral overlap  
787 was confirmed using single fluorescing specimens and antibody specificity via secondary controls.  
788 Macrophages were identified via iba-1 and the presence and location of p-Tyr signal was quantified in 7 fields  
789 of view using Imaris software. Brightness and contrast were adjusted for each image individually. Gaussian  
790 filters were applied using Imaris software.

791

#### 792 *Chemokine-driven $T_{RM}$ accumulation*

793 After 6 weeks of reconstitution with CD11c-YFP or CD11c-DTR BM, we transferred  $10^4$  GFP<sup>+</sup> OT-I T cells and  
794 infected the day after with  $10^5$  pfu LCMV-OVA. After  $\geq 30$  days p.i., mice were anesthetized one day after i.p.  
795 injection of DTx (4 ng/g) as for 2PM imaging. To block immigration of cells from blood, we treated mice with  
796 integrin blocking antibodies anti- $\alpha$ L (FD441.8) and anti- $\alpha$ 4 (PS/2) (each at 50  $\mu$ g/mouse; nanotools). SMG  
797 was surgically exposed. Using thin glass capillaries as for Wharton's duct injection, we injected 2  $\mu$ l of a 1:1  
798 mix of mCXCL10 (100  $\mu$ g/ml; R&D 466-CR-010) and Qdots<sub>655</sub> (0.16  $\mu$ M; Thermo Fisher Q2152MP), to obtain  
799 a final mCXCL10 amount of 0.5  $\mu$ g per site of injection. After 4 h, we sacrificed the mice and harvest the SMG  
800 for vibratome sectioning. Mosaic images were taken of 100  $\mu$ m-thick sections, and lobes with the highest  
801 Qdot signal were analyzed by transforming the 3D image into extended 2D image, using the Z-projection  
802 function of ImageJ.  $T_{RM}$  density in the surrounding area and injection area (defined as an octagon with 500  
803  $\mu$ m diameter) was calculated using Imaris 8.4.1.

804

#### 805 *Statistical analysis*

806 Two-tailed, unpaired Student's t-test, Mann-Whitney U-test, one-way ANOVA with Dunnett's multiple  
807 comparisons test, Kruskal-Wallis test, or a Wilcoxon rang test was used to determine statistical significance  
808 (Prism, GraphPad). Significance was set at  $p < 0.05$ .

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820

821 **Author contributions**

822 BS, FT and XF performed most experiments with support by LMA and NR. LMA and KI carried out SUSHI  
823 imaging under supervision of UVN. PG carried out computational analysis under supervision of JS. AR and FM  
824 performed correlative electron microscopy of SMG sections under supervision of MI. NP, KAK, FB, DM and  
825 OTF provided vital material and support. SMSF, MSD and CS analyzed human SMG sections. BS, FT, XF and  
826 JVS designed experiments and wrote the manuscript with input from all coauthors.

827

828 **Competing interests**

829 The authors declare no competing interests.

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- 1012

1013 **Figure legends**

1014 **Fig. 1. Dynamic motility parameters of memory CD8<sup>+</sup> T cells in PLN versus SMG.** **A.** Experimental layout for  
1015 CD8<sup>+</sup> T cell analysis in SMG and PLN. **B.** Immunofluorescent sections of GFP<sup>+</sup> OT-I T cells in PLN and SMG in  
1016 memory phase ( $\geq$  day 30 p.i.). Scale bar, 100  $\mu$ m (left panels) and 20  $\mu$ m (right panel). **C.** Time-lapse 2PM  
1017 image sequences showing OT-I CD8<sup>+</sup> T<sub>PLN-M</sub> cell motility in PLN in memory phase ( $\geq$  day 30 p.i.). **D and E.** Time-  
1018 lapse 2PM image sequences showing OT-I CD8<sup>+</sup> T<sub>RM</sub> cell motility in SMG in memory phase ( $\geq$  day 30 p.i.).  
1019 Arrowheads indicate protrusions (**D**) and the arrow indicates squeezing behavior (**E**) of OT-I CD8<sup>+</sup> T<sub>RM</sub>. Scale  
1020 bar in C-E, 10  $\mu$ m. Time in min:s. **F.** Time-coded shapes of exemplary T<sub>PLN-M</sub> and T<sub>RM</sub> tracks. **G.** Shape factor  
1021 distribution of T<sub>PLN-M</sub> and T<sub>RM</sub> with exemplary cell shapes. **H.** Speed frequency distribution of OT-I CD8<sup>+</sup> T cells  
1022 in PLN and SMG. Arrows indicate median values ( $\mu$ m/min). **I.** Arrest coefficient frequency distribution of OT-  
1023 I CD8<sup>+</sup> T cells in PLN and SMG (cut-off < 2.5  $\mu$ m/min). **J.** Mean displacement versus time of OT-I T<sub>PLN-M</sub> (left)  
1024 and T<sub>RM</sub> (right) before and after OVA<sub>257-264</sub> injection with motility coefficients ( $\mu$ m<sup>2</sup>/min). **K.** IFN- $\gamma$  expression  
1025 in OT-I T<sub>PLN-M</sub> and T<sub>RM</sub> 24 h after OVA<sub>257-264</sub> injection (mean  $\pm$  SD). Data in G are from 2-3 independent  
1026 experiments and 3 mice total for each group. Data in H and I are pooled from 5 to 6 mice from 4 independent  
1027 experiments with at least 194 tracks analyzed per organ. Data in J are pooled of 3-4 mice from 2 independent  
1028 experiments. Data in K show one of two independent experiments. Data in G and I were analyzed with Mann-  
1029 Whitney-test and data in H with Student's t-test. \*\*\*, p < 0.001.

1030

1031 **Fig. 2. SMG T<sub>RM</sub> move alongside tissue macrophages.** **A.** Immunofluorescent section showing localization of  
1032 SMG T<sub>RM</sub> adjacent to tissue macrophages (arrows). Scale bars, 1 mm (left), 100  $\mu$ m (middle) and 20  $\mu$ m (right).  
1033 **B.** Percent of SMG T<sub>RM</sub> adjacent to tissue macrophages. Data are pooled from 105 FOV with a total of 3270  
1034 T<sub>RM</sub> and shown as box and whisker graph with 2.5 – 97.5 percentiles. **C.** Correlative light and electron  
1035 microscopy sections (left; confocal image; middle and right, TEM image) showing close spatial association of  
1036 SMG T<sub>RM</sub> and tissue macrophages. M, tissue macrophages; E, epithelial cell; ME, myoepithelial cell; ECM,  
1037 extracellular matrix. Scale bar, 5 (left), 2 (middle) and 1  $\mu$ m (right). **D.** TEM images showing attachment of  
1038 epithelial cells to ECM (top) and through intercellular junctions (white arrows; bottom). Scale bar, 800 nm.  
1039 **E.** 2PM time-lapse image sequence showing overlap of OT-I T<sub>RM</sub> tracks with tissue macrophages in SMG in

1040 memory phase ( $\geq$  day 30 p.i.). Scale bar, 20  $\mu\text{m}$ . Time in min:s. The right panels show the time accumulated  
1041 overlays of images with or without OT-I  $T_{\text{RM}}$ . **F.** Immunofluorescent section of WT and CXCR3<sup>-/-</sup> OT-I T cells  
1042 and macrophages. Magnified image shows association of CXCR3<sup>-/-</sup> OT-I  $T_{\text{RM}}$  to tissue macrophages (arrows).  
1043 Scale bar, 100  $\mu\text{m}$  (left) and 20  $\mu\text{m}$  (right).

1044

1045 **Fig. 3. Confinement induces SMG  $T_{\text{RM}}$  motility through chemokine- and adhesion-mediated signals and**  
1046 **bivalent cation-dependent friction. A.** Experimental layout of under agarose assay. Arrows indicate F-actin  
1047 flow. **B.** Representative  $T_{\text{N}}$  (n = 75) and  $T_{\text{RM}}$  (n = 58) tracks in presence of chemokine and ICAM-1. **C.** Speeds  
1048 of  $T_{\text{N}}$  and  $T_{\text{RM}}$ . Data are presented as Tukey box and whiskers plot. **D.** Time-lapse image sequence showing  
1049  $T_{\text{RM}}$  motility among immotile  $T_{\text{N}}$ .  $T_{\text{RM}}$  displacement shown by segmented line. Scale bar, 20  $\mu\text{m}$ . Time in min:s.  
1050 **E.** Time-lapse image sequence in under agarose plates coated with HSA showing  $T_{\text{PLN-M}}$  (top) and  $T_{\text{RM}}$  (bottom)  
1051 motility. Cell displacement shown by segmented line. Scale bar, 10  $\mu\text{m}$ . Time in min:s. **F.** Representative  $T_{\text{N}}$   
1052 (n = 75),  $T_{\text{PLN-M}}$  (n = 226) and  $T_{\text{RM}}$  (n = 379) tracks. **G.**  $T_{\text{N}}$ ,  $T_{\text{PLN-M}}$  and  $T_{\text{RM}}$  speeds in under agarose plates coated  
1053 with HSA. Numbers indicate percentage of tracks > 3  $\mu\text{m}/\text{min}$  (boxed). Lines indicate median. **H.** Meandering  
1054 index of  $T_{\text{N}}$ ,  $T_{\text{PLN-M}}$  and  $T_{\text{RM}}$  tracks. **I.**  $T_{\text{RM}}$  speeds after treatment with PTx, RGD peptide, anti-Mac1 mAb, or in  
1055 presence of EDTA. Numbers indicate percentage of tracks > 3  $\mu\text{m}/\text{min}$  (boxed). Lines indicate median. **J.**  
1056 Image sequence of  $T_{\text{RM}}$  protrusions in presence of EDTA. Scale bar, 10  $\mu\text{m}$ . **K.** Representative  $T_{\text{RM}}$  cell tracks  
1057 in presence of EDTA (n = 75). **L.** Mean displacement over time of  $T_{\text{RM}}$  tracks. Numbers indicate motility  
1058 coefficients ( $\mu\text{m}^2/\text{min}$ ). Data in C, G, H, I and L were pooled from at least 2 independent experiments each.  
1059 Statistical analysis was performed with unpaired t-test (C) or Kruskal-Wallis with Dunn's multiple comparison  
1060 in G - I (as compared to " $T_{\text{RM}}$ "). \*\*, p < 0.01; \*\*\*, p < 0.001.

1061

1062 **Fig. 4.  $T_{\text{RM}}$  insert protrusions for cell displacement in absence of external chemoattractants and friction. A.**  
1063 Experimental layout. Arrows indicate protrusion direction. **B.** Image sequences of  $T_{\text{RM}}$  within  $T_{\text{N}}$  clusters in  
1064 presence of EDTA. Arrowheads show membrane protrusions; segmented line indicates cell track. Scale bar,  
1065 10  $\mu\text{m}$ . Time in min:s. **C.** Graphical representation of  $T_{\text{RM}}$  inside  $T_{\text{N}}$  cluster (i) or dispersed (ii). **D.**  $T_{\text{RM}}$  track  
1066 speeds according to their location. Numbers indicate percentage of tracks > 3  $\mu\text{m}/\text{min}$  (boxed). Lines indicate

1067 median. **E.** Meandering index of  $T_{RM}$  tracks sorted according to their location. Lines indicate median. **F.** Image  
1068 sequences of  $T_{RM}$  alone (top) and with 7  $\mu\text{m}$  polystyrene beads (bottom) in presence of EDTA. Arrowheads  
1069 show membrane protrusions. Segmented line indicates cell track. Scale bar, 10  $\mu\text{m}$ , time in min:s. **G.**  $T_{RM}$  track  
1070 speeds according to their association with or without beads. Numbers indicate percentage of tracks > 3  
1071  $\mu\text{m}/\text{min}$  (boxed). Lines indicate median. **H.** Meandering index of  $T_{RM}$  tracks sorted according to their location.  
1072 Lines indicate median. Data in D, E, G and H are pooled from 4 - 5 independent experiments. Statistical  
1073 analysis was performed with Mann-Whitney test. \*\*\*,  $p < 0.001$ .

1074

1075 **Fig. 5. Residual *in vivo* SMG  $T_{RM}$  motility during inhibition of  $G\alpha_i$  and integrins.** **A.** Experimental layout. **B.**  
1076 OT-I  $T_{PLN-M}$  and  $T_{RM}$  speeds after combined anti- $\alpha_L$ ,  $\alpha_4$  and  $\alpha_E$  integrin mAb ( $\alpha_{Itg}$ ) inhibition. Arrows indicate  
1077 median values ( $\mu\text{m}/\text{min}$ ). **C.** 2PM image of  $T_{RM}$  – tissue macrophage colocalization in  $\alpha_{Itg}$ -treated SMG.  
1078 Arrows indicate T cell – tissue macrophage contacts. Scale bar, 20  $\mu\text{m}$ . **D.** OT-I  $T_{RM}$  speeds in SMG after WD  
1079 administration of RAD or RGD peptide. **E.** WT and  $CXCR3^{-/-}$  OT-I  $T_{RM}$  speeds in SMG in memory phase ( $\geq$  day  
1080 30 p.i.). Arrows indicate median values ( $\mu\text{m}/\text{min}$ ). **F.** OT-I  $T_{PLN-M}$  and  $T_{RM}$  speeds after systemic treatment with  
1081 active PTx or inactive (mutant) PTx ( $PTx_{mut}$ ). Arrows indicate median values ( $\mu\text{m}/\text{min}$ ). **G.** 2PM image of  $T_{RM}$  –  
1082 tissue macrophage colocalization in PTx-treated SMG. Arrows indicate T cell – tissue macrophage contacts.  
1083 Scale bar, 20  $\mu\text{m}$ . **H.** Flow cytometry plot of mixed  $T_{RM}$  and macrophages. **I.** Quantification of cluster formation  
1084 as shown in H. **J.** Example image sequences showing  $T_{RM}$  in transient contact with macrophages under agarose  
1085 on fibronectin-coated plates.  $T_{RM}$  displacement is shown by segmented line. Scale bar, 50  $\mu\text{m}$ . Time in min:s.  
1086 **K.**  $T_{RM}$  – macrophage contact duration for individual tracks. Data in B, D, E and F are pooled from 2-5  
1087 independent experiments with a total of 2-7 mice with at least 111 tracks per condition and analyzed with  
1088 unpaired Student's t-test. Data in I are pooled from 2 independent experiments and analyzed using unpaired  
1089 Student's t-test. \*\*\*,  $p < 0.001$ .

1090

1091 **Fig. 6. Tissue macrophage attachment in SMG.** **A.** Experimental layout of super-resolution shadow imaging  
1092 (SUSHI) of SMG slices. **B.** Example of SUSHI image for determination of extracellular space. E, epithelium; BV,  
1093 blood vessel. Scale bar, 10  $\mu\text{m}$ . **C.** Overview of ECS signal with SMG epithelium (E) and  $CD11c\text{-YFP}^+$  tissue

1094 macrophages. Scale bar, 10  $\mu\text{m}$ . **D.** Example of epithelial attachment before and after hyperosmotic  
1095 challenge. Arrows show interepithelial junctions. Scale bar, 5  $\mu\text{m}$ . **E.** Example of macrophage detachment  
1096 before and after hyperosmotic challenge. Arrowheads indicate detachment. Scale bar, 5  $\mu\text{m}$ . **F.**  
1097 Quantification of gap size between macrophage and epithelium before and after hyperosmotic challenge. **G.**  
1098 Immunofluorescent SMG section showing macrophages and epithelial cells in acini and ducts (identified by  
1099 luminal ZO-1 labelling). Yellow dashed lines indicate outlines of acini and ducts. Scale bar, 10  $\mu\text{m}$ . **H.** Confocal  
1100 image of SMG section with macrophage protrusions traversing a basement membrane below an epithelial  
1101 acinus (indicated by arrow). Scale bar, 20  $\mu\text{m}$  (overview) and 5  $\mu\text{m}$  (insert). **I.** Electron microscopy image of  
1102 macrophages creating a discontinuation of the basement membrane of an acinus (indicated by arrow).  
1103 Numbers mark two neighboring macrophages. Arrowheads indicate lack of tight adhesion between  
1104 macrophages and neighboring cells. Scale bar, 2  $\mu\text{m}$ . All images are representative of at least 2 independent  
1105 experiments. Data in F were analyzed using a paired t-test. \*\*\*,  $p < 0.001$ .

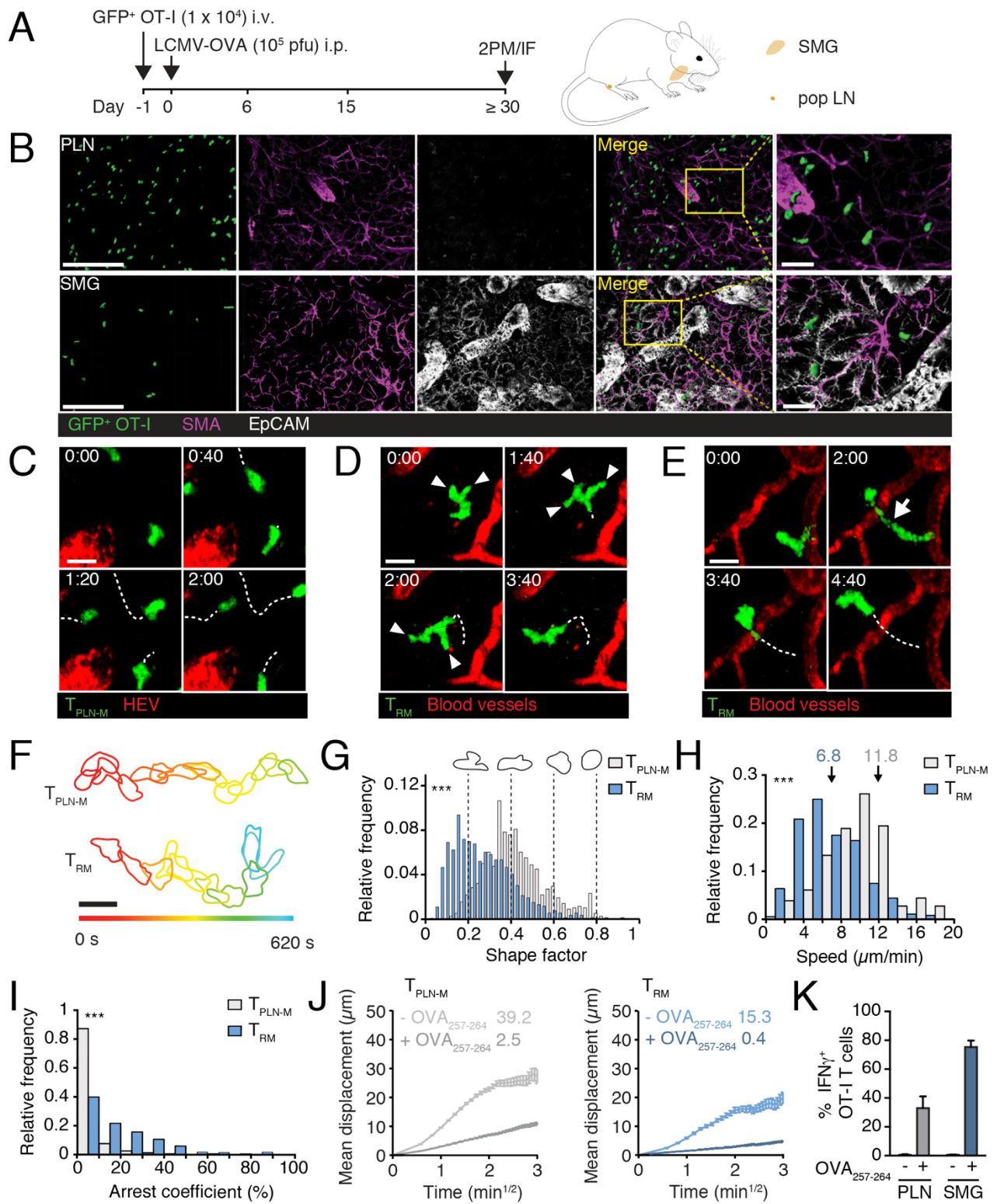
1106

1107 **Fig. 7. Tissue macrophages assist  $T_{RM}$  patrolling of SMG.** **A.** 2PM time-lapse image sequence of  $T_{RM}$  in DTx-  
1108 treated CD11c-YFP or CD11c-DTR  $\rightarrow$  Ubi-GFP chimeras. Magenta lines indicate outlines of acini, white  
1109 segmented lines indicate cell tracks. Scale bar, 50  $\mu\text{m}$  (overview) and 20  $\mu\text{m}$  (insert). Time in min:s. **B.** Example  
1110  $T_{RM}$  tracks in presence or absence of macrophage. Scale bar, 10  $\mu\text{m}$ . **C.** Frequency distribution of  $T_{RM}$  speeds  
1111 in DTx-treated CD11c-YFP or CD11c-DTR bone marrow chimera. Arrows indicate median ( $\mu\text{m}/\text{min}$ ). **D.** Track  
1112 analysis outline. Top panel. U-turns (red) describe tracks reversing direction while excluding continuous  
1113 turns. Bottom panel. Synthetic tracks were generated to assess dwell time in an 80  $\mu\text{m}$ -diameter sphere  
1114 (black). One example track is shown for control (light blue) and macrophage-depleted (dark blue) condition.  
1115 **E.** Percent of tracks making U-turn. Bars indicate 95% confidence intervals. **F.** *In silico* dwell times for  $T_{RM}$   
1116 tracks in 80  $\mu\text{m}$ -spheres based on measured track parameters. **G.** 2PM time-lapse image sequences of  $T_{RM}$   
1117 crawling along a macrophage to enter acini. Epithelial signal was manually masked to show an isolated acinus  
1118 in zoomed panels. Dashed white line indicates area displayed in xz-view, and arrow indicates  $T_{RM}$  -  
1119 macrophage contact. Top: Scale bar, 50  $\mu\text{m}$  (overview) and 20  $\mu\text{m}$  (insert); bottom: Scale bar, 20  $\mu\text{m}$   
1120 (overview) and 10  $\mu\text{m}$  (insert). Time in min:s. **H.** Percentage of  $T_{RM}$  transitions into or out of acini and ducts



1121 in CD11c-YFP -> Ubi-GFP chimeras (n = 42) with and without contact to macrophages. **I, J.** 2PM time-lapse  
1122 image sequence of CD11cYFP -> Ubi-GFP and DTx-treated CD11cDTR->Ubi-GFP chimeras were analyzed for  
1123  $T_{RM}$  crossing events (leaving or entering acini). **I** shows average transitions per hour track duration, and **J**  
1124 depicts transitions per 1000  $\mu\text{m}$  total distance migrated. Data points represent individual image sequences.  
1125 Line indicates mean. **K.** Experimental layout for analysis of  $T_{RM}$  response to local chemokine. CXCL10 was  
1126 injected with a fluorescent tracer for 4 h to allow  $T_{RM}$  accumulation. Integrin blocking mAbs prevent  
1127 recruitment of circulating T cells. **L.**  $T_{RM}$  per  $\text{cm}^2$  at sites of CXCL10 injection in presence or absence of  
1128 macrophages. Numbers indicate mean  $\pm$  SD. Data in C, I, J and L are pooled from 2-4 independent  
1129 experiments with 4-6 mice total. Data in C, I and J were analyzed with Mann-Whitney and data in L were  
1130 analyzed with Wilcoxon rank test. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ .





1131

Figure 1

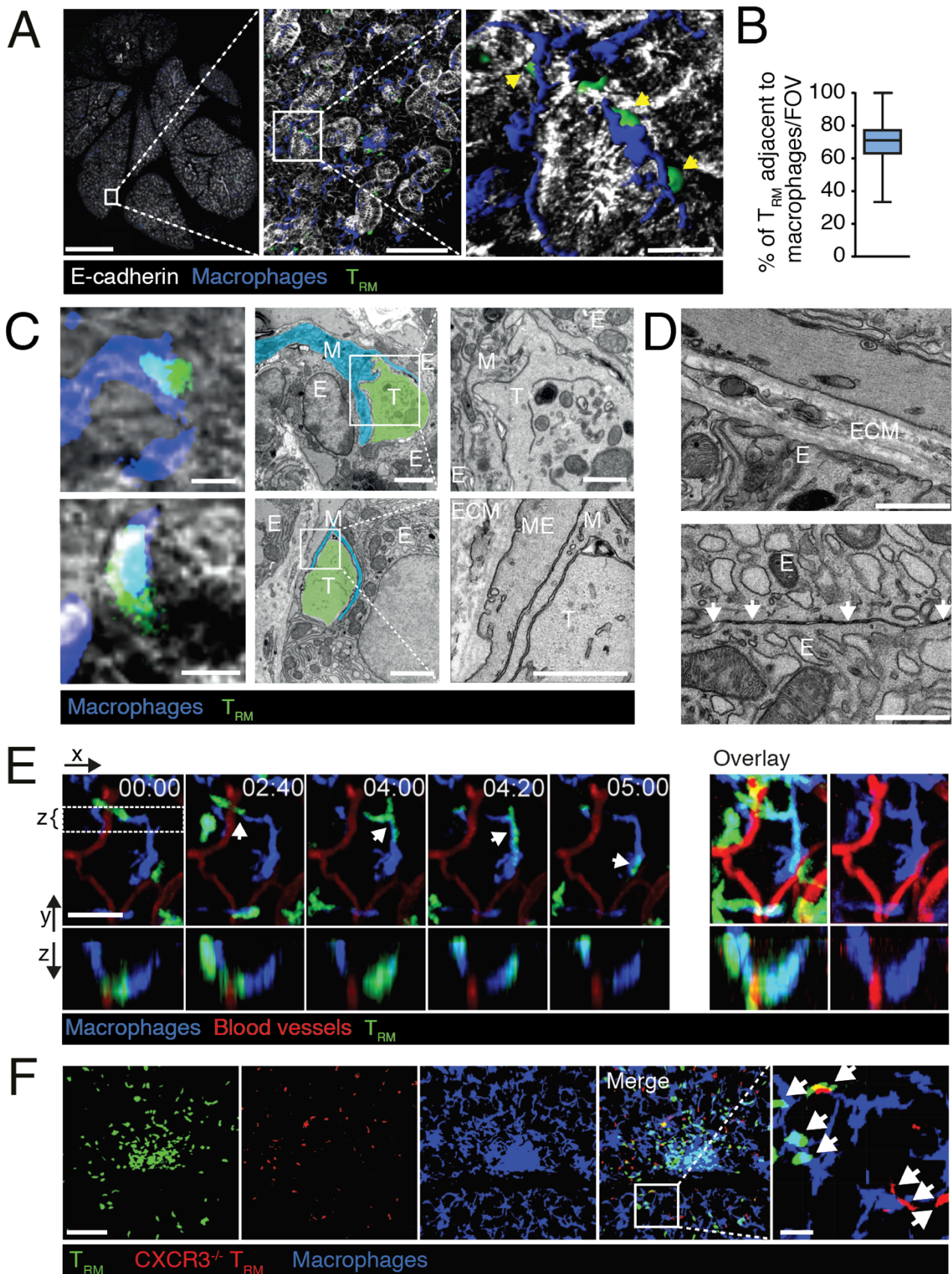
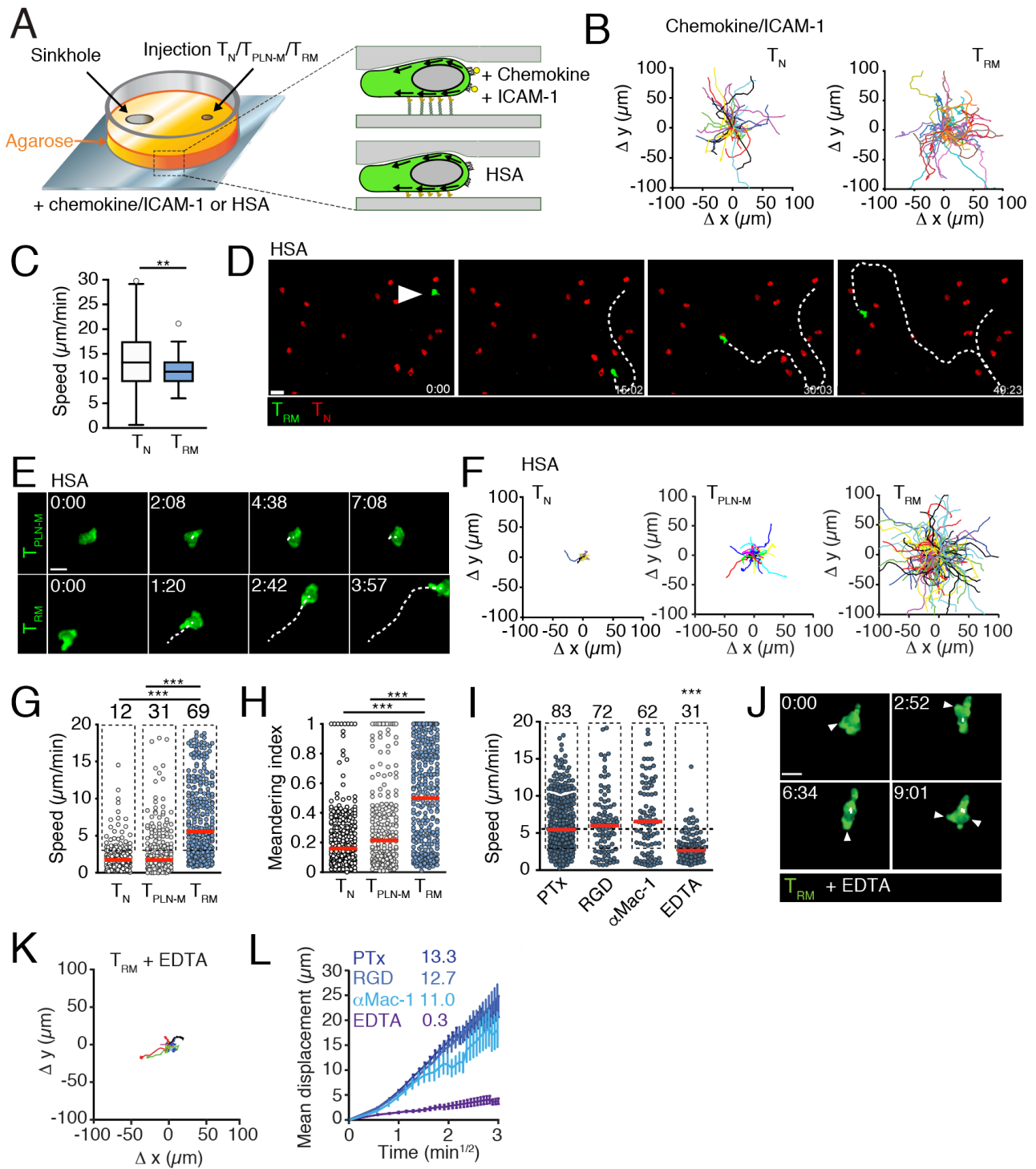


Figure 2

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1133

Figure 3



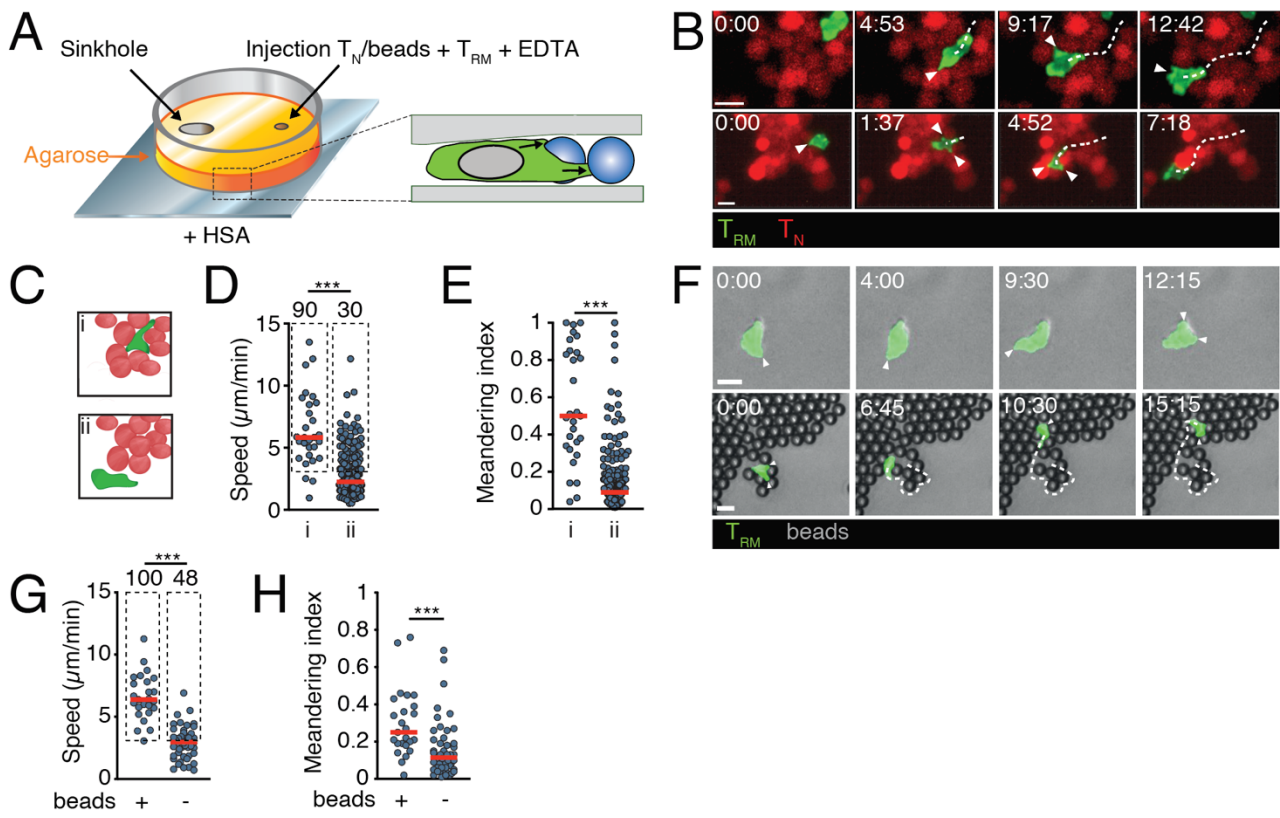


Figure 4

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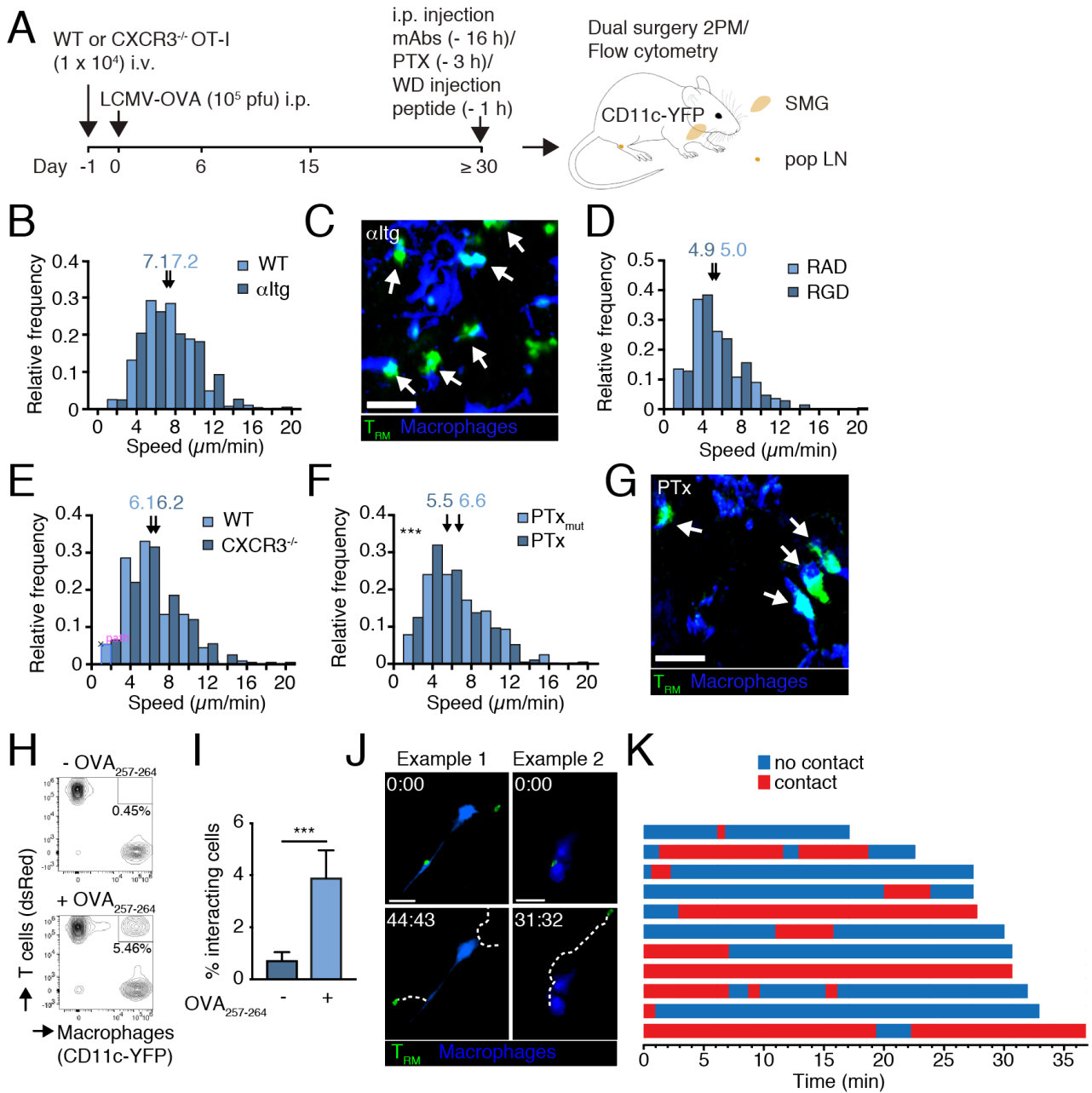
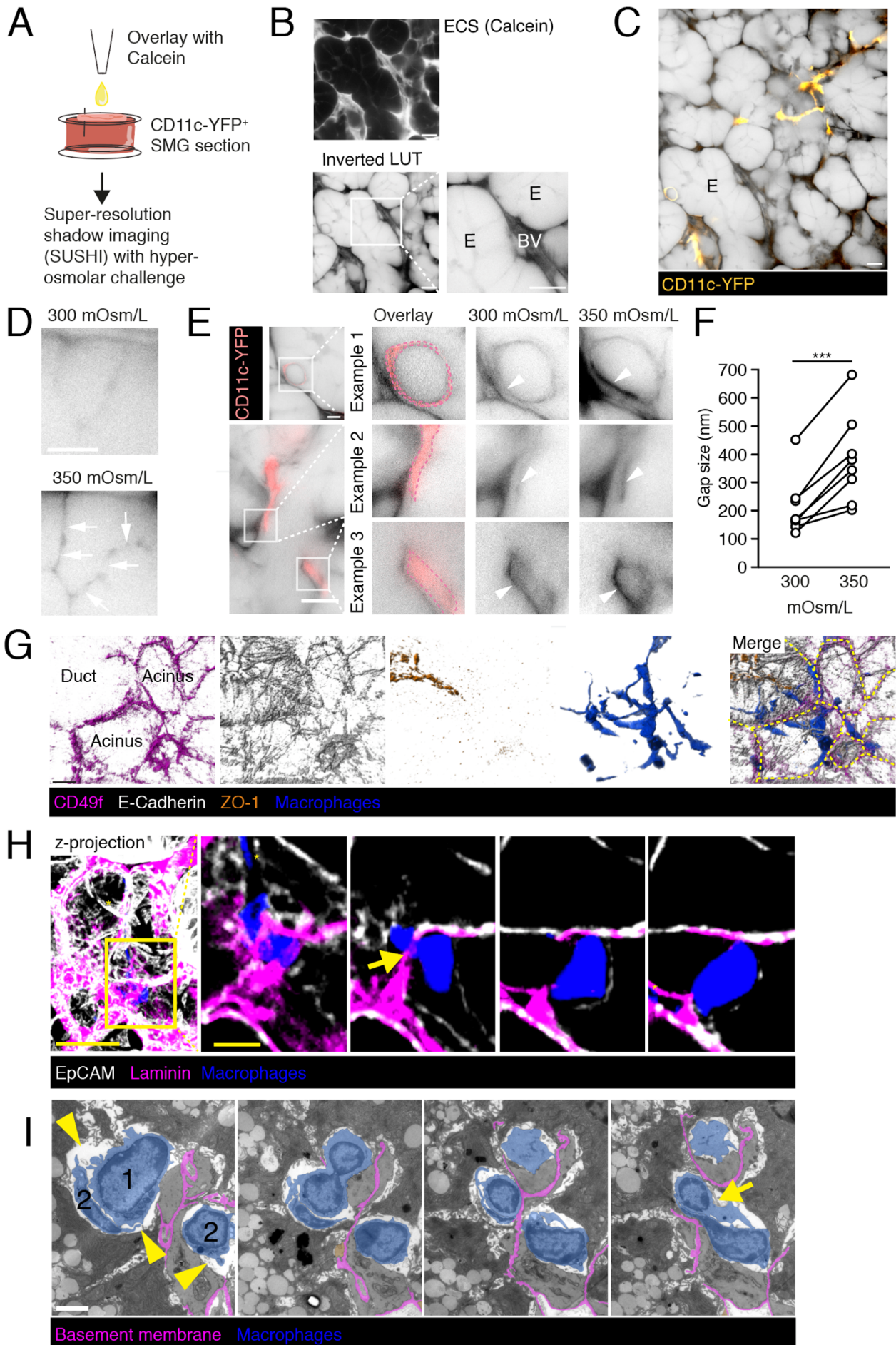


Figure 5

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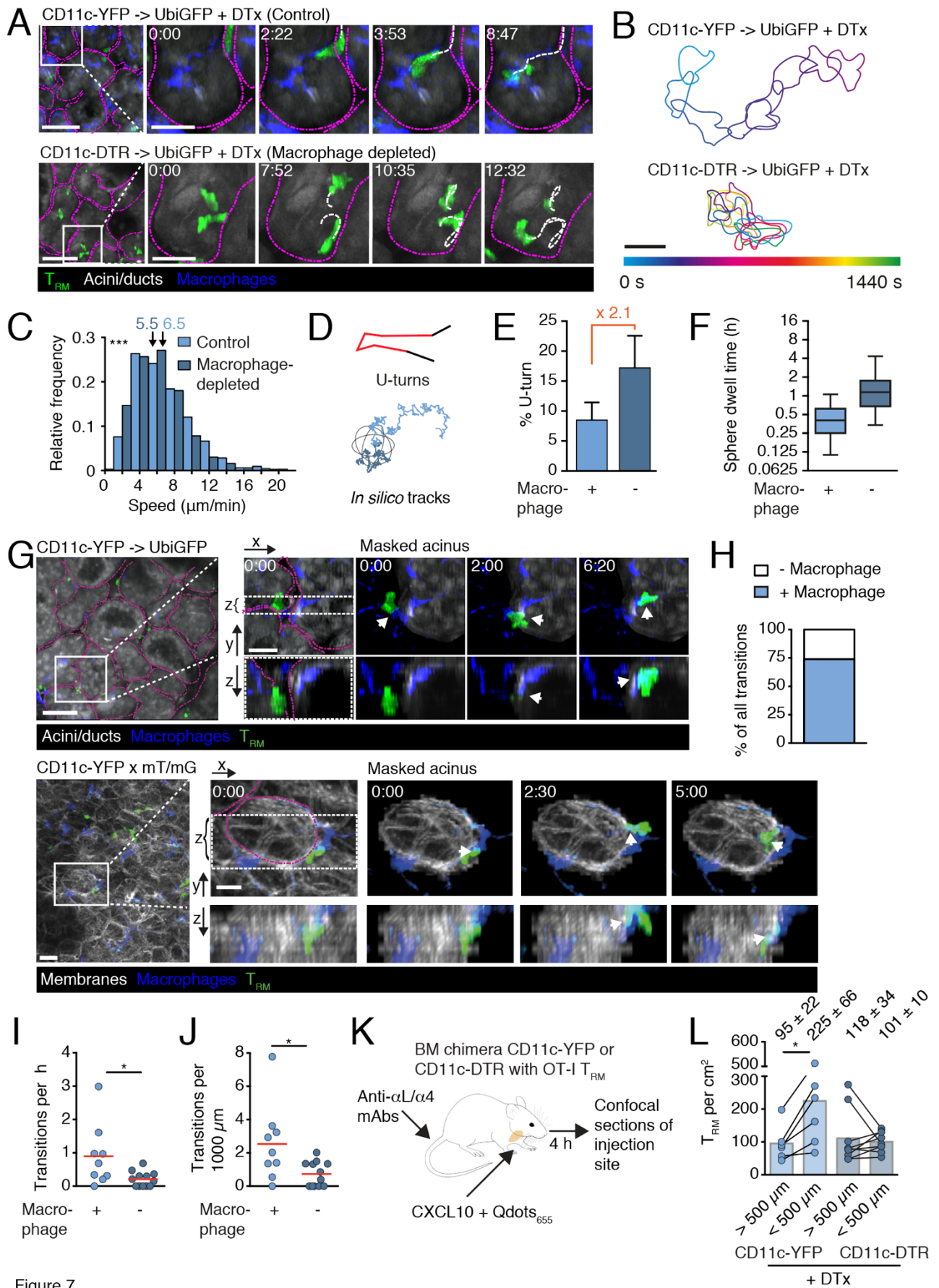


Figure 7

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