1 **Salivary gland macrophages and tissue-resident CD8+ T cells cooperate for homeostatic organ surveillance**

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32 **One sentence summary**

33 Salivary gland tissue resident memory CD8$^+$ T cells (T$_{RM}$) integrate complementary motility modes and

34 cooperate with tissue macrophages for homeostatic organ surveillance.

35

Abstract

It is well-established that tissue macrophages and tissue resident memory CD8$^+$ T cells (T$_{RM}$) play important roles for pathogen sensing and rapid protection of barrier tissues. In contrast, it is incompletely understood how these two cell types cooperate for homeostatic organ surveillance after clearance of infections. Here, we used intravital imaging to show that T$_{RM}$ dynamically followed tissue macrophage topology in non-inflamed murine submandibular salivary glands (SMG). Depletion of tissue macrophages interfered with SMG T$_{RM}$ motility and caused a reduction of interepithelial T cell crossing. Accordingly, in the absence of macrophages, SMG T$_{RM}$ failed to cluster in response to local inflammatory chemokines. A detailed analysis of the SMG microarchitecture uncovered discontinuous attachment of tissue macrophages to neighboring epithelial cells, with occasional macrophage protrusions bridging adjacent acini and ducts. When dissecting the molecular mechanisms that drive homeostatic SMG T$_{RM}$ motility, we found that these cells exhibit a wide range of migration modes: in addition to chemokine-and adhesion receptor-driven motility, resting SMG T$_{RM}$ displayed a remarkable capacity for autonomous motility in the absence of chemoattractants and adhesive ligands. Autonomous SMG T$_{RM}$ motility was mediated by friction and insertion of protrusions into gaps offered by the surrounding microenvironment. In sum, SMG T$_{RM}$ display a unique continuum of migration modes, which are supported *in vivo* by tissue macrophages to allow homeostatic patrolling of the complex SMG architecture.

Introduction

Naïve CD8$^+$ T cells (T$_N$) continuously traffic through lymphoid tissue including peripheral lymph nodes (PLN) and spleen, where they screen antigen presenting dendritic cells (DC) for the presence of cognate peptide-MHC (pMHC) complexes. Intravital two-photon microscopy (2PM) of PLN uncovered a high amoeboid-like T$_N$ motility of 12-15 µm/min ([1-4]), facilitating their search for rare cognate pMHC-presenting DC interspersed on a 3D stromal scaffold of fibroblastic reticular cells (FRC) ([5-10]). Intranodal motility is mediated by the CCR7 ligands CCL19 and CCL21 that drive F-actin polymerization at the leading edge in a Gαi-dependent manner to generate a retrograde cortical actin flow. The integrin LFA-1 conveys cortical actin flow into forward movement without generating substantial substrate adhesion ([11]). In the absence of homeostatic chemokine signaling, T$_N$ remain essentially immotile in *ex vivo* assays and show reduced scanning of lymphoid tissue *in vivo* ([11-16]).

During viral infections, effector CD8$^+$ T cells (T$_{EFF}$) generated in reactive lymphoid tissue disseminate into non-lymphoid tissues (NLT) including gut, lung, genitourinary tract, and skin. T$_{EFF}$ recruitment into NLT is mediated by inflammatory chemokines and integrin ligands that guide these cells for killing of infected cells ([17-19]). After clearance of viral antigens, T$_{EFF}$ differentiate into CCR7$^+$ central memory T cells (T$_{CM}$) and continue to patrol lymphoid organs, or into effector memory T cells (T$_{EM}$) that lack CCR7 and CD62L expression and recirculate through NLT ([20]). In addition, a subset of T$_{EFF}$ differentiates into tissue-resident memory T cells (T$_{RM}$), which stably reside in NLT and to a minor extent in lymphoid tissue as non-recirculating, self-renewing population. T$_{RM}$ continuously patrol NLT in search of cognate pMHC on local cells and act as "first line" sentinels to eliminate infected cells and to trigger an organ-wide alert status through cytokine secretion upon pathogen re-encounter ([19], [21-25]).

The steady-state patrolling behavior of epidermal T$_{RM}$ after clearance of infections has been extensively studied in mouse models. Epidermal T$_{RM}$ display characteristically elongated, dendritic shapes and move with speeds of 1-2 µm/min in proximity to the extracellular matrix (ECM)-rich basement membrane (BM) separating epidermis from dermis, i.e. in plane with the bottom keratinocyte layer ([26], [27]). A similar pattern was recently confirmed in explanted human skin ([28]). Inhibition of Gαi receptor signaling induces epidermal T$_{RM}$ to round up and stop migrating, suggesting that similar to T$_N$, chemoattractants are essential for their

84   homeostatic search strategy (*29*). In addition, epidermal CD8^{+} T cells follow local chemokine signals to

85   accumulate around infected cells upon pathogen reencounter (*30*). Recent work has shown that such CD8^{+} T

86   cell accumulation is a critical step for cooperative elimination of infected stromal cells through repeated

87   cytotoxic attacks (*31*).

88   T_{RM} are also present in exocrine glands of the head and neck region, including submandibular salivary glands

89   (SMG). Salivary glands are targeted by several bacteria and viruses including human beta- and gamma-

90   herpesviruses, which can cause disease, mostly in immunocompromised individuals (*32, 33*). Like skin and

91   gut, SMG contains epithelial tissue basally anchored onto connective tissue. However, while skin and gut

92   consist of "layered" connective and epithelial tissue, the SMG epithelium displays a more complex 3D

93   "arborized" geometry. SMG acini secrete saliva into intermediate and collecting ducts, from where it is

94   channeled into the oral cavity via the Wharton's duct (WD). The glandular epithelium is separated by a BM

95   from the supporting interstitium containing blood and lymphatic vasculature, fibroblasts and tissue

96   macrophages, which are dispersed throughout tissue (*34*). In tissue sections, most CD8^{+} T_{RM} in SMG are

97   localized within the abundant acini and ducts, implying a mechanism that allows T_{EFF} arriving in interstitial

98   venules to cross the BM underneath the epithelial compartment and develop into memory T cells (*35, 36*).

99   Yet, the cellular dynamics of homeostatic tissue surveillance by SMG T_{RM} and their interactions with other

100   local cell types have not been examined to date.

101   Here, we used intravital microscopy of mouse SMG in the memory phase following a systemic viral infection

102   to uncover a high baseline motility of resting T_{RM} of 6-7 μm/min. Remarkably, SMG T_{RM} followed tissue

103   macrophage topology during surveillance of SMG, and tissue macrophage depletion resulted in a significant

104   disruption of T_{RM} patrolling behavior. Using super-resolution microscopy to explore extracellular space

105   distribution in SMG, we observed discontinuous attachment of tissue macrophages to the surrounding tightly

106   packed epithelium, offering paths of least resistance to migrating T_{RM}. When examining the molecular

107   mechanism driving steady-state T_{RM} motility, we did not find compelling evidence for a decisive chemokine-

108   or integrin-driven contribution, in contrast to T_{CM} or epidermal T_{RM}. Instead, confinement alone was sufficient

109   to trigger SMG T_{RM} migration in the absence of chemoattractants and specific adhesion receptor

110   engagement. Mechanistically, the autonomous motility of resting SMG T_{RM} was mediated by friction- and

111   protrusion insertion-driven migration modes, while leaving cells responsive to extrinsic chemokine signals.

112   Taken together, our data uncover a new accessory role for tissue macrophages to support T_{RM} surveillance

113   of salivary glands. Our observations suggest a continuum of extrinsic factor-driven and cell-intrinsic motility

114   modes, which exploit topographic features of tissue macrophages to scan the complex 3D architecture of

115   exocrine glands during homeostatic surveillance.

Results

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

We used a viral infection model for a comparative analysis of CD8$^+$ T cell populations in lymphoid tissue and SMG (**Fig. S1A**). Systemic infection with lymphocytic choriomeningitis virus (LCMV)-OVA, a replication-competent, attenuated LCMV mutant expressing ovalbumin (OVA) as model antigen (*37*), led to transient and low viral titers in spleen on day 3 p.i. that remained below the detection limit in PLN and SMG (**Fig. S1B**). Adoptively transferred GFP$^+$ OT-I CD8$^+$ TCR tg T cells (which recognize the OVA$_{257-264}$ peptide in the context of H2-K^b) (*38*) underwent a prototypic expansion – contraction kinetic in spleen and PLN over the course of 30 days (**Fig. S1, C and D**). Despite the lack of detectable viral titers, OT-I T cells accumulated in SMG from day 6 p.i. onwards, with a stable population maintained until at least day 30 p.i. (**Fig. S1, C and D**). By day 30 p.i., OT-I T cells isolated from SMG but not PLN or spleen showed increased expression of CD103 and CD69, while losing the KLRG-1$^+$ population present on day 6 p.i. (**Fig. S1, E and F**). These data suggest that most SMG CD8$^+$ T cells had developed into *bona fide* T_{RM} at day 30 p.i. (*39*). Memory OT-I CD8$^+$ T cells isolated from PLN were approximately 65% CD62L$^+$ CD44^{high} T_{CM}, with the remaining population being CD62L$^-$ CD44^{high} memory T cells. To take this heterogeneity into account, we refer to memory T cells isolated from PLN as T_{PLN-M}. T_{RM} establishment in SMG was also observed at ≥ 30 day after systemic VSV-OVA infection. In sum, systemic viral infection led to the recruitment and retention of CD8$^+$ T cell populations in SMG.

Homeostatic SMG T_{RM} migration is characterized by dynamic cell shape changes

We next determined the localization of T_{PLN-M} and T_{RM} in their target organs during the memory phase after LCMV-OVA infection (**Fig. 1A**). Immunofluorescent sections showed that most GFP$^+$ OT-I T cells in PLN and SMG were dispersed evenly throughout tissue at day 30 p.i. (**Fig. 1B**). In PLN, OT-I T_{PLN-M} cells localized mainly with smooth muscle actin (SMA)$^+$ FRCs, whereas most OT-I T_{RM} cells in SMG were within or adjacent to EpCAM$^+$ acini and ducts (**Fig. 1B**). We developed a sequential surgery method to visualize homeostatic tissue surveillance of T_{PLN-M} and T_{RM} in PLN and SMG, respectively, in the same host by 2PM (*40*). T_{PLN-M} displayed characteristic amoeboid shapes and moved with high speeds similar to values reported for T_N (11.8 ± 4.0 μm/min, median ± SD) (**Fig. 1, C and F to H; movie S1**). Compared to T_{PLN-M}, SMG T_{RM} displayed more

7

143 pronounced shape changes, with several protrusions probing the microenvironment during migration, at

144 times with thin and elongated cell bodies (**Fig. 1, D to G; movie S2**). While T_{PLN-M} and T_{RM} covered large

145 distances throughout the observation period of intravital imaging sequences (20-60 min), both populations

146 differed in their speed and arrest coefficients, i.e. percentage of track segments with speeds < 2.5 μm/min.

147 Thus, SMG T_{RM} were significantly slower than T_{PLN-M} (**Fig. 1H**) and had higher arrest coefficients (**Fig. 1I**).

148 Nonetheless, SMG T_{RM} retained a relatively high motility coefficient, which is a proxy of a cell's ability to scan

149 the environment during random migration, of > 15 μm^2/min (**Fig. 1J**). Accordingly, their median speed of 6.8

150 ± 3.4 μm/min was notably higher than values reported for epidermal T_{RM} (1-2 μm/min) (*26*), with some cells

151 achieving speeds of > 12 μm/min. Both T_{PLN-M} and SMG T_{RM} retained a fast response to antigenic stimulation,

152 as systemic administration of cognate peptide resulted in immediate arrest and secretion of IFN-γ (**Fig. 1, J**

153 **and K**).

154 To examine a distinct CD8$^+$ T cell population, we transferred GFP$^+$ P14 CD8$^+$ TCR tg T cells (which recognize

155 the LCMV epitope gp$_{33-41}$ in the context of H2-D^b) (*41*) and performed 2PM of PLN and SMG at > 30 days p.i.

156 with the LCMV Armstrong strain. We measured similar speeds and meandering indices for GFP$^+$ P14 T_{PLN-M}

157 and T_{RM} as with OT-I CD8$^+$ T cells, both before and after cognate peptide administration (**Fig. 1, L to N**).

158 Furthermore, GFP$^+$ OT-I T cells patrolled the structurally comparable lacrimal gland (LG) in the same speed

159 range as in SMG (7.6 ± 4.3 μm/min, median ± SD; **Fig 1O**). Collectively, these data suggest that migration

160 parameters of T_{RM} patrolling exocrine glands during homeostasis are independent of TCR specificity and may

161 reflect tissue properties.

162

163 *T_{RM} colocalize with tissue macrophages in salivary and lacrimal glands*

164 To explore the microenvironmental context of exocrine gland T_{RM} migration, we used a CD11c-YFP reporter

165 strain that preferentially labels CD64$^+$ SMG macrophages (**Fig. S2A**), as previously described (*34*). In tissue

166 sections, CD11c-YFP$^+$ cells were also positive for the macrophage marker Iba-1, whereas some Iba-1$^+$ cells

167 were CD11c-YFP$^{low/negative}$, indicating that most but not all tissue macrophages were labeled in CD11c-YFP

168 mice (**Fig. S2 B**). Confocal analysis of thick tissue sections showed that CD11c-YFP$^+$ tissue macrophages

169 extended numerous protrusions from their cell bodies throughout the SMG tissue and were located within

170   EpCAM$^+$ ducts and acini, as well as SMA$^+$ perivascular structures of the interstitium (**Fig. S2C**). We further

171   investigated the spatial arrangement of macrophage protrusions with regard to epithelial BM markers. The

172   laminin ligand CD49f (α6) was prominent on the basal side of acini and to a lesser extent on ducts, which

173   were identified by the presence of the tight junction protein ZO-1 on the luminal side. In some cases, tissue

174   macrophage protrusions appeared to project between adjacent acini and ducts (**Fig. S2D**).

175   To assess the spatial relationship between tissue macrophages and T$_{RM}$, we transferred GFP$^+$ OT-I T cells into

176   CD11c-YFP recipients one day prior to infection with LCMV-OVA and analyzed tissue sections by confocal

177   microscopy in memory phase ($\geq$ day 30 p.i.). We observed a striking spatial proximity of T$_{RM}$ and tissue

178   macrophages in SMG, with approximately 70% of OT-I T cells directly in contact with CD11c-YFP$^+$ cells (**Fig. 2,**

179   **A and B; movie S3**). A comparable association of T$_{RM}$ and tissue macrophages was observed in LG after LCMV-

180   OVA infection (**Fig. 2C**). The close spatial association between SMG tissue macrophages and T$_{RM}$ was

181   confirmed by correlative light and electron microscopy imaging, with both cell membranes adjacent to each

182   other (**Fig. 2D**). Electron microscopy images also highlighted the compact tissue structure of acinar and ductal

183   epithelium linked by tight junctions and surrounded by a dense ECM (**Fig. 2E**). Occasionally, we observed

184   small T$_{RM}$ clusters around tissue macrophages (**Fig. 2F**). CXCR3$^{-/-}$ OT-I T$_{RM}$ failed to accumulate at tissue

185   macrophage clusters, suggesting the existence of local CXCL9/CXCL10 "hotspots" at these sites (**Fig. 2F**).

186   Similarly, CD3$^+$ T cells colocalized with CD68$^+$ macrophages in human parotid gland sections, both as

187   dispersed individual cells and in clusters (**Fig. 2, G and H**). Taken together, T$_{RM}$ displayed a prominent

188   colocalization with tissue macrophages in non-inflamed salivary glands across species.

189

190   *Migrating T$_{RM}$ follow tissue macrophage topology during SMG surveillance*

191   The spatial proximity of T$_{RM}$ to SMG macrophages in tissue sections raised the question whether patrolling

192   T$_{RM}$ migrate alongside macrophages. 2PM imaging of GFP$^+$ T$_{RM}$ in LCMV-OVA memory phase CD11c-YFP

193   recipients indeed confirmed that T$_{RM}$ crawled along CD11c-YFP$^+$ macrophages during most of the observation

194   period, with T$_{RM}$ shapes often closely matching the underlying macrophage topology. This was particularly

195   evident along thin macrophage protrusions, which T$_{RM}$ often followed (**Fig. 3A; movie S4**). At the same time,

196   T$_{RM}$ protrusions occasionally detached from macrophages, apparently scanning the surrounding

197 environment. Accordingly, we identified occasional T_{RM} track segments that were not associated with tissue

198 macrophages, with a minor reduction in T_{RM} speeds (7.0 ± 5.3 with versus 6.1 ± 4.8 µm/min without

199 macrophages; $p < 0.001$).

200

201 *Depletion of tissue macrophages disrupts T_{RM} patrolling within and between acini and ducts*

202 Our observations prompted us to examine T_{RM} motility in the absence of tissue macrophages. To this end,

203 we generated bone marrow chimera by reconstituting C57BL/6 or Ubi-GFP mice with control CD11c-YFP or

204 CD11c-DTR bone marrow. At 6 weeks of reconstitution, we adoptively transferred GFP$^+$ or DsRed$^+$ OT-I T cells,

205 followed by LCMV-OVA infection. In some experiments, we directly transferred OT-I T cells into CD11c-DTR

206 mice and infected mice with LCMV-OVA. Both approaches allowed us to deplete CD11c$^+$ macrophages by

207 diphtheria toxin (DTx) treatment in the memory phase without affecting the unfolding of the adaptive

208 immune response.

209 2PM imaging in DTx-treated mice revealed that SMG T_{RM} patrolling behavior was disrupted when

210 macrophages were depleted (**Fig. 3B**). T_{RM} motility was decreased, reflected by less displacement (**Fig. 3C**)

211 and slower speeds (**Fig. 3D**). Also, we observed cells that returned and migrated back the same path within

212 acini and ducts after macrophage depletion (**Fig. 3B; movie S5**). To quantify this behavior, we developed a

213 method to specifically retrieve U-turns from track parameters (**Fig. 3E**). This analysis confirmed that the

214 percent of T cell tracks showing U-turns was doubled in DTx-treated CD11c-DTR SMG from 8.1 to 16.7 % of

215 tracks (**Fig. 3F**). We observed a similar impact of macrophage depletion on T_{RM} speeds in LG (7.6 ± 4.3 and

216 5.5 ± 3.2 µm/min in control and macrophage-depleted LG; $p < 0.001$), with a 2.5 fold increase in U-turns (**Fig.

217 S3, A and B**).

218 We next asked how impaired motility impacts organ surveillance. We generated tracks *in silico* from the data

219 sets obtained by 2PM imaging of DTx- and control-treated SMG and assessed the average T_{RM} dwell time in

220 a sphere of 80 µm diameter as surrogate epithelial structure (**Fig. 3E**). This analysis uncovered a nearly

221 threefold increased sphere dwell time from 24 ± 1.8 min for control SMG to 69 ± 6.5 min (median ± SEM) for

222 DTx-treated CD11c-DTR SMG (**Fig. 3G**). A similar finding was made for control and macrophage-depleted LG

223   (**Fig. S3C**). Taken together, macrophage depletion disrupted motility parameters and increased the

224   propensity of T_{RM} to make U-turns.

225

226   *Absence of macrophages reduces T_{RM} movements into and out of epithelium*

227   We investigated whether lack of macrophages may also affect T_{RM} transitions into and out of epithelium as

228   part of the impaired motility pattern, as suggested by **movie S5**. To address this point, we reconstituted

229   irradiated Ubi-GFP mice expressing GFP in all cells with CD11c-YFP bone marrow before transfer of DsRed[+]

230   OT-I T cells and systemic LCMV-OVA infection. We found that in these chimera, acini and ducts of surgically

231   prepared SMG were GFP[bright] and readily identifiable by their glandular shapes, whereas connective tissue

232   was GFP[low]. Using case-by-case 3D rendering of 2PM image sequences in memory phase ($\geq$ 30 days p.i. with

233   LCMV-OVA), we observed that DsRed[+] T_{RM} were not restricted to individual epithelial structures but

234   occasionally crossed between adjacent acini or between epithelial and connective tissue compartments in a

235   bidirectional manner along macrophage protrusions (**Fig. 4A; movie S6**). We confirmed this observation in a

236   mouse model expressing membrane tomato and CD11c-YFP (**Fig. 4B**). In total, 75% of T_{RM} transits (n = 42)

237   into and out of epithelial structures occurred along macrophage protrusions (**Fig. 4C**). Given that not all tissue

238   macrophages are YFP[+] (**Fig. S2B**), the actual percentage of macrophage-assisted transitions may still be

239   higher. DTx treatment of CD11c-DTR SMG reduced, but did not abolish, T_{RM} transit into or out of acini and

240   ducts. In total, we observed 55 T_{RM} crossing events into or out of acini in CD11c-YFP versus 12 events in

241   CD11c-DTR chimera SMG. These data corresponded to a 77% fewer crossing events per h track duration (**Fig.**

242   **4D**). Since this defect is more pronounced than the relatively mild decrease in T_{RM} speeds after macrophage

243   depletion (**Fig. 3D**), it is unlikely to be fully explained by decreased motility. Accordingly, when we normalized

244   T_{RM} transitions to migrated distance, we continued to observe impaired epithelial crossing in absence of

245   macrophages (**Fig. 4E**). Reduced T_{RM} crossing into and out of epithelial structures was also observed when we

246   prolonged DTx treatment for 5 days (**movie S7**).

247

248   *Impaired clustering of SMG T_{RM} after macrophage depletion*

We set out to interrogate the impact of macrophage – T_{RM} cooperativity in a local recall response. We infected CD11c-YFP or CD11c-DTR mice with LCMV-OVA one day after transfer of GFP[+] OT-I T cells and depleted macrophages after 4 weeks using DTx (**Fig. S4A**). One day later, we re-challenged mice with an mCherry- and OVA-expressing mouse cytomegalovirus (MCMV-OVAmCherry) (*42, 43*) administered locally via retrograde WD injection. After 48 h, we analyzed SMG tissue sections for the presence and intensity of viral foci identified by mCherry[+] signal. This analysis confirmed the strictly local reinfection since no mCherry signal was observed in the adjacent sublingual gland (**Fig. S4B**). We observed close proximity of OT-I T cells and CD11c-YFP^{+} cells with MCMV-infected cells after SMG infection (**Fig. S4C**), with macrophages occasionally engulfing infected cells (**Fig. S4D**). This observation is in line with the well-described macrophage core function of engulfing apoptotic cells (efferocytosis) (*44-47*). Accordingly, we observed massively increased numbers of infected cell foci in macrophage-depleted SMG after WD infection with MCMV-OVAmCherry as compared to SMG containing tissue macrophages, irrespective of the presence of T_{RM} (**Fig. S4E**). Of note, in the absence of tissue macrophages, T_{RM} partially suppressed viral replication as assessed by decreased mCherry intensity in viral foci (**Fig. S4F**). Given the increased viral load after DTx treatment, tissue macrophage depletion did not permit to directly assess the functional impact of reduced T_{RM} migration patterns in presence and absence of these cells.

We therefore designed an experiment to examine T_{RM} cluster formation in response to exogenously added inflammatory chemokine as a prerequisite for efficient elimination of infected cells (*31*). We treated LCMV-OVA-immunized CD11c-YFP and CD11c-DTR BM chimera mice with DTx, followed one day later by local injection of the CXCR3 ligand CXCL10 and quantum dots into SMG (**Fig. 4F**). We also administered anti-α4 and LFA-1 blocking mAbs, which inhibit recruitment of circulating T cells to SMG (*48*). At 4 h after CXCL10 administration, we isolated SMG and quantified T_{RM} enrichment in thick confocal SMG sections according to the area marked by the co-injected fluorescent quantum dots. CXCR3^{-/-} OT-I T cells did not show accumulation in CXCL10 injection sites, supporting the specificity of chemokine-triggered clustering (49 - 63 > 500 μm versus 60 - 68 cells/cm^2 cells < 500 μm from injection site; range from two SMG). WT OT-I T_{RM} were twofold enriched at CXCL10 injection sites, suggesting that these cells had followed a CXCL10 gradient or became retained during their surveillance path (**Fig. 4G**). In contrast, local accumulation of T_{RM} was lost when

276 macrophages had been depleted, although T_{RM} numbers outside the site of chemokine injection remained

277 comparable to macrophage-containing SMG (**Fig. 4G**). These data suggested that presence of SMG

278 macrophages permitted T_{RM} clustering in response to local inflammatory chemokines.

279

280 *Macrophages show discontinuous attachment to neighboring cells within SMG*

281 These observations prompted us to examine macrophage embedding within salivary gland tissue in more

282 detail. In immunofluorescent sections, most macrophage protrusions contained phosphotyrosine-positive

283 patches (**Fig. 5, A and B**), suggesting the presence of focal adhesion-like structures at these sites. We

284 investigated how these local signals correlated with macrophage attachment *in situ*. We applied the super-

285 resolution shadow imaging microscopy (SUSHI) technique, which was originally developed to visualize the

286 complex topology of the extracellular space (ECS) in living brain slices (*49*). SUSHI can also be used to follow

287 dynamic changes in ECS in response to a hyperosmotic challenge, which leads to cell shrinkage and ECS

288 widening in brain tissue. We adapted SUSHI imaging to acutely sliced SMG sections, which were superfused

289 with the cell-impermeable fluorescent dye Calcein in order to examine macrophage anchorage to adjacent

290 epithelium (**Fig. 5C**). Steady-state imaging revealed that the interstitium contained more ECS as compared to

291 the tightly packed epithelium (**Fig. 5, D and E**). We reasoned that SUSHI in combination with hyperosmotic

292 challenge could be applied to explore attachment between neighboring cells. Performing time-lapse ECS

293 imaging, we gradually increased the osmolarity to induce cell shrinkage, which led to a strong increase of ECS

294 in the interstitium (**movie S8**). In turn, interepithelial junctions remained relatively stable and only mildly

295 increased their spacing under osmotic challenge, reflecting the presence of adherens and tight junctions

296 known to link epithelial cells (**Fig. 5F**). In contrast, hyperosmolarity induced intraepithelial CD11c-YFP$^+$

297 macrophages detachment from the adjacent epithelium (**Fig. 5, G and H**). This finding confirms previous

298 observations that tissue macrophages do not form continuous adhesive contacts with the epithelium, unlike

299 the extensive cell-to-cell contacts between acinar epithelial cells (*50*).

300 For an examination of macrophage protrusions and epithelial BM, we analyzed laminin-stained tissue

301 sections from immunized CD11c-YFP mice. We observed in some cases macrophage protrusions penetrating

302 between adjacent acini, or between epithelium and connective tissue, thus bridging adjacent compartments

303   separated by BM (**Fig. 5I; movie S9**). Using correlative confocal and transmission electron microscopy, we

304   validated that some macrophage protrusions transversed BM (**Fig. 5J**). Taken together, our data uncover

305   discontinuous attachment of tissue macrophages to neighboring cells and occasional incursions of

306   macrophage protrusions across the epithelial BM, creating paths of least resistance for migrating T_{RM}. These

307   observations offer an explanation for T_{RM} migration in accordance with tissue macrophage topography.

308

309   *In vivo motility of SMG T_{RM} remains largely intact upon integrin and chemokine receptor blockade*

310   Our data established a correlation between macrophage topography and T_{RM} migration patterns in non-

311   inflamed salivary and lacrimal glands. Next, we set out to dissect the molecular mechanisms that drive

312   homeostatic T_{RM} motility in SMG, focusing on well-described canonical chemoattractant- and integrin-

313   signaling pathways. We performed quantitative PCR analysis of cytokine and chemokine expression by

314   CD11c-YFP$^+$ cells sorted from SMG in steady-state, acute (day 6 p.i.) and memory phase (day 30 p.i.) of LCMV-

315   OVA infection. We observed detectable mRNA levels of the cytokines IL-1 and TNF, as well as the chemokines

316   CCL3, CCL4, CXCL2, CXCL9, CXCL10 and CXCL16. Expression levels were similar at all time points analyzed,

317   reflecting the lack of detectable viral spread to this organ (**Fig. S1B**). Given the expression of promigratory

318   chemokines and adhesion receptors including ICAM-1 on tissue macrophages (*51*), we examined their

319   influence on T_{RM} migration parameters. <u>Integrins mediate attachment and force transmission through</u>

320   <u>engagement of their ligands expressed by many cell types including macrophages.</u> SMG T_{RM} express $\alpha 1$, $\alpha 4$,

321   αE, αL, $\beta 1$, $\beta 2$ and $\beta 7$ integrins, and low levels of αV (**Fig. S1 and S5A**). To assess their involvement in SMG

322   T_{RM} immune surveillance, we administered a mix of integrin-blocking mAbs against the major lymphocyte

323   integrin αL (CD11a/CD18, LFA-1), the E-cadherin receptor αE (CD103), and $\alpha 4$ (VLA-4 and $\alpha 4\beta 7$) to LCMV-

324   OVA memory phase mice containing T_{PLN-M} and T_{RM} (**Fig. 6A**). We confirmed that mAbs were saturating

325   surface integrins at the time point analyzed (**Fig. S5B**). We then followed OT-I T cell motility in PLN and SMG

326   on $\geq$ day 30 p.i., using dual surgery 2PM of the same recipient mouse as above. Integrin blockade significantly

327   lowered T_{PLN-M} speeds from 11.7 to 8.8 µm/min (**Fig. S5C**), similar to the decreased cell speeds of CD18-

328   deficient T_N in lymphoid stroma (*16*). In contrast, T_{RM} speeds and crawling along tissue macrophages

329   remained unaltered by this treatment (**Fig. 6, B and C; movie S10**). We did not detect Mac-1 (CD11b/CD18)

expression on SMG T_{RM} by flow cytometry, and addition of anti-Mac1 mAb to the integrin blocking mix did

not decrease T_{RM} speeds or guidance by tissue macrophages (7.1 ± 2.9 µm/min). Similarly, inclusion of

blocking mAbs against α1 and αV together with αL, α4 and αE had no impact on T_{RM} cells speeds or

association with macrophages (6.5 ± 2.7 µm/min).

Poor surface saturation of blocking anti-β1 mAbs on OT-I T cells preempted us to assess the role of β1

integrins for T_{RM} motility by this approach. As alternative, we directly administered the β1-blocking peptide

RGD or the control peptide RAD through the Wharton's duct (WD) into SMG and followed its impact on T_{RM}

motility parameters by 2PM. The WD channels saliva from SMG into the oral cavity and can be used to

administer reagents or pathogens through retrograde duct cannulation (*52*). Control experiments using WD

administration of OVA$_{257-264}$ peptide led to instantaneous arrest of T_{RM} similar to systemic injection,

suggesting efficient peptide permeation of SMG by this route. WD injection of either peptide slightly lowered

T_{RM} speeds; yet, we did not observe an impact on RGD administration on T_{RM} motility parameters as

compared to control peptide (**Fig. 6D**). This reflected low β1 integrin levels in the interface between

macrophages and T_{RM} (**Fig. S5D**). Furthermore, E-cadherin levels on macrophages and T_{RM} were barely

detectable in SMG tissue sections, arguing against a role for this cadherin in mediating close spatial

association with tissue macrophages (**Fig. S5E**).

Cytokine-driven chemoattractant production plays a key role for T cell trafficking. Since the CXCR3 ligands

CXCL9 and CXCL10 play a role in T_{RM} clustering in SMG (**Fig. 2F**), we co-transferred WT and CXCR3$^{-/-}$ OT-I T

cells one day prior to LCMV-OVA infection. Consistent with a recent report (*48*), we found that absence of

CXCR3 did not impair T_{RM} formation in SMG after viral infection. Non-clustered CXCR3$^{-/-}$ OT-I T_{RM} showed no

significant differences in speeds as compared to WT T_{RM} (**Fig. 6E**), and lack of CXCR3 did not prevent T_{RM}

patrolling along tissue macrophages (**Fig. 2F**). These data argue against a role for CXCR3 in mediating baseline

homeostatic motility of SMG T_{RM}, despite its function in facilitating local clustering in response to

proinflammatory CXCR3 ligands (**Fig. 4F**).

To comprehensively assess a function for potential chemoattractants, we inhibited Gαi signaling by systemic

PTx treatment (*53*) and performed 2PM analysis of OT-I T cell motility parameters on ≥ day 30 after LCMV-

OVA infection. To control for inhibitor efficacy, we took advantage of the dual surgery of PLN and SMG in the

357   same recipient. Systemic PTx administration significantly slowed T_{PLN-M} down from 11.3 µm/min in control to

358   8.6 µm/min in PTx-treated recipients (**Fig. S5F**), resembling observations made with PTx-treated T_N in PLN

359   (*12*, *15*). Speeds were also decreased in SMG T_{RM} (from 6.6 to 5.5 µm/min) by PTx treatment (**Fig. 6F**),

360   suggesting a role for chemoattractants in mediating high T_{RM} speeds. Nonetheless, we observed a robust

361   residual motility and continued T_{RM} crawling along tissue macrophages in presence of PTx (**Fig. 6G, movie**

362   **S11**). Furthermore, PTx treatment had essentially no impact on U-turn frequency (1.15 fold increase as

363   compared to PTx$_{mut}$), in contrast to the absence of macrophages (**Fig. 3F**). These data suggest that while Gαi-

364   coupled receptors contribute to SMG T_{RM} motility, they are not required for T_{RM} association with tissue

365   macrophages. Finally, we interfered with MMP activity using the broad MMP-9, MMP-1, MMP-2, MMP-14

366   and MMP-7 inhibitor marimastat as described (*87*). Yet, MMP inhibition did not reduce T_{RM} migration speeds

367   (6.9 ± 3.0 µm/min). Taken together, with exception of a minor effect by PTx, the *in vivo* inhibitor treatments

368   examined here did not alter T_{RM} motility and close spatial proximity to tissue macrophages.

369   To directly assess intercellular adhesion, we co-incubated freshly isolated tissue macrophages and T_{RM} *ex vivo*

370   and analyzed cluster formation by flow cytometry (**Fig. 6H**). As positive control, we pre-incubated

371   macrophages with cognate OVA$_{257-264}$ peptide. While addition of OVA$_{257-264}$ to tissue macrophages induced

372   detectable binding to T_{RM}, the baseline association between both populations remained low (**Fig. 6, H and I**).

373   Taken together, within the technical limitations of our experimental approach, our *in vivo* and *in vitro*

374   observations did not identify specific molecules that provide strong adhesion of resting SMG T_{RM} to tissue

375   macrophages. Our data rather suggested that T_{RM} association to macrophages occurred preferentially in the

376   context of the SMG microanatomy. Importantly, our data do not exclude the presence of unidentified

377   adhesion receptors mediating T_{RM} association to tissue macrophages *in vivo*.

378

379   *SMG T_{RM} motility is induced by confinement and can be tuned by extrinsic factors*

380   To address the molecular mechanisms underlying homeostatic T_{RM} motility, we employed under agarose

381   assays that allow to precisely control environmental factors and provide confinement required for T cell

382   motility (**Fig. 7A**) (*11*). To benchmark our system, we transferred T_N on CCL21 - and ICAM-1-coated plates as

383   surrogate lymphoid tissue microenvironment. We observed high chemokinetic T_N motility with similar speeds

as measured *in vivo* (13.3 ± 5.9 µm/min) (**Fig. 7, B and C; movie S12**) (*11, 16*). Similarly, T_{RM} showed a high

motility (11.4 ± 3.0 µm/min) when migrating on CXCL10 + CXCL12- and ICAM-1-coated plates, which was only

slightly lower than that of T_N (**Fig. 7C; movie S13**). These observations show that SMG T_{RM} respond to

inflammatory chemokines and adhesion molecules with high speeds.

Since we found that *in vivo* motility of resting T_{RM} was largely refractory to inhibition by PTx and integrins,

we examined T cell displacement on plates coated with fatty acid-free human serum albumin (HSA) and thus

free of chemoattractants and specific adhesion ligands. In line with previous findings (*11*), T_N and T_{PLN-M}

remained essentially immobile throughout the observation period (**Fig. 7, D to F; movies S14 and S15**). Under

these conditions, only 12% of T_N and 31% of T_{PLN-M} migrated faster than 3 µm/min, and showed low

directionality (**Fig. 7, G and H**). Remarkably, most SMG T_{RM} showed robust intrinsic motility on HSA-coated

plates despite the absence of chemoattractants and adhesion molecules (**Fig. 7, D to F; movies S14 and S15**).

Almost 70% of SMG T_{RM} migrated faster than 3 µm/min with high directionality, with their median speed of

5.5 µm/min approaching values observed *in vivo* (**Fig. 7, G and H**). High temporal resolution imaging revealed

that migratory T_{RM} often formed several protrusions along the leading edge that appeared to probe the

environment, followed by rapid displacement of the cell body along one of the protrusions (**movie S16**).

Next, we performed under agarose assays in presence of SMG tissue macrophages to assess their influence

on T_{RM} motility. On the few occasions when motile T_{RM} contacted co-plated tissue macrophages, these

contacts were mostly transient (**Fig. 7, I and J**). Furthermore, T_{RM} did not crawl along macrophage protrusions

as observed *in vivo* (**movie S17**). These observations support the notion that T_{RM} - macrophage association

occurred preferentially in the SMG microenvironment.

We examined whether spontaneous motility was a common feature of all T_{RM} populations. To this end, we

isolated epidermal OT-I T cells from > 30 day LCMV-OVA-infected mice, which had been recruited to DNFB +

$OVA_{257-264}$-treated skin during the expansion phase. In line with the reported sensitivity to *in vivo* PTx

treatment (*29*), epidermal T_{RM} did not show spontaneous motility in the absence of chemokine (**Fig. 7, K and L; movie S18**), although these cells remained responsive to exogenous chemokines (5.6 ± 4.2 µm/min in

presence of CXCL10 and CXCL12). Thus, confinement alone was sufficient to induce spontaneous SMG but

not epidermal T_{RM} migration, representing to the best of our knowledge the first observation of such a

411 motility mode in resting T cells. Their speeds were increased in presence of chemokines and adhesion

412 molecules, suggesting that extrinsic promigratory factors tune intrinsic cell motility.

413

414 *Friction mediates SMG T$_{RM}$ migration in the absence of chemokines and ICAM-1*

415 We set out to characterize the requirements for autonomous SMG T$_{RM}$ motility using under agarose assays.

416 Reflecting the absence of chemoattractants and integrin ligands, pertussis toxin (PTx) treatment or addition

417 of the β1-blocking peptide RGD did not affect T$_{RM}$ speeds in this setting (**Fig. 7M**). Although Mac-1 binds

418 weakly to serum albumin (*54*), addition of anti-Mac-1 mAb did not cause a significant reduction in T$_{RM}$ speeds

419 (**Fig. 7M**). These observations suggested a friction-based migration mechanism (*55*). Friction is the resisting

420 force when two elements slide against each other and may be composed of a number of fundamental forces.

421 While the nature of the weak interactions between T$_{RM}$ and migratory surface causing friction are not

422 defined, we hypothesized that these might in part involve bivalent cations. Indeed, chelation of bivalent

423 cations by EDTA caused a strong decline of T$_{RM}$ speeds under agarose (**Fig. 7M**). High temporal resolution

424 imaging showed that despite the lack of translocation in presence of EDTA, SMG T$_{RM}$ continued to probe the

425 environment via transient protrusion formation, essentially "running on the spot" (**Fig. 7, N and O; movie**

426 **S19 left**). This behavior precipitated a loss in the motility coefficient (**Fig. 7P**). In sum, our data suggest that

427 bivalent cation-dependent friction between SMG T$_{RM}$ and the confining 2D surfaces generated sufficient

428 traction for translocation in the absence of considerable surface binding.

429

430 *SMG T$_{RM}$ insert protrusions between adjacent structures for translocation*

431 In addition to friction-based migration, protrusion insertion has emerged in recent years as a complementary

432 mechanism to allow cell migration without specific adhesions (*55*). The continuous probing of T$_{RM}$ in presence

433 of EDTA (**Fig. 7N**) provided an opportunity to test whether topographic features of the environment such as

434 narrow intercellular spaces may rescue cell motility by permitting insertion of pseudopods as mechanical

435 "footholds" (*56, 57*). As a surrogate approach to re-introduce a "2.5D" environmental geometry in under

436 agarose assays, we co-transferred a surplus of T$_N$ together with T$_{RM}$ and performed time-lapse imaging in the

437 presence of EDTA and in the absence of chemoattractants and adhesion molecules (**Fig. 8A**). Remarkably,

438 T_{RM} localized within T_N clusters frequently showed lateral displacement despite the presence of EDTA (**movies**

439 **S19, right, and S20**). Under these conditions, T_{RM} displacement occurred through insertion of protrusions

440 between adjacent T_N and subsequent translocation of the cell body accompanied by dynamic cell shape

441 changes (**Fig. 8B**). T_{RM} within T_N clusters were significantly faster than isolated T_{RM} (5.8 ± 3.0 and 2.3 ± 1.7

442 μm/min, respectively), displayed higher directionality, and resembled in cell shape and speeds T_{RM} migrating

443 *in vivo* (**Fig. 8, C to E**). Once T_{RM} had traversed T_N clusters, they returned to their probing behavior without

444 efficient translocation, indicating a close interdependence on physical contact and motility (**movie S19 right**).

445 We then examined whether potential residual molecular interactions between naive T cells and T_{RM} might

446 act as drivers of migration. We therefore transferred uncoated polystyrene beads with T_{RM} in under agarose

447 assays. These beads replaced T_N as surrogate 2.5D structures and allowed to examine protrusion insertion in

448 the absence of potential adhesive interactions. In this setting, T_{RM} recapitulated the behavior observed within

449 T_N clusters, showing effective cell displacement only when in contact with clusters of beads for protrusion

450 insertion (**Fig. 8F; movie S21**). T_{RM} speeds increased to 6.4 ± 1.9 μm/min and became more directional when

451 in contact with beads, whereas isolated T_{RM} showed no displacement (**Fig. 8, G and H**). This was also seen

452 when beads were passivated with pluronic acid, which prevents any unspecific residual adhesion. We further

453 observed that T_{RM} moved around dense bead areas, in line with a search for permissive gaps for locomotion

454 (**movie S21**). In sum, our data uncover the remarkable ability of resting SMG T_{RM} to migrate by adapting to

455 topographic features of the environment through protrusion insertion and shape deformation, even in the

456 absence of considerable friction, chemoattractants and adhesion receptors (**Fig. 8I**). At the same time, these

457 data are consistent with the notion that these cells retain responsiveness to chemokine signals, follow

458 macrophage topology to short-cut SMG epithelium and accumulate at local inflammatory hot-spots upon

459 pathogen re-encounter (**Fig. 8J**).

Discussion

After clearing of pathogens, T_{RM} display a remarkable capacity to patrol heterogeneous tissues without impairing vital organ functions (*21-25*). Their scanning behavior evolved because T cells are MHC-restricted and hence need to physically probe membrane surfaces of immotile stromal cells. In this study, we examined how these cells achieve this feat in the complex arborized epithelial structure of SMG during homeostatic immune surveillance. Our main finding is that T_{RM} preferentially moved along tissue macrophages, and that depletion of macrophages impaired T_{RM} patrolling. These observations assign a new accessory role to tissue macrophages in addition to their core functions for tissue homeostasis and sentinels of infection. Our data suggest two non-exclusive options to explain macrophage guidance of T_{RM}: first, through unidentified specific adhesive interaction(s) independent of ICAM-1 and other canonical adhesion molecules such as CD103; and second, by offering paths of least resistance within the exocrine gland microenvironment for protrusion insertion by autonomously moving T cells. Our data provide support for the second option without discarding the first one. Thus, reductionist *in vitro* experiments revealed that SMG T_{RM} respond to exogenous cues from chemoattractant and adhesion molecules. At the same time, confinement alone suffices to trigger friction- and protrusion insertion-based motility without exogenous chemoattractants or adhesion molecule. The continuum of intrinsic motility and integration of external factors may permit T_{RM} to patrol these exocrine glands in homeostasis and rapidly respond to inflammatory stimuli.

Macrophages and T cells closely cooperate during the onset of inflammation, the effector phase and contraction through antigen presentation, cytokine secretion and effector functions such as phagocytosis. Yet, little is known whether and how these two cell types collaborate for surveillance of NLT during homeostasis. Tissue macrophages are best characterized for their core function of maintenance or restoration of tissue homeostasis by engulfing apoptotic cells, clearing debris, initiation of repair and cloaking of microlesions (*44-47, 58, 59*). Furthermore, tissue macrophages serve as sentinels of infection, leading to cytokine secretion and leukocyte recruitment (*21*, *60*, *61*). In recent years, several non-phagocytic and non-sentinel functions were assigned to macrophages, as it was recognized that core functions of parenchymal parts of organs became outsourced to accessory cells. Accessory macrophage functions include blood vessel and mammary duct morphogenesis, hematopoietic stem cell maintenance, pancreatic cell specification, lipid

487 metabolism, relay of long-distance signals during zebrafish patterning and electric conduction in the heart

488 (*62, 63*). Our data suggest a novel accessory function, which is to facilitate T_{RM} patrolling within and between

489 acini and ducts of arborized secretory epithelium.

490 Our initial assumption was that specific adhesion receptors drive T cell association with tissue macrophages,

491 while chemoattractants fuel their high baseline motility. Tissue macrophage express ICAM-1 and other

492 adhesion molecules that can serve as ligands for T cell adhesion receptors, as well as chemoattractants (*51*).

493 It was therefore startling that - against our expectations - we were unable to find evidence for strong adhesive

494 contacts between salivary gland macrophages and T_{RM}. The experimental systems we have used to address

495 this point encompass *in vivo* inhibition of adhesion receptors in combination with reductionist *in vitro*

496 adhesion assays. Such assays have previously been employed to identify intercellular adhesion through

497 specific molecular interactions, such as ICAM-1-driven binding between T cells and DCs (*64*). It is important

498 to note our data do not rule out the presence of specific adhesive and/or promigratory interactions between

499 T_{RM} and tissue macrophages *in situ*. For instance, low T_{RM} binding to tissue macrophages *in vitro* may be owing

500 to altered gene expression patterns after macrophage isolation (*65*). Along the same line, we have not

501 examined talin-deficient T cells lacking functional integrins, since these cells are unable to attach to

502 endothelium for extravasation. Poor surface mAb saturation preempted a complete analysis of CD44 for SMG

503 T_{RM} motility (*66*). Finally, PTx treatment induced a minor but significant reduction in T_{RM} speeds *in vivo*. Yet,

504 PTx treatment had essentially no impact on U-turn frequency and movement along tissue macrophages. In

505 line with this, recent observations suggest that guidance and adhesion do not necessarily correlate, as T_N

506 migrate along the FRC network even in the absence of LFA-1 and CCR7 (*11*). Finally, the fact that isolated T_{RM}

507 can move between polystyrene beads or other lymphocytes for efficient translocation, correlating with

508 spontaneous formation of intercalating protrusions, suggests that any structure with permissive gaps can

509 serve for topological guidance. Nonetheless, owing to a lack of a suitable experimental system, our data do

510 not provide unambiguous evidence for or against specific molecular interactions between T_{RM} and

511 macrophages *in vivo*. Therefore, we cannot definitely clarify to which extent specific interactions and

512 microenvironmental architecture contribute to macrophage – T_{RM} association *in situ*.

The canonical model of leukocyte migration postulates chemoattractant-stimulated F-actin polymerization at the leading edge (*4*). The resulting retrograde F-actin flow in turn generates traction and cell body translocation via an integrin "clutch" that binds to adhesion receptors of the ECM or on the surface of neighboring cells. Although integrin-independent migration in 3D matrices has become a widely accepted concept in cell biology based on studies with cell lines and DCs (*55*), several studies uncovered integrin involvement during immune surveillance of skin T cells (*29, 67*). Thus, it remained unclear to which extent integrin-free motility occurs in primary lymphocytes, which have less cytoplasm and surface area as compared to DCs and cell lines. Another open question was whether memory T cells from distinct anatomical locations would employ similar or tissue-specific mechanisms of host surveillance. Work by Zaid et al. has identified a critical role of G-protein-coupled receptor signaling during scanning by epidermal T_{RM} (*29*). Our own observations confirm that similar to T_N and T_{PLN-M}, *ex vivo* confined epidermal T_{RM} do not migrate in the absence of integrin ligands or chemoattractants. Spontaneous motility under 2D confinement appears to constitute therefore a distinctive hallmark of SMG T_{RM} not shared by other resting T cells. Isolated T_{RM} showed high intrinsic protrusive activity *in vitro*, which may reflect high F-actin turnover and/or increased Rho-ROCK-mediated actomyosin contractility. <u>Constitutive protrusion formation could be driven by increased activity of actin nucleation factors at the leading edge, supported by forward cytoplasma flow driven by uropod contractions.</u> In fact, low adhesiveness under confinement induces spontaneous amoeboid motility via cortical contractility in adherent mesenchymal cell lines (*68, 69*), suggesting that T_{RM} may use a similar mechanism for autonomous migration *in vitro* and *in vivo*. Yet, it remains currently unknown how this unique motility program is imprinted in SMG T_{RM} and whether it is shared by tissue-resident cells from other exocrine glands.

Adhesion-free motility in 2D conditions has been proposed for large, blebbing carcinoma cells, based on friction mediated by a large interface between migrating cells and substrates (*70*). We show that 2D confinement suffices to induce T_{RM} motility through cation-dependent friction, since these cells become unable to translocate their cell bodies in presence of EDTA. Friction is composed of multiple nanoscale forces between two interfaces. For instance, electrostatic and van der Waals forces have been implicated in cell migration and non-specific adherence to substrate (*71-73*). As chelation of bivalent cations reduced friction

540 below a threshold for cell translocation in our setting, electrostatic forces are likely to be relevant. In this

541 context, transmembrane molecules may require bivalent cations for force transmission in the absence of

542 specific counter-ligands. In principle, cells may compensate for a lower friction by increasing the contacting

543 surface area (*74*). However, lymphocytes are likely too small to generate a sufficiently large interface under

544 these conditions. In turn, T_{RM} regained the capability to translocate in presence of EDTA when narrow spaces

545 are created by immotile neighboring cells or beads that lack strong adhesion to each other. This motility

546 mode correlated with continuous changes in cell shapes owing to the intrinsic protrusion formation capacity

547 of T_{RM}. Thus, T_{RM} continuously formed multiple simultaneous protrusions that probed the environment,

548 leading to their insertion into permissive gaps and subsequent cell body translocation. How T_{RM} protrusions

549 generated tractive force for cell translocation under these conditions remains incompletely understood. One

550 possibility is that protrusions insert into gaps of the 3D environment akin to cogs of a cogwheel and transmit

551 the necessary force for translocation through retrograde actin flow along irregularly shaped surfaces, even

552 in the absence of any adhesive interaction. In fact, this translocation mode is reminiscent of the "squeezing

553 and flowing" mechanism proposed for DCs (*75*), although SMG T_{RM} do not require a chemokine gradient for

554 displacement. We also observed that T_{RM} avoided areas of high bead density, thus choosing the path of least

555 resistance in this mode.

556 The efferocytic function of tissue macrophages conceivably requires physical contact with surrounding cells

557 to detect and phagocytose senescent or infected cells. Our data are in line with a model where the flexible

558 anchorage of macrophage protrusions between epithelial cells facilitates the insertion of F-actin-rich

559 pseudopods by T_{RM} before squeezing of the nucleus as biggest organelle (*55, 57, 76*). T_{RM} migration along

560 macrophages may be further assisted by unknown adhesion receptors or other molecular interactions

561 between these cells. In any event, non-proteolytic path finding is beneficial to preserve the integrity of the

562 target tissue, as it does not require constant repair of newly generated discontinuities in the ECM (*77*). The

563 scanning strategy adopted by T_{RM} resembles the migration pattern of T cell blasts in 3D collagen networks,

564 where these cells routinely bypass dense collagen areas, while probing the environment for permissive gaps

565 for cell body translocation (*78*). The influence of the physical properties of the microenvironment is

566 increasingly recognized to play a central role for decision-taking by migrating leukocytes (*71*). Thus,

leukocytes have recently been shown to use the nucleus to identify the path of least resistance in complex 3D environments with different pore sizes (*79*). This migration mode preserves tissue integrity and is energetically favorable by avoiding ECM degradation. Yet, technical limitations in recreating the complex tissue microenvironment of exocrine glands under controlled *in vitro* conditions limit the experimental scope to address this issue in depth.

Our local CXCL10 deposition experiment suggests that macrophages facilitate local T_{RM} accumulation at sites where inflammatory chemokines are produced. This resembles observations made in skin infection models where CXCR3 promotes CD8$^+$ T cell accumulation at sites of viral replication necessary for efficient elimination of infected cells (*18*, *19*). A recent study by Förster and colleagues has uncovered an unexpectedly low killing rate of cytotoxic T cells against viral-infected stromal cells (*80*). Thus, effective stromal cell elimination requires cooperativity through repeated cytotoxic attacks by multiple CD8$^+$ T cells. Conceivably, the promigratory accessory function of tissue macrophages described here helps to cluster a quorum of T_{RM} for successful stromal cell killing. Furthermore, unlike the monoclonal T_{RM} population created in our experimental setting, not all T_{RM} recognize the same pathogen under physiological conditions. This imposes a requirement for T cells to scan local sites of pathogen re-emergence and to form clusters for timely elimination of fast-replicating microbes. In line with these observations, presence of macrophages and T_{RM} cooperatively reduced viral load in the MCMV challenge model through efferocytosis of infected cells and reduction of viral replication, partially owing to presence of T_{RM}. Given that SMG macrophages are poor cross-presenters (*34*), these data are consistent with the concept of macrophage-aided tissue scanning by T_{RM}. Furthermore, both cell types are likely to enhance antiviral response through secretion of interferons and other cytokines.

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

Materials and Methods

Mice

OT-I TCR (*38*) and P14 TCR transgenic mice (*41*) were backcrossed to Tg(UBC-GFP)30Scha "Ubi-GFP" (*81*) or hCD2-dsRed (*82*) mice. Ubi-GFP (GFP$^+$) OT-I mice backcrossed to CXCR3$^{-/-}$ mice have been described (*83*). Tg(Itgax-Venus)1Mnz CD11c-YFP (*84*) and Tg(Itgax-DTR/EGFP)57Lan CD11c-DTR mice were used as recipients or bone marrow donors for lethally irradiated C57BL/6 or Ubi-GFP mice. C57BL/6 mice were purchased from Janvier (AD Horst). All mice were maintained at the Department of Clinical Research animal facility of the University of Bern, at the Theodor Kocher Institute and the University of Fribourg. All animal work has been approved by the Cantonal Committees for Animal Experimentation and conducted according to federal guidelines.

T cell transfer and viral infections

CD8$^+$ T cells were negatively isolated from spleen, peripheral and mesenteric lymph nodes of GFP$^+$ or dsRed$^+$ OT-I or GFP$^+$ P14 mice, using the EasySep^{TM} Mouse CD8$^+$ T cell Isolation Kit (Stem Cell Technologies). CD8$^+$ T cell purity was confirmed to be > 95% by flow cytometry prior to cell transfer. 10^4 OT-I T cells were i.v. transferred into recipient mice 24 h before i.p. infection with 10^5 pfu LCMV-OVA (*37*). For epidermal T$_{RM}$, we injected 5 x 10^4 OT-I as above, and 15 μl 0.3% DNFB (in acetone/oil 4:1) was applied to the right flank on day 3 p.i., followed by 500 ng SIINFEKL peptide days 4 and 5 p.i.. Experimental read-outs for the acute, cleared and memory phase of viral infection were performed 6, 15 and ≥ 30 days p.i., respectively.

LCMV virus titer

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

Antibodies and reagents

620   Alexa633-conjugated anti-PNAd MECA79, αL-integrin FD441.8 and anti-α4-integrin PS/2 mAbs were from

621   nanotools (Freiburg, Germany). Anti-α1-integrin Ha31/8 was from BD Bioscience, anti-α4 integrin PS/2 and

622   anti-αE integrin M290 were from BioXCell, anti-αV integrin RMV-7 was from BioLegend, and anti-Mac1 mAb

623   M1/70 was purified from hybridoma supernatant. TexasRed-Dextran 70 kDa was from Molecular Probes.

624   Cascade Blue (MW 10 kDA) was purchased from Invitrogen. TRITC-Dextran (MW 70 kDa) and Diphtheria Toxin

625   whereas purchased from Sigma. Pertussis toxin (PTx) and enzymatically inactive mutant PTx (PTx$_{mut}$) were

626   obtained from List Biological Laboratories. Sodium Pyruvate (100 mM; #11360-039), HEPES buffer (1M;

627   #15630-056), Minimum essential Medium Non-essential amino acids (MEM NEAA, #11140-035), L-Glutamine

628   (200 mM; #25030-024), PenStrep (#15140-122) and RPMI-1640 (#21875-034) were purchased from Gibco

629   and Fetal Bovine Serum (FCS, #SV30143.03) was purchased from HyClone.

630

631   *Flow cytometry analysis*

632   PLN and spleen were harvested at the indicated time points and single cell suspensions were obtained by

633   passing organs through cell strainers (70 µm; Bioswisstec). Red blood cell lysis was performed on splenocytes

634   in some experiments. For analysis of SMG and LG, organs were minced and treated with 2 U/µl collagenase

635   II (Worthington Biochem), 2 U/µl bovine DNAse I (Calbiochem) and - only for intracellular stainings of

636   cytokines – 5 µg/ml Brefeldin A (B6542, Sigma-Aldrich) in CMR (RPMI/10% FCS/1% HEPES/1% PenStrep/2

637   mM L-Glutamine/1 mM Sodium Pyruvate) for 30 min at 37°C, passed through a 70 µm cell strainer and

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

armenian hamster IgG-PE	-	Biolegend	400907

639   Single cell suspensions were stained for surface antigens on ice for 30 min with the indicated antibodies and

640   washed in FACS buffer (FB; PBS/2% FCS/1 mM EDTA) or FB with 5 µg/ml Brefeldin A for intracellular cytokine

641   stainings. All sample were washed in FB after staining, and for intracellular stainings, cells were permeabilized

642   and fixed in Cytofix/Cytoperm (#51-2090KZ, BD Biosciences) for 20 min on ice. Fixative was removed by

643   washing with Perm/Wash buffer (#51-2091KZ, BD Biosciences) and subsequent intracellular staining steps

644   were performed in Perm/Wash buffer. Cells were washed again prior to acquisition and at least 10^5 cells in

645   the lymphocyte FSC/SSC gate were acquired using a FACSCalibur (BD Bioscience), LSR II (BD Bioscience), LSR

646   II SORP Upgrade (BD Bioscience) or Attune NxT Flow cytometer (ThermoFisher). Total cell counts were

647   obtained by measuring single cell suspensions in PKH26 reference microbeads (Sigma) for 1 min at high

648   speed. Gating for CD103$^+$ and KLRG1$^+$ was set according to isotype controls. For CD69 staining, positive and

649   negative gates were set according to distinguishable populations and FMO was subtracted from the final %

650   of CD69$^+$ cells as background.

651

652   *Immunofluorescence*

653   Mice were anesthetized with i.p. injection of ketamine and xylazine and perfused with ice-cold 1% PFA.

654   Organs were harvested and fixed overnight in 2% PFA prior to embedding in TissueTek O.C.T. compound

655   (Sakura) for cryostat sectioning or 5% low-melting-point agarose (Sigma) for vibratome (Microslicer™ DTK-

656   1000) sectioning. 6 µm-thick frozen cryostat sections were permeabilized, blocked and stained with 0.05%

657   Triton-X 100 in 5% skimmed milk or 0.05% Tween 20 and 3% BSA for 1h, washed 3 times with PBS/1%

658   BSA/0.05% Tween (TBPBS) and stained with goat-anti-Iba1 1/200 (ab5076, Abcam) and anti-phosphotyrosine

659   (pTyr) (ab179530, Abcam) for 2 h at RT prior to mounting with Fluoromount-G (Electron Microscopy

660   Sciences).

661   For vibratome sections, 100 µm-thick section were collected in a 48-well plate and blocked with TBPBS for 2

662   h, then blocked with F$_C$-block o.n. at 4°C (hybridoma supernatant; 2.4 mg/ml diluted 1/800 in TBPBS). After

663   washing once with TBPBS for 1 h, sections were stained in TBPBS for 2-3 days at 4°C (in 100 µl, 3 sections per

664   well) with Alexa647-conjugated anti-EpCAM (1/160 dilution; clone G8.8, 118212, Biolegend), eFluor660-

665 conjugated anti-E-cadherin (1/200 dilution; clone DECMA-1, 50-3249-1633, eBioscience), polyclonal rabbit

666 anti-Laminin (1/1000 dilution; Z0097, Dako) or Cy3-conjugated anti-α-smooth muscle cell actin (clone 1A4,

667 C6198, Sigma). Sections were washed 3 times for 1 h with TBPBS and incubated with secondary Cy3-

668 conjugated anti-rabbit Ig (1/400 in TBPBS; 111-165-144, Jackson Immune Research), then washed 3 times 1

669 h with TBPBS and one time with PBS. Images were acquired with a Zeiss LSM510 or Leica SP5 confocal

670 microscope and processed using Adobe Photoshop CS6 and Imaris 8.4.1. We used Imaris software for surface

671 rendering and channel masking function to separate fluorophores with close emission spectra (i.e. GFP and

672 YFP).

673

674 *2PM image acquisition and analysis*

675 2PM intravital imaging of the popliteal lymph node was performed as described (*85*). In brief, mice were

676 anesthetized with ketamine/xylazine/acepromazine. The right popliteal lymph node was surgically exposed.

677 Prior to recording, Alexa 633-conjugated MECA-79 (10 μg/mouse) was injected i.v. to label HEV. 2PM was

678 performed with an Olympus BX50WI microscope equipped with a 20X Olympus (NA 0.95) or 25X Nikon (NA

679 1.0) objective and a TrimScope 2PM system controlled by ImSpector software (LaVisionBiotec). Some of the

680 image series were acquired using an automated system for real-time correction of tissue drift (*86*). For 2-

681 photon excitation, a Ti:sapphire laser (Mai Tai HP) was tuned to 780 or 840 nm. For 4-dimensional analysis

682 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

683 for 20-60 min; the field of view was 150-350 x 150-350 μm. Emitted light and second harmonic signals were

684 detected through 447/55-nm, 525/50-nm, 593/40-nm and 655/40-nm bandpass filters with non-descanned

685 detectors in case of C57BL/6 recipient mice. For CD11c-YFP[+] recipient mice or bone marrow chimera, we used

686 447/55-nm, 513/20-nm, 543/30-nm and 624/30-nm bandpass filters.

687 For imaging of the SMG, neck and thorax of the mouse were shaved, and residual hair removed with hair

688 removal cream (Veet). Subsequently, the animal was fixed on its back onto a custom-built SMG imaging stage

689 and stereotactic holders were attached to the head for stabilization. A 10 x 5 mm piece of skin on the right

690 side of the neck was excised to expose the right SMG lobe, which was micro-surgically loosened from

691 surrounding tissue. The right SMG lobe was flipped to the right and gently immobilized in between 2 cover

692 glasses to minimize motion artifacts from heartbeat and breathing. During the whole operation and imaging

693 procedure tissue was kept moist. During imaging, the temperature at the SMG was monitored and kept at

694 37°C by a heating ring. In most experiments, mice were operated twice (for PLN and SMG) in alternating

695 order to directly compare behavior of cells in different organs of the same recipient. Prior to imaging, blood

696 vessels were labeled by i.v. injection of 400 – 600 µg of 10 kDa Cascade-blue dextran or 70 kDa TexasRed

697 Dextran. Surgical exposure of the LG was essentially performed as for the SMG, with the mouse fixed on its

698 left flank onto the custom-built SMG imaging stage and a 10 x 5 mm piece of skin excised between the right

699 ear and eye of the mouse.

700 Sequences of image stacks were transformed into volume-rendered four-dimensional videos with Volocity

701 6.0 or Imaris 6.00-9.00 (Bitplane), which was also used for semi-automated tracking of cell motility in three

702 dimensions. Drift in image sequences was corrected using a MATLAB script recognizing 3D movement in a

703 reference channel or by using the correct drift function of Imaris. Since our filter set up does not allow

704 complete separation of GFP and YFP signals, we performed spectral unmixing of GFP and YFP using the Image

705 J plugin "Spectral_Unmixing" from Joachim Walter. Cellular motility parameters were calculated from x, y,

706 and z coordinates of cell centroids using Volocity, Imaris and MATLAB protocols. The motility coefficient, a

707 measure of the ability of a cell to move away from its starting position, was calculated from the gradient of

708 a graph of mean displacement against the square root of time. We defined U-turns as the steepest turn over

709 five steps of a track, if it is over more than 166 degrees and has a skew line distance between the first and

710 last step smaller than one mean step of the respective track (**Fig. 7D**) to exclude continuous turns. The given

711 binomial proportion 95% confidence intervals are Wilson Score intervals. We generated 100 synthetic tracks

712 of 12 h duration for each condition using a sampling strategy, which was designed to preserve the correlation

713 between velocity and turning angle and the autocorrelation of velocity and turning angle (*10*). We then took

714 the first timestep further than 40 µm away from the origin of each track as simulated dwelling time in an

715 acinus of 80 µm diameter. These analyses were performed using scientific computing packages for Python.

716 For the image series depicted in the Figures, raw 2PM data was filtered with a fine median filter (3x3x1), and

717 brightness and contrast were adjusted. Shape factors were determined by rendering and tracking cells in

718 Imaris, and manually excluding all cells that did not move along a horizontal axis. The signal from the filter

719 cells was projected into a single z-slice and the shape-factor of the 2D image calculated with Volocity.

720

721 *In vivo inhibitor treatment*

722 Gαi signaling by chemokines was blocked as described previously (*53*). Briefly, mice were treated with 3 μg

723 PTx or PTx$_{mut}$ by i.p. injection 3 h prior to imaging. For depletion of CD11c-positive cells in CD11c-DTR mice

724 or BM chimera, 4 ng/g Diphtheria Toxin (DTx) was i.p. injected 24 h prior to imaging. Depletion efficiency of

725 CD11c$^+$ cells was determined by flow cytometry and was above 98% in all organs analyzed. For the

726 synchronous blocking of integrins, 100 μg each of the purified mAbs M290, FD441.8, M1/70 and PS/2 were

727 injected i.v. 16 h prior to imaging. Surface saturation of blocking mAbs in PLN and SMG suspensions was

728 determined at the end of the experiment by sample staining with or without the same mAb clones used for

729 blocking, followed by a fluorescently labeled secondary mAb and flow cytometry. RGD peptide or as control

730 GRADSP (RAD) peptide (SIGMA) was injected via Wharton's duct cannulation (approximately 600 nmol of

731 either peptide in 30 μl per lobe in PBS), as described previously (*52*). For this procedure, mice were

732 anesthetized with ketamine/xylazine and their upper incisors rested on a metal rod and the lower incisors

733 pulled down with string, which kept the mouth open. With the aid of a stereomicroscope, we located the

734 orifice of the Wharton's duct in the sublingual caruncle and inserted a pointed glass-capillary (Untreated

735 Fused Silica Tubing - L × I.D. 3 m × 0.10 mm, #25715, Sigma). The glass capillary was connected to a Hamilton

736 Micro-syringe (Hamilton) via fine bore polythene tubing (0.28 mm, #800/100/100, Smiths), which allowed

737 the injection of small volumes. For inhibition of MMPs, Marimastat (#S7156) was obtained from Sellcheck

738 and diluted in PBS/10% DMSO (0.2 mg/g) or the corresponding volume of PBS/10% DMSO was injected i.p.

739 90 min before starting imaging (*87*). OVA$_{257-264}$ (#BAP-201) and gp$_{33-41}$ (#BAP-206) peptides were obtained

740 from ECM microcollections and 200 μg/100 μl saline injected i.v. immediately prior to imaging or 6 - 12 h

741 prior to organ harvest for FACS staining.

742

743 *Viral infection via Wharton's duct cannulation*

744 Wharton's duct cannulation was prepared as described above. Approximately 12500 pfu MCMV-

745 OVAmCherry (*42*) were injected into the Wharton's duct (WD) of DTx-treated CD11c-DTR or CD11c-YFP mice

746 in memory phase of LCMV-OVA infection. Mice were euthanized 48 h post infection and SMG tissue fixed in

747 4% PFA at 4°C for 12 h.

748

749 *Under agarose assays*

750 T_N were isolated from spleen and PLN of a naive mouse using CD8$^+$ T cell isolation kit from Stemcell. For

751 epidermal T_{RM}, skin on the right flank was harvested, minced and digested in 1 ml 0.25% Trypsin-EDTA

752 (25200056, gibco) 30 min at 37°C. Trypsin was inactivated with FCS and the skin was passed through a 70 µm

753 cell strainer and washed with PBS/5 mM EDTA. For under agarose experiments comparing SMG versus

754 epidermal T_{RM}, SMG T_{RM} were treated in exactly the same way (digestion with Trypsin-EDTA). SMG-derived

755 macrophages were isolated from uninfected CD11c-YFP mice and sorted for CD11c-YFP$^+$ cells. T_{RM} and T_{CM}

756 were isolated from SMG and PLN, respectively, from LCMV-OVA-infected C57BL/6 mice in memory phase.

757 Single cells suspension of SMG and PLN were stained with APC-conjugated anti-KLRG1 mAb and sorted for

758 GFP$^+$ or DsRED$^+$ KLRG1$^-$ T_{RM} and T_{PLN-M}, respectively. A 17-mm diameter circle was cut into the center of 60-

759 mm dishes. The hole was sealed from the bottom part of the dish using aquarium silicone (Marina) and a 24-

760 mm glass coverslip. After the silicone dried, we overlaid a 5 mm-high ring cut from a 15-ml falcon tube and

761 sealed the borders with low melting point paraffin. Coverslips were washed with PBS and coated with 3%

762 human serum albumin (HSA; A1653, Sigma) o.n. at 4°C or for 3 h at 37°C. In some experiments, coverslips

763 were coated with 10 µg/ml fibronectin (11080938001, Roche315-02, PeproTech). Fresh medium was added

764 every 2 days and macrophages were cultured for 6-7 days. For naïve T cell migration, coverslips were coated

765 with 20 µg/ml Protein A (6500-10, BioVision) for 1 h at 37°C, washed 3 times with PBS and blocked with 1.5

766 % BSA for 2 h at 37°C or o.n. at 4°C. After washing once with PBS, cover glasses were coated for 2 h at 37°C

767 with 100 nM recombinant ICAM1-F$_C$ (796IC, R&D Systems) and washed 2 times with PBS. Five ml of 2 x HBSS

768 and 10 ml of 2 x RPMI containing 1% HSA for T_{RM} and T_{CM} and 20% FBS for macrophage and naïve T cell

769 experiments, were mixed and heated in a water bath to 56°C. Golden agarose (100 mg; 50152, Lonza) was

770 dissolved and heated in 5 ml distilled water before adding to the prewarmed medium to give a 1% agarose

771   mix. After cooling to 37°C, 500 µl of the agarose mix was added on top of the coverslip. In some cases,

772   inhibitors were added (200 µg/ml PTx, 5 µg/ml anti-Mac1 mAb, 10 µM RGD or GRADSP, 2.5 mM EDTA, or 5

773   µg/ml Hoechst (H21492, Invitrogen). After incubation for 30 min at 4°C, the dish was warmed up to 37°C

774   before adding 1 ml of PBS outside the ring to prevent agarose drying. We punched a sink hole (diameter

775   approximately 2 mm) at the side of the agarose. Sorted T cell populations were suspended in RPMI/0.5% HSA

776   and in some cases treated with 5 µg anti-Mac1, 10 µM RGD or GRADSP, and pelleted in an Eppendorf tube.

777   Cells were resuspended in the smallest possible achievable volume (ca. 5-10 µl) and 0.3 µl were injected in

778   the opposite side from the sink hole using a 2.5-µl Eppendorf pipette. In some experiments, polystyrene

779   beads (Sigma-Aldrich, LB30 or 78462) were co-injected with the cells. From the sink hole surplus of medium

780   was collected to confine cells between the agarose and the glass slide. Time-lapse images were taken from

781   the center of the dish using a Zeiss fluorescent microscope (AxioObserver, Zeiss).

782

783   *Correlative Confocal and Transmission Electron Microscopy*

784   Correlative confocal and transmission electron microscopy (TEM) was carried out as described (*88*). Briefly,

785   CD11c-YFP mice were perfused with PBS and SMG were fixed *in situ* by left ventricle injection of 1.5%

786   glutaraldehyde/2% PFA in 0.1 M sodium cacodylate buffer (pH 7.4). SMG were harvested and immersed in

787   the same solution for 16 h. Fixed samples were cryoprotected in 30% sucrose prior to embedding in OCT and

788   freezing. Thirty µm sections were cut with a CM1520 cryostat (Leica) and collected on Superfrost Plus slides

789   (Thermo Fisher Scientific). Sections were processed for confocal imaging using PBS as mounting medium to

790   prevent dehydration. After confocal image acquisition, the coverslips were gently removed and sections

791   adherent to the slide were processed for TEM as described (*88*). Briefly, sections were postfixed using the

792   ferrocyanide-reduced osmium-thiocarbohydrazide-osmium (R-OTO) procedure, *en bloc* stained in 1% uranyl

793   acetate and dehydrated through increasing concentration of ethanol. Finally, sections were embedded by

794   overlaying a BEEM capsule filled with Epoxy resin. The BEEM capsules containing the embedded sections

795   were detached by immerging the slides in liquid nitrogen, leaving the section facing up on the resin block.

796   The specimens were mounted on a Leica Ultracut UCT and 70-90 nm thick serial sections were collected on

797   formvar-coated copper slot grids and imaged with a ZEISS Leo912AB Omega fitted with a 2k × 2k bottom-

33

798 mounted slow-scan Proscan camera controlled by the EsivisionPro 3.2 software. Using the florescent confocal

799 and bright field images, the same areas were relocated in the electron microscope and several images were

800 acquired through the different serial section. Acquired TEM images were then aligned and overlaid with the

801 confocal images by means of the eC-CLEM Icy plugin (*89*).

802

803 *Super-resolution shadow imaging (SUSHI)*

804 After euthanizing mice with CO_2, submandibular salivary glands (SMG) were isolated from 8-11 weeks old

805 C57BL6 or CD11c-YFP mice and submerged in ice-cold PBS. SMG were embedded in 4% low gelling agarose

806 (Sigma), cut in 300 μm-thick transversal slices and submerged in cold complete RPMI medium containing:

807 10% FCS (Hyclone). Slices were left to recover at room temperature for 15-30 min before entering the imaging

808 chamber of a custom-built 3D-STED microscopy setup (*49*). First, the positively labelled (YFP) macrophages

809 were identified at a depth of 20-30 μm below the surface and imaged in STED mode (excitation 485 nm,

810 depletion 597 nm, objective HC PL APO 63X/1.30 NA, Leica) with the following acquisition parameters: field

811 of view: 200 μm X 200 μm; pixel size: 48 nm X 48 nm; pixel dwell time: 30 μs, frame acquisition time: 20 min.

812 The medium was exchanged in the chamber with the complete RPMI containing 400 μM Calcein dye, which

813 was allowed for 20 - 30 min to disperse throughout the extracellular space of the tissue. Subsequently, we

814 acquired a SUSHI image to identify a region of interest around the macrophages. We performed a

815 hyperosmolar challenge by exchanging the chamber solution (300 μl) with high osmolar solution (350

816 mOsm/L), and acquired time lapse images to track changes in ECS topology with a 20-min interval between

817 the image frames. All image analysis including morphological measurements were done on raw images using

818 the "Plot Line Profile" function in ImageJ on structures of interest. Brightness and contrast were adjusted

819 using the "Brightness and Contrast" function in ImageJ. It was applied for illustration purposes only and did

820 not affect the quantitative analysis. No filtering or any other image processing was applied, other than

821 inverting the look-up-tables (LUT). The YFP signal was used only to identify macrophages and was not

822 recorded during subsequent imaging. Only unambiguously recognizable macrophages and ECS were

823 analyzed. Image analysis was performed on SMG slices from two mice in two independent experiments.

824

Confocal imaging of the p-Tyr signal and quantification

Mice were perfused with PBS containing 4% paraformaldehyde, SMG were isolated and fixed in the same solution at 4°C for 18 h, followed by at least 5 h dehydration in 30% sucrose. Glands were embedded in OCT (Tissue-Tek) and cut at a thickness of 6 and 20 μm at the cryostat, flash dried and fixed with 4% PFA for 10 min at room temperature. Sections were permeabilized using 0.2% Triton X-100, blocked in 10% serum of the secondary antibody and 2% BSA containing PBS and stained with antibodies for 18 h at 4°C in the same solution, after being washed in PBS and mounted in Prolong Gold containing DAPI (Invitrogen, Carlsbad, CA). Fluorescence microscopy was performed using the LSM880 confocal microscopes with 40x oil (Plan-Apochromat 40x 1.3 Oil DIC M27) or 63x oil (Plan-Apochromat 63x 1.3 Oil DIC M27) objectives (Zeiss, Oberkochen, Germany). All images were recorded using sequential excitation. The lack of spectral overlap was confirmed using single fluorescing specimens and antibody specificity via secondary controls. Macrophages were identified via iba-1 and the presence and location of p-Tyr signal was quantified in 7 fields of view using Imaris software. Brightness and contrast were adjusted for each image individually. Gaussian filters were applied using Imaris software.

Chemokine-driven T_{RM} accumulation

After 6 weeks of reconstitution with CD11c-YFP or CD11c-DTR BM, we transferred 10^4 GFP$^+$ OT-I T cells and infected the day after with 10^5 pfu LCMV-OVA. After $\geq$ 30 days p.i., mice were anesthetized one day after i.p. injection of DTx (4 ng/g) as for 2PM imaging. To block immigration of cells from blood, we treated mice with integrin blocking antibodies anti-αL (FD441.8) and anti-α4 (PS/2) (each at 50 μg/mouse; nanotools). SMG was surgically exposed. Using thin glass capillaries as for Wharton's duct injection, we injected 2 μl of a 1:1 mix of mCXLC10 (100 μg/ml; R&D 466-CR-010) and Qdots$_{655}$ (0.16 μM; Thermo Fisher Q2152MP), to obtain a final mCXCL10 amount of 0.5 μg per site of injection. After 4 h, we sacrificed the mice and harvest the SMG for vibratome sectioning. Mosaic images were taken of 100 μm-thick sections, and lobes with the highest Qdot signal were analyzed by transforming the 3D image into extended 2D image, using the Z-projection function of ImageJ. T_{RM} density in the surrounding area and injection area (defined as an octagon with 500 μm diameter) was calculated using Imaris 8.4.1.

852

Statistical analysis

Two-tailed, unpaired Student's t-test, Mann-Whitney U-test, one-way ANOVA with Dunnett's multiple

comparisons test, Kruskal-Wallis test, or a Wilcoxon rang test was used to determine statistical significance

(Prism, GraphPad). Significance was set at $p < 0.05$.

Author contributions

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

Competing interests

The authors declare no competing interests.

880   **References**

881   1.   R. N. Germain, M. J. Miller, M. L. Dustin, M. C. Nussenzweig, Dynamic imaging of the immune system:

882   progress, pitfalls and promise. *Nature Publishing Group*. **6**, 497–507 (2006).

883   2.   C. Sumen, T. R. Mempel, I. B. Mazo, U. H. von Andrian, Intravital microscopy: visualizing immunity in

884   context. *Immunity*. **21**, 315–329 (2004).

885   3.   B. Breart, P. Bousso, Cellular orchestration of T cell priming in lymph nodes. *Current Opinion in*

886   *Immunology*. **18**, 483–490 (2006).

887   4.   P. Friedl, B. Weigelin, Interstitial leukocyte migration and immune function. *Nat Immunol*. **9**, 960–969

888   (2008).

889   5.   M. Bajénoff *et al.*, Highways, byways and breadcrumbs: directing lymphocyte traffic in the lymph

890   node. *Trends in Immunology*. **28**, 346–352 (2007).

891   6.   T. Katakai, K. Habiro, T. Kinashi, Dendritic Cells Regulate High-Speed Interstitial T Cell Migration in the

892   Lymph Node via LFA-1/ICAM-1. *J. Immunol.* **191**, 1188–1199 (2013).

893   7.   M. Lee, J. N. Mandl, R. N. Germain, A. J. Yates, The race for the prize: T-cell trafficking strategies for

894   optimal surveillance. *Blood*. **120**, 1432–1438 (2012).

895   8.   G. Bogle, P. R. Dunbar, Agent-based simulation of T-cell activation and proliferation within a lymph

896   node. *Immunol Cell Biol*. **88**, 172–179 (2009).

897   9.   J. Textor *et al.*, Random Migration and Signal Integration Promote Rapid and Robust T Cell

898   Recruitment. *PLoS Comput Biol*. **10**, e1003752–16 (2014).

899   10.   M. Ackerknecht *et al.*, Antigen Availability and DOCK2-Driven Motility Govern CD4+ T Cell Interactions

900   with Dendritic Cells In Vivo. *The Journal of Immunology*. **199**, 520–530 (2017).

901   11.   M. Hons *et al.*, Chemokines and integrins independently tune actin flow and substrate friction during

902   intranodal migration of T cells. *Nat Immunol*. **19**, 606–616 (2018).

903 12. T. Okada, J. G. Cyster, CC chemokine receptor 7 contributes to Gi-dependent T cell motility in the
904 lymph node. *J. Immunol.* **178**, 2973–2978 (2007).

905 13. T. Worbs, T. R. Mempel, J. Bölter, U. H. von Andrian, R. Förster, CCR7 ligands stimulate the intranodal
906 motility of T lymphocytes in vivo. *J. Exp. Med.* **204**, 489–495 (2007).

907 14. I.-Y. Hwang, C. Park, J. H. Kehrl, Impaired trafficking of Gnai2+/- and Gnai2-/- T lymphocytes:
908 implications for T cell movement within lymph nodes. *J. Immunol.* **179**, 439–448 (2007).

909 15. F. Asperti-Boursin, E. Real, G. Bismuth, A. Trautmann, E. Donnadieu, CCR7 ligands control basal T cell
910 motility within lymph node slices in a phosphoinositide 3-kinase-independent manner. *J. Exp. Med.*
911 **204**, 1167–1179 (2007).

912 16. E. Woolf *et al.*, Lymph node chemokines promote sustained T lymphocyte motility without triggering
913 stable integrin adhesiveness in the absence of shear forces. *Nat Immunol*. **8**, 1076–1085 (2007).

914 17. U. H. von Andrian, C. R. Mackay, T-cell function and migration. Two sides of the same coin. *New
915 England Journal of Medicine*. **343**, 1020–1034 (2000).

916 18. H. D. Hickman *et al.*, CXCR3 chemokine receptor enables local CD8(+) T cell migration for the
917 destruction of virus-infected cells. *Immunity*. **42**, 524–537 (2015).

918 19. S. Ariotti *et al.*, Subtle CXCR3-Dependent Chemotaxis of CTLs within Infected Tissue Allows Efficient
919 Target Localization. *The Journal of Immunology*. **195**, 5285–5295 (2015).

920 20. F. Sallusto, J. Geginat, A. Lanzavecchia, Central memory and effector memory T cell subsets: function,
921 generation, and maintenance. *Annu Rev Immunol*. **22**, 745–763 (2004).

922 21. N. Iijima, A. Iwasaki, A local macrophage chemokine network sustains protective tissue-resident
923 memory CD4 T cells. *Science*. **346**, 93–98 (2014).

924 22. J. M. Schenkel *et al.*, Resident memory CD8 T cells trigger protective innate and adaptive immune
925 responses. *Science*. **346**, 98–101 (2014).

926   23.   G. Stary *et al.*, A mucosal vaccine against Chlamydia trachomatis generates two waves of protective

927   memory T cells. *Science*. **348**, aaa8205–aaa8205 (2015).

928   24.   S. Ariotti *et al.*, Skin-resident memory CD8$^+$ T cells trigger a state of tissue-wide pathogen alert.

929   *Science*. **346**, 101–105 (2014).

930   25.   M. Kadoki *et al.*, Organism-Level Analysis of Vaccination Reveals Networks of Protection across

931   Tissues. *Cell*, 1–38 (2017).

932   26.   A. Zaid *et al.*, Persistence of skin-resident memory T cells within an epidermal niche. *Proc. Natl. Acad.*

933   *Sci. U.S.A.* **111**, 5307–5312 (2014).

934   27.   S. Ariotti *et al.*, Tissue-resident memory CD8+ T cells continuously patrol skin epithelia to quickly

935   recognize local antigen. *Proceedings of the National Academy of Sciences*. **109**, 19739–19744 (2012).

936   28.   F. E. Dijkgraaf *et al.*, Tissue patrol by resident memory CD8+ T cells in human skin. *Nat Immunol*. **20**,

937   756–764 (2019).

938   29.   A. Zaid *et al.*, Chemokine Receptor-Dependent Control of Skin Tissue-Resident Memory T Cell

939   Formation. *The Journal of Immunology*. **199**, 2451–2459 (2017).

940   30.   J. W. Griffith, C. L. Sokol, A. D. Luster, Chemokines and Chemokine Receptors: Positioning Cells for

941   Host Defense and Immunity. *Annu Rev Immunol*. **32**, 659–702 (2014).

942   31.   S. Halle, O. Halle, R. Förster, Mechanisms and Dynamics of T Cell-Mediated Cytotoxicity In Vivo. *Trends*

943   *Immunol*. **38**, 432–443 (2017).

944   32.   C. S. Miller *et al.*, High Prevalence of Multiple Human Herpesviruses in Saliva from Human

945   Immunodeficiency Virus-Infected Persons in the Era of Highly Active Antiretroviral Therapy. *Journal*

946   *of Clinical Microbiology*. **44**, 2409–2415 (2006).

947   33.   M. J. Cannon *et al.*, Repeated measures study of weekly and daily cytomegalovirus shedding patterns

948   in saliva and urine of healthy cytomegalovirus-seropositive children. *BMC Infect. Dis.* **14**, 569 (2014).

949   34.   J. T. Thom, S. M. Walton, N. Torti, A. Oxenius, Salivary gland resident APCs are Flt3L- and CCR2-
950   independent macrophage-like cells incapable of cross-presentation. *Eur. J. Immunol.* **44**, 706–714
951   (2013).

952   35.   J. T. Thom, T. C. Weber, S. M. Walton, N. Torti, A. Oxenius, The Salivary Gland Acts as a Sink for Tissue-
953   Resident Memory CD8+ T Cells, Facilitating Protection from Local Cytomegalovirus Infection.
954   *CellReports*, 1–13 (2015).

955   36.   C. J. Smith, S. Caldeira-Dantas, H. Turula, C. M. Snyder, Murine CMV Infection Induces the Continuous
956   Production of Mucosal Resident T Cells. *CellReports*, 1–13 (2015).

957   37.   S. M. Kallert *et al.*, Replicating viral vector platform exploits alarmin signals for potent CD8+ T cell-
958   mediated tumour immunotherapy. *Nat Commun*. **8**, 15327 (2017).

959   38.   K. A. Hogquist *et al.*, T cell receptor antagonist peptides induce positive selection. *Cell*. **76**, 17–27
960   (1994).

961   39.   E. M. Steinert *et al.*, Quantifying Memory CD8 T Cells Reveals Regionalization of
962   Immunosurveillance. *Cell*. **161**, 737–749 (2015).

963   40.   X. Ficht, F. Thelen, B. Stolp, J. V. Stein, Preparation of Murine Submandibular Salivary Gland for Upright
964   Intravital Microscopy. *J Vis Exp*, 1–8 (2018).

965   41.   D. Brändle *et al.*, T cell development and repertoire of mice expressing a single T cell receptor alpha
966   chain. *Eur J Immunol*. **25**, 2650–2655 (1995).

967   42.   A. Marquardt *et al.*, Single cell detection of latent cytomegalovirus reactivation in host tissue. *Journal*
968   *of General Virology*. **92**, 1279–1291 (2011).

969   43.   F. R. Stahl *et al.*, Nodular Inflammatory Foci Are Sites of T Cell Priming and Control of Murine
970   Cytomegalovirus Infection in the Neonatal Lung. *PLoS Pathog*. **9**, e1003828–18 (2013).

971 44. F. Ginhoux, S. Jung, Monocytes and macrophages: developmental pathways and tissue homeostasis.

972   *Nat Rev Immunol*. **14**, 392–404 (2014).

973 45. S. Epelman, K. J. Lavine, G. J. Randolph, Origin and Functions of Tissue Macrophages. *Immunity*. **41**,

974   21–35 (2014).

975 46. Y. Lavin, A. Mortha, A. Rahman, M. Merad, Regulation of macrophage development and function in

976   peripheral tissues. *Nat Rev Immunol*. **15**, 731–744 (2015).

977 47. A. W. Roberts *et al.*, Tissue-Resident Macrophages Are Locally Programmed for Silent Clearance of

978   Apoptotic Cells. *Immunity*. **47**, 913–927.e6 (2017).

979 48. S. Woyciechowski, M. Hofmann, H. Pircher, α 4β 1integrin promotes accumulation of tissue-resident

980   memory CD8 +T cells in salivary glands. *Eur. J. Immunol.* **47**, 244–250 (2016).

981 49. J. Tønnesen, V. V. G. K. Inavalli, U. V. Nägerl, Super-Resolution Imaging of the Extracellular Space in

982   Living Brain Tissue. *Cell*. **172**, 1108–1121.e15 (2018).

983 50. A. Le, M. Saverin, A. R. Hand, Distribution of Dendritic Cells in Normal Human Salivary Glands. *Acta

984   Histochem. Cytochem.* **44**, 165–173 (2011).

985 51. E. L. Gautier *et al.*, Gene-expression profiles and transcriptional regulatory pathways that underlie the

986   identity and diversity of mouse tissue macrophages. *Nat Immunol*. **13**, 1118–1128 (2012).

987 52. M. Bombardieri *et al.*, Inducible Tertiary Lymphoid Structures, Autoimmunity, and Exocrine

988   Dysfunction in a Novel Model of Salivary Gland Inflammation in C57BL/6 Mice. *J. Immunol.* **189**, 3767–

989   3776 (2012).

990 53. E. Russo *et al.*, Intralymphatic CCL21 Promotes Tissue Egress of Dendritic Cells through Afferent

991   Lymphatic Vessels. *CellReports*. **14**, 1723–1734 (2016).

992 54. G. E. Davis, The Mac-1 and p150,95 beta 2 integrins bind denatured proteins to mediate leukocyte

993   cell-substrate adhesion. *Exp Cell Res*. **200**, 242–252 (1992).

994   55.   E. K. Paluch, I. M. Aspalter, M. Sixt, Focal Adhesion–Independent Cell Migration. *Annu. Rev. Cell Dev.*

995   *Biol.* **32**, 469–490 (2016).

996   56.   J. T. H. Mandeville, M. A. Lawson, F. R. Maxfield, Dynamic imaging of neutrophil migration in three

997   dimensions: mechanical interactions between cells and matrix. *J Leukoc Biol*. **61**, 188–200 (1997).

998   57.   S. Nourshargh, P. L. Hordijk, M. Sixt, Breaching multiple barriers: leukocyte motility through venular

999   walls and the interstitium. *Nat Rev Mol Cell Biol*. **11**, 366–378 (2010).

1000   58.   M. Baratin *et al.*, T Cell Zone Resident Macrophages Silently Dispose of Apoptotic Cells in the Lymph

1001   Node. *Immunity*, 1–20 (2017).

1002   59.   S. Uderhardt, A. J. Martins, J. S. Tsang, T. Lämmermann, R. N. Germain, Resident Macrophages Cloak

1003   Tissue Microlesions to Prevent Neutrophil-Driven Inflammatory Damage. *Cell*. **177**, 541–555.e17

1004   (2019).

1005   60.   Y. Natsuaki *et al.*, Perivascular leukocyte clusters are essential for efficient activation of effector T cells

1006   in the skin. *Nat Immunol*, 1–8 (2014).

1007   61.   K. W. Cho *et al.*, An MHC II-Dependent Activation Loop between Adipose Tissue Macrophages and

1008   CD4+ T Cells Controls Obesity-Induced Inflammation. *CellReports*. **9**, 605–617 (2014).

1009   62.   T. A. Wynn, A. Chawla, J. W. Pollard, Macrophage biology in development, homeostasis and disease.

1010   *Nature*. **496**, 445–455 (2013).

1011   63.   M. Hulsmans *et al.*, Macrophages Facilitate Electrical Conduction in the Heart. *Cell*. **169**, 510–513.e20

1012   (2017).

1013   64.   A. Scholer, S. Hugues, A. Boissonnas, L. Fetler, S. Amigorena, Intercellular adhesion molecule-1-

1014   dependent stable interactions between T cells and dendritic cells determine CD8+ T cell memory.

1015   *Immunity*. **28**, 258–270 (2008).

1016 65. Z. Haimon *et al.*, Re-evaluating microglia expression profiles using RiboTag and cell isolation

1017   strategies. *Nat Immunol*, 1–13 (2018).

1018 66. P. Mrass *et al.*, CD44 Mediates Successful Interstitial Navigation by Killer T Cells and Enables Efficient

1019   Antitumor Immunity. *Immunity*. **29**, 971–985 (2008).

1020 67. M. G. Overstreet *et al.*, Inflammation-induced interstitial migration of effector CD4+ T cells is

1021   dependent on integrin αV. *Nat Immunol*. **14**, 949–958 (2013).

1022 68. Y.-J. Liu *et al.*, Confinement and Low Adhesion Induce Fast Amoeboid Migration of Slow Mesenchymal

1023   Cells. *Cell*. **160**, 659–672 (2015).

1024 69. V. Ruprecht *et al.*, Cortical Contractility Triggers a Stochastic Switch to Fast Amoeboid Cell Motility.

1025   *Cell*. **160**, 673–685 (2015).

1026 70. M. Bergert *et al.*, Force transmission during adhesion-independent migration. *Nat Cell Biol*. **17**, 524–

1027   529 (2015).

1028 71. G. Charras, E. Sahai, Physical influences of the extracellular environment on cell migration. *Nat Rev

1029   Mol Cell Biol*. **15**, 813–824 (2014).

1030 72. K. Kendall, A. D. Roberts, van der Waals forces influencing adhesion of cells. *Philosophical Transactions

1031   of the Royal Society B: Biological Sciences*. **370**, 20140078–20140078 (2014).

1032 73. M. H. Lee, D. A. Brass, R. Morris, R. J. Composto, P. Ducheyne, The effect of non-specific interactions

1033   on cellular adhesion using model surfaces. *Biomaterials*. **26**, 1721–1730 (2005).

1034 74. R. J. Hawkins *et al.*, Pushing off the Walls: A Mechanism of Cell Motility in Confinement. *Phys. Rev.

1035   Lett.* **102**, 1567–4 (2009).

1036 75. T. Lämmermann *et al.*, Rapid leukocyte migration by integrin-independent flowing and squeezing.

1037   *Nature*. **453**, 51–55 (2008).

1038 76. K. Wolf *et al.*, Physical limits of cell migration: Control by ECM space and nuclear deformation and

1039 tuning by proteolysis and traction force. *J Cell Biol*. **201**, 1069–1084 (2013).

1040 77. R. G. Rowe, S. J. Weiss, Breaching the basement membrane: who, when and how? *Trends in Cell*

1041 *Biology*. **18**, 560–574 (2008).

1042 78. K. Wolf, Amoeboid shape change and contact guidance: T-lymphocyte crawling through fibrillar

1043 collagen is independent of matrix remodeling by MMPs and other proteases. *Blood*. **102**, 3262–3269

1044 (2003).

1045 79. J. Renkawitz *et al.*, Nuclear positioning facilitates amoeboid migration along the path of least

1046 resistance. *Nature*. **568**, 546–550 (2019).

1047 80. S. Halle *et al.*, In Vivo Killing Capacity of Cytotoxic T Cells Is Limited and Involves Dynamic Interactions

1048 and T Cell Cooperativity. *Immunity*. **44**, 233–245 (2016).

1049 81. B. C. Schaefer, M. L. Schaefer, J. W. Kappler, P. Marrack, R. M. Kedl, Observation of antigen-dependent

1050 CD8+ T-cell/ dendritic cell interactions in vivo. *Cell Immunol*. **214**, 110–122 (2001).

1051 82. A. C. Kirby, M. C. Coles, P. M. Kaye, Alveolar Macrophages Transport Pathogens to Lung Draining

1052 Lymph Nodes. *J. Immunol.* **183**, 1983–1989 (2009).

1053 83. A. J. Ozga *et al.*, pMHC affinity controls duration of CD8 +T cell–DC interactions and imprints timing of

1054 effector differentiation versus expansion. *J. Exp. Med.* **213**, 2811–2829 (2016).

1055 84. R. L. Lindquist *et al.*, Visualizing dendritic cell networks in vivo. *Nat Immunol*. **5**, 1243–1250 (2004).

1056 85. B. Stolp *et al.*, HIV-1 Nef interferes with T-lymphocyte circulation through confined environments in

1057 vivo. *Proceedings of the National Academy of Sciences*. **109**, 18541–18546 (2012).

1058 86. M. Vladymyrov, J. Abe, F. Moalli, J. V. Stein, A. Ariga, Real-time tissue offset correction system for

1059 intravital multiphoton microscopy. *Journal of Immunological Methods*. **438**, 35–41 (2016).

1060   87.   D. C. Marshall *et al.*, Selective Allosteric Inhibition of MMP9 Is Efficacious in Preclinical Models of

1061   Ulcerative Colitis and Colorectal Cancer. *PLoS ONE*. **10**, e0127063–26 (2015).

1062   88.   L. G. Guidotti *et al.*, Immunosurveillance of the Liver by Intravascular Effector CD8+ T Cells. *Cell*. **161**,

1063   486–500 (2015).

1064   89.   P. Paul-Gilloteaux *et al.*, eC-CLEM: flexible multidimensional registration software for correlative

1065   microscopies. *Nat Meth*. **14**, 102–103 (2017).

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Figure legends

1068 **Fig. 1. Dynamic motility parameters of memory CD8$^+$ T cells in PLN versus SMG. A.** Experimental layout for

1069 CD8$^+$ T cell analysis in SMG and PLN. **B.** Immunofluorescent sections of GFP$^+$ OT-I T cells in PLN and SMG in

1070 memory phase ($\geq$ day 30 p.i.). Scale bar, 100 µm (left panels) and 20 µm (right panel). **C.** Time-lapse 2PM

1071 image sequences showing OT-I CD8$^+$ T$_{PLN-M}$ cell motility in PLN in memory phase ($\geq$ day 30 p.i.). **D and E.** Time-

1072 lapse 2PM image sequences showing OT-I CD8$^+$ T$_{RM}$ cell motility in SMG in memory phase ($\geq$ day 30 p.i.).

1073 Arrowheads indicate protrusions (**D**) and the arrow indicates squeezing behavior (**E**) of OT-I CD8$^+$ T$_{RM}$. Scale

1074 bar in C-E, 10 µm. Time in min:s. **F.** Time-coded shapes of exemplary T$_{PLN-M}$ and T$_{RM}$ tracks. **G.** Shape factor

1075 distribution of T$_{PLN-M}$ and T$_{RM}$ with exemplary cell shapes. **H.** Speed frequency distribution of OT-I CD8$^+$ T cells

1076 in PLN and SMG. Arrows indicate median values (µm/min). **I.** Arrest coefficient frequency distribution of OT-

1077 I CD8$^+$ T cells in PLN and SMG (cut-off < 2.5 µm/min). **J.** Mean displacement versus time of OT-I T$_{PLN-M}$ (left)

1078 and T$_{RM}$ (right) before and after OVA$_{257-264}$ injection with motility coefficients (µm^2/min). **K.** IFN-γ expression

1079 in OT-I T$_{PLN-M}$ and T$_{RM}$ 24 h after OVA$_{257-264}$ injection (mean ± SD). **L, M.** Speeds (L) and meandering index (M)

1080 of P14 CD8$^+$ T cells in PLN and SMG in memory phase ($\geq$ day 30 p.i.) of LCMV Armstrong infection. **N.** Speeds

1081 of P14 CD8$^+$ T cells SMG T$_{RM}$ cells after injection of irrelevant (OVA$_{257-264}$) or cognate (gp$_{33-41}$) peptide. Red

1082 lines indicate median. **O.** Speeds of OT-I CD8$^+$ T$_{RM}$ cells in LG in memory phase ($\geq$ day 30 p.i.) after LCMV-OVA

1083 infection. Data in G are from 2-3 independent experiments and 3 mice total for each group. Data in H and I

1084 are pooled from 5 to 6 mice from 4 independent experiments with at least 194 tracks analyzed per organ.

1085 Data in J are pooled of 3-4 mice from 2 independent experiments. Data in K show one of two independent

1086 experiments. Data in L and M are from 4-7 mice and in N from 1-4 mice in 1-3 independent experiments.

1087 Data in G, I and M were analyzed with Mann-Whitney-test and data in H, L and N with Student's t-test. ***,

1088 p < 0.001.

1089

1090 **Fig. 2. T$_{RM}$ colocalize with tissue macrophages in SMG and LG. A.** Immunofluorescent section showing

1091 localization of SMG T$_{RM}$ adjacent to tissue macrophages (arrows). Scale bars, 1 mm (left), 100 µm (middle)

1092 and 20 µm (right). **B.** Percent of SMG T$_{RM}$ adjacent to tissue macrophages. Data are pooled from 105 FOV

1093 with a total of 3270 T$_{RM}$ and shown as box and whisker graph with 2.5 – 97.5 percentiles. **C.**

1094   Immunofluorescent LG section in memory phase ($\geq$ 30 days p.i. with LCMV-OVA) showing GFP$^+$ OT-I T$_{RM}$

1095   adjacent to CD11c-YFP$^+$ tissue macrophages (indicated by yellow arrowheads). Scale bars, 1 mm (left), 100

1096   µm (middle) and 20 µm (right). **D.** Correlative light and electron microscopy sections (left; confocal image;

1097   middle and right, TEM image) showing close spatial association of SMG T$_{RM}$ and tissue macrophages. M, tissue

1098   macrophages; E, epithelial cell; ME, myoepithelial cell; ECM, extracellular matrix. Scale bar, 5 (left), 2 (middle)

1099   and 1 µm (right). **E.** TEM images showing attachment of epithelial cells to ECM (top) and through intercellular

1100   junctions (white arrows; bottom). Scale bar, 800 nm. **F.** Immunofluorescent section of WT and CXCR3$^{-/-}$ OT-I

1101   T cells and macrophages. Magnified image shows association of CXCR3$^{-/-}$ OT-I T$_{RM}$ to tissue macrophages

1102   (arrows). Scale bar, 100 µm (left) and 20 µm (right). **G.** Dispersed T cells (brown) in human parotid salivary

1103   gland associate with macrophage cell bodies or thin protrusions (red), indicated by arrowheads. Scale bar,

1104   20 µm. **H.** Example of colocalization of CD68$^+$ macrophage (red) and CD3$^+$ T cell clusters (brown) in human

1105   parotid salivary gland. Scale bar, 50 µm.

1106

1107   **Fig. 3. Macrophage depletion disrupts T$_{RM}$ patrolling. A.** 2PM time-lapse image sequence showing overlap

1108   of OT-I T$_{RM}$ tracks with tissue macrophages in SMG in memory phase ($\geq$ day 30 p.i.). Scale bar, 20 µm. Time

1109   in min:s. The right panels show the time accumulated overlays of images with or without OT-I T$_{RM}$. **B.** 2PM

1110   time-lapse image sequence of T$_{RM}$ in DTx-treated CD11c-YFP or CD11c-DTR -> Ubi-GFP chimeras. Magenta

1111   lines indicate outlines of acini, white segmented lines indicate cell tracks. Scale bar, 50 µm (overview) and 20

1112   µm (insert). Time in min:s. **C.** Example T$_{RM}$ tracks in presence or absence of macrophage. Scale bar, 10 µm.

1113   **D.** Frequency distribution of T$_{RM}$ speeds in DTx-treated CD11c-YFP or CD11c-DTR bone marrow chimera.

1114   Arrows indicate median (µm/min). Data are pooled from 2-4 independent experiments with 4-6 mice total

1115   and analyzed with Mann-Whitney test; ***, $p < 0.001$. **E.** Track analysis outline. Top panel. U-turns (red)

1116   describe tracks reversing direction while excluding continuous turns. Bottom panel. Synthetic tracks were

1117   generated to assess dwell time in an 80 µm-diameter sphere (black). One example track is shown for control

1118   (light blue) and macrophage-depleted (dark blue) condition. **F.** Percent of tracks making U-turn. Bars indicate

1119   95% confidence intervals. **G.** *In silic*o dwell times for T$_{RM}$ tracks in 80 µm-spheres based on measured track

1120   parameters.

1121

1122 **Fig. 4. Absence of macrophages reduces T$_{RM}$ movement into and out of epithelium. A and B.** 2PM time-

1123 lapse image sequences of T$_{RM}$ crawling along a macrophage to enter acini in UbiGFP (A) and mT/mG (B) bone

1124 marrow recipients. Epithelial signal was manually masked to show an isolated acinus in zoomed panels.

1125 Dashed white line indicates area displayed in xz-view, and arrow indicates T$_{RM}$ - macrophage contact. A. Scale

1126 bar, 50 μm (overview) and 20 μm (insert); B. Scale bar, 20 μm (overview) and 10 μm (insert). Time in min:s.

1127 **C.** Percentage of T$_{RM}$ transitions into or out of acini and ducts in CD11c-YFP -> Ubi-GFP chimeras (n = 42) with

1128 and without contact to macrophages. **D and E.** 2PM time-lapse image sequence of CD11cYFP -> Ubi-GFP and

1129 DTx-treated CD11cDTR->Ubi-GFP chimeras were analyzed for T$_{RM}$ crossing events (leaving or entering acini).

1130 **D** shows average transitions per hour track duration, and **E** depicts transitions per 1000 μm total distance

1131 migrated. Data points represent individual image sequences. Line indicates mean. **F.** Experimental layout for

1132 analysis of T$_{RM}$ response to local chemokine. CXCL10 was injected with a fluorescent tracer (Qdots$_{65}$5) for 4 h

1133 to allow T$_{RM}$ accumulation. Integrin blocking mAbs prevent recruitment of circulating T cells. **G.** T$_{RM}$ per cm^2

1134 at sites of CXCL10 injection in presence or absence of macrophages. Numbers indicate mean $\pm$ SD. Data in D

1135 and F are pooled from 2-4 independent experiments with 4-6 mice total. Data in D was analyzed with Mann-

1136 Whitney and data in F was analyzed with Wilcoxon rank test. *, p < 0.05.

1137

1138 **Fig. 5. SMG macrophages show discontinuous attachment to neighboring cells. A.** Confocal SMG section

1139 showing pTyr signal in tissue macrophages. Left panel shows macrophage/pTyr/DAPI signal, right panels

1140 show three consecutive z-stacks (spacing 1 μm) of macrophage/pTyr signal. Scale bar, 3 μm. **B.** Quantification

1141 of pTyr$^+$ protrusions. **C.** Experimental layout of super-resolution shadow imaging (SUSHI) of SMG slices. **D.**

1142 Example of SUSHI image for determination of extracellular space. E, epithelium; BV, blood vessel. Scale bar,

1143 10 μm. **E.** Overview of ECS signal with SMG epithelium (E) and CD11c-YFP$^+$ tissue macrophages. Scale bar, 10

1144 μm. **F.** Example of epithelial attachment before and after hyperosmotic challenge. Arrows show

1145 interepithelial junctions. Scale bar, 5 μm. **G.** Examples of macrophage detachment before and after

1146 hyperosmotic challenge. Arrowheads indicate detachment. Scale bar, 5 μm. **H.** Quantification of gap size

1147 between macrophage and epithelium before and after hyperosmotic challenge. **I.** Confocal image of SMG

49

section with macrophage protrusions traversing a basement membrane below an epithelial acinus (indicated by arrow). Scale bar, 20 μm (overview) and 5 μm (insert). **J.** Electron microscopy image of macrophages creating a discontinuation of the basement membrane of an acinus (indicated by arrow). Numbers mark two neighboring macrophages. Arrowheads indicate lack of tight adhesion between macrophages and neighboring cells. Scale bar, 2 μm. All images are representative of at least 2 independent experiments. Data in H were analyzed using a paired t-test. ***, p < 0.001.

Fig. 6. Robust *in vivo* SMG T_{RM} motility after inhibition of Gαi and integrins. A. Experimental layout. **B.** OT-I T_{PLN-M} and T_{RM} speeds after combined anti-αL, α4 and αE integrin mAb (αItg) inhibition. Arrows indicate median values (μm/min). **C.** 2PM image of T_{RM} – tissue macrophage colocalization in αItg-treated SMG. Arrows indicate T cell – tissue macrophage contacts. Scale bar, 20 μm. **D.** OT-I T_{RM} speeds in SMG after WD administration of RAD or RGD peptide. **E.** WT and CXCR3$^{-/-}$ OT-I T_{RM} speeds in SMG in memory phase (≥ day 30 p.i.). Arrows indicate median values (μm/min). **F.** OT-I T_{PLN-M} and T_{RM} speeds after systemic treatment with active PTx or inactive (mutant) PTx (PTx$_{mut}$). Arrows indicate median values (μm/min). **G.** 2PM image of T_{RM} – tissue macrophage colocalization in PTx-treated SMG. Arrows indicate T cell – tissue macrophage contacts. Scale bar, 20 μm. **H.** Flow cytometry plot of mixed T_{RM} and macrophages. **I.** Quantification of cluster formation as shown in H. Data in B, D, E and F are pooled from 2-5 independent experiments with a total of 2-7 mice with at least 111 tracks per condition and analyzed with unpaired Student's t-test. Data in I are pooled from 2 independent experiments and analyzed using unpaired Student's t-test. ***, p < 0.001.
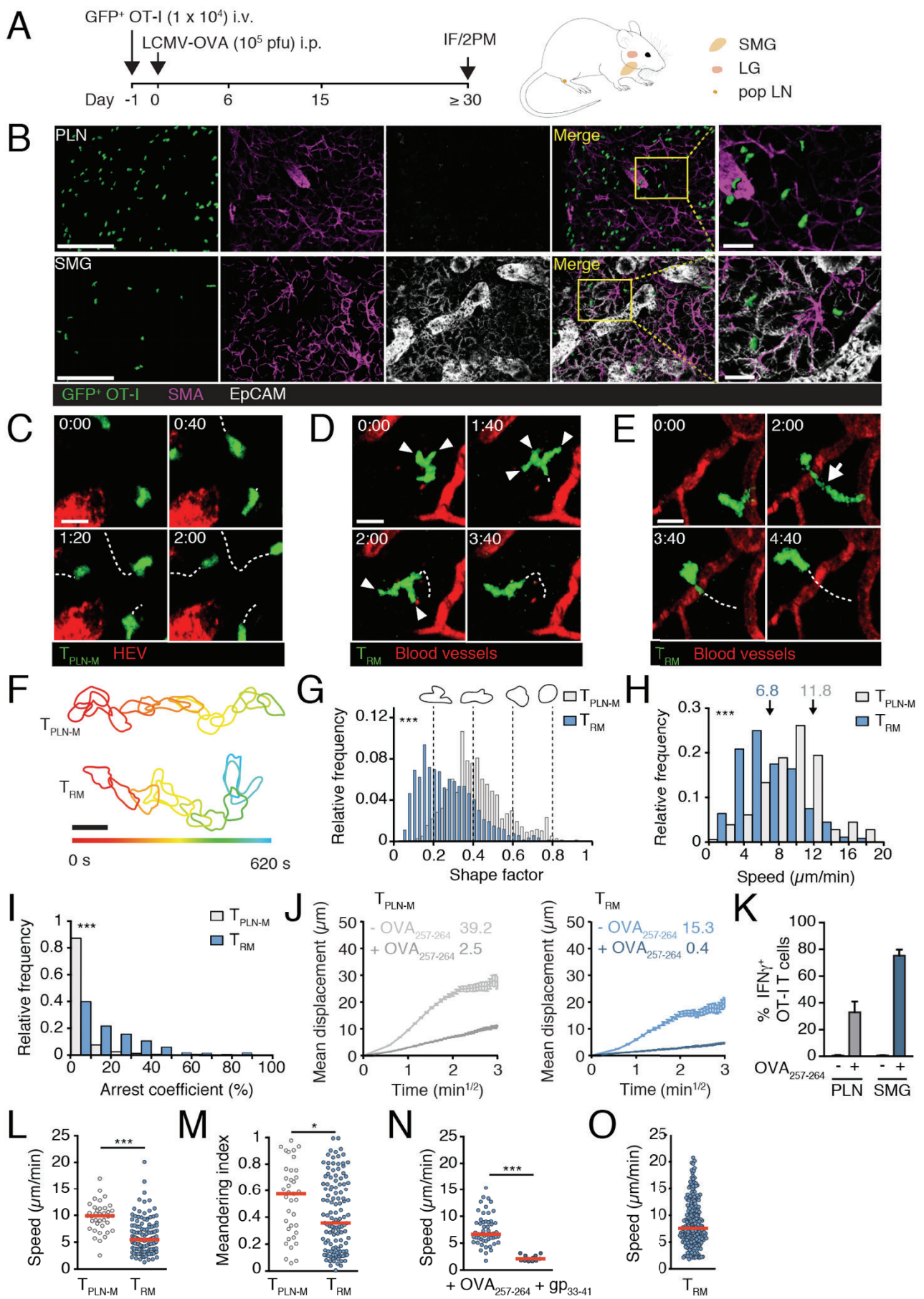
Fig. 7. Confinement induces autonomous SMG T_{RM} motility through friction. A. Experimental layout of under agarose assay. Arrows indicate F-actin flow. **B.** Representative T_N (n = 75) and T_{RM} (n = 58) tracks in presence of chemokine and ICAM-1. **C.** Speeds of T_N and T_{RM}. Data are presented as Tukey box and whiskers plot. **D.** Time-lapse image sequence showing T_{RM} motility among immotile T_N. T_{RM} displacement shown by segmented line. Scale bar, 20 μm. Time in min:s. **E.** Time-lapse image sequence in under agarose plates coated with HSA showing T_{PLN-M} (top) and T_{RM} (bottom) motility. Cell displacement shown by segmented line. Scale bar, 10 μm. Time in min:s. **F.** Representative T_N (n = 75), T_{PLN-M} (n = 226) and T_{RM} (n = 379) tracks. **G.** T_N, T_{PLN-M} and T_{RM}

speeds in under agarose plates coated with HSA. Numbers indicate percentage of tracks > 3 μm/min (boxed). Lines indicate median. **H**. Meandering index of T_N, T_{PLN-M} and T_{RM} tracks. **I.** Example image sequences showing T_{RM} in transient contact with macrophages under agarose on fibronectin-coated plates. T_{RM} displacement is shown by segmented line. Scale bar, 50 μm. Time in min:s. **J.** T_{RM} – macrophage contact duration for individual tracks. **K.** Time-lapse image sequence showing SMG (green) epidermal T_{RM} (red) under agarose on HSA. SMG T_{RM} displacement shown by segmented line. Scale bar, 20 μm. Time in min:s. **L.** Speeds of SMG and epidermal T_{RM} in under agarose plates coated with HSA. **M.** T_{RM} speeds after treatment with PTx, RGD peptide, anti-Mac1 mAb, or in presence of EDTA. Numbers indicate percentage of tracks > 3 μm/min (boxed). Lines indicate median, dotted line represents median speed of control. **N.** Image sequence of T_{RM} protrusions in presence of EDTA. Scale bar, 10 μm. **O.** Representative T_{RM} cell tracks in presence of EDTA (n = 75). **P.** Mean displacement over time of T_{RM} tracks. Numbers indicate motility coefficients ($μm^2$/min). Data in C, G, H, M and P were pooled from at least 2 independent experiments each. Statistical analysis was performed with unpaired t-test (C) or Kruskal-Wallis with Dunn's multiple comparison in G,H and M (as compared to "T_{RM}"). **, $p < 0.01$; ***, $p < 0.001$.

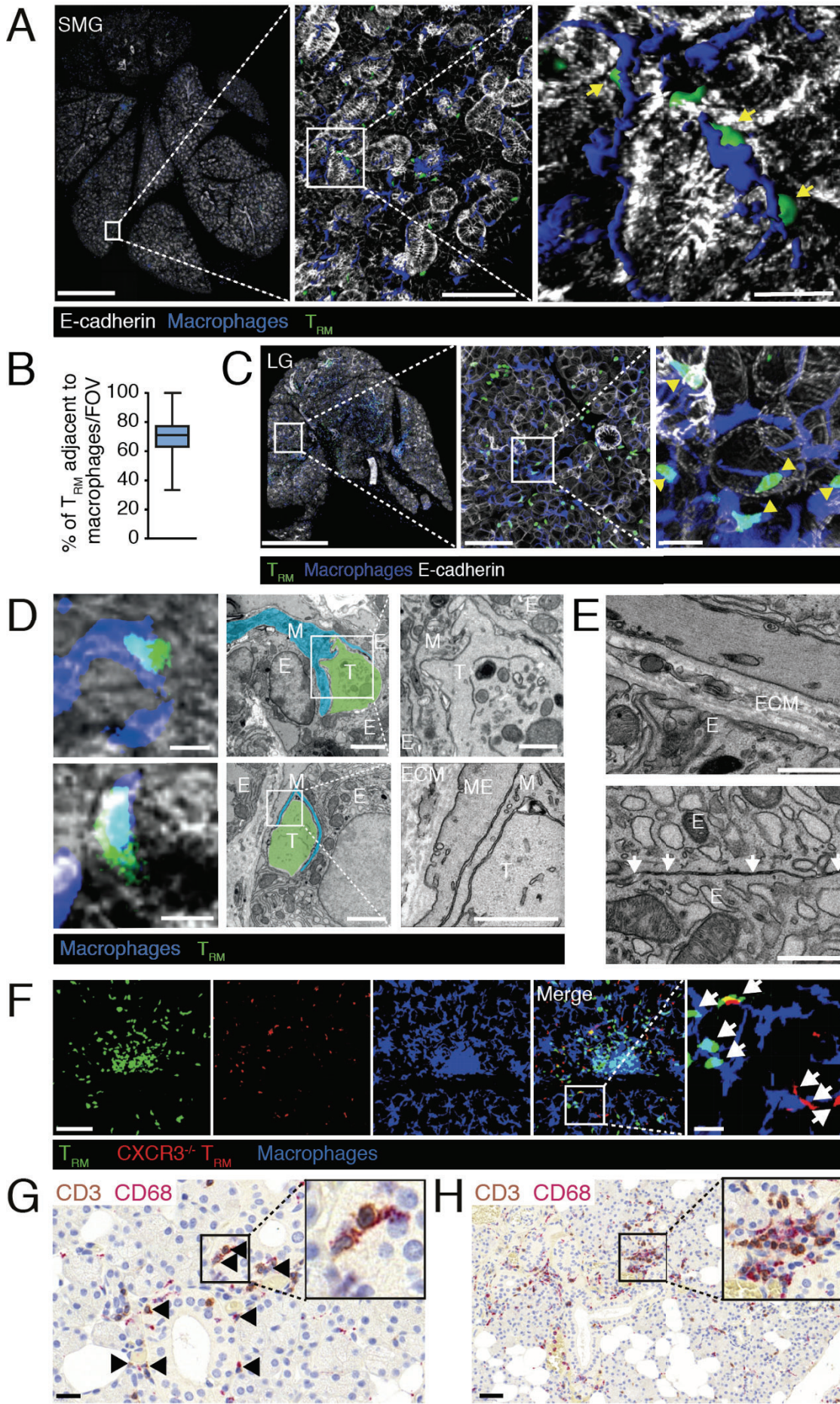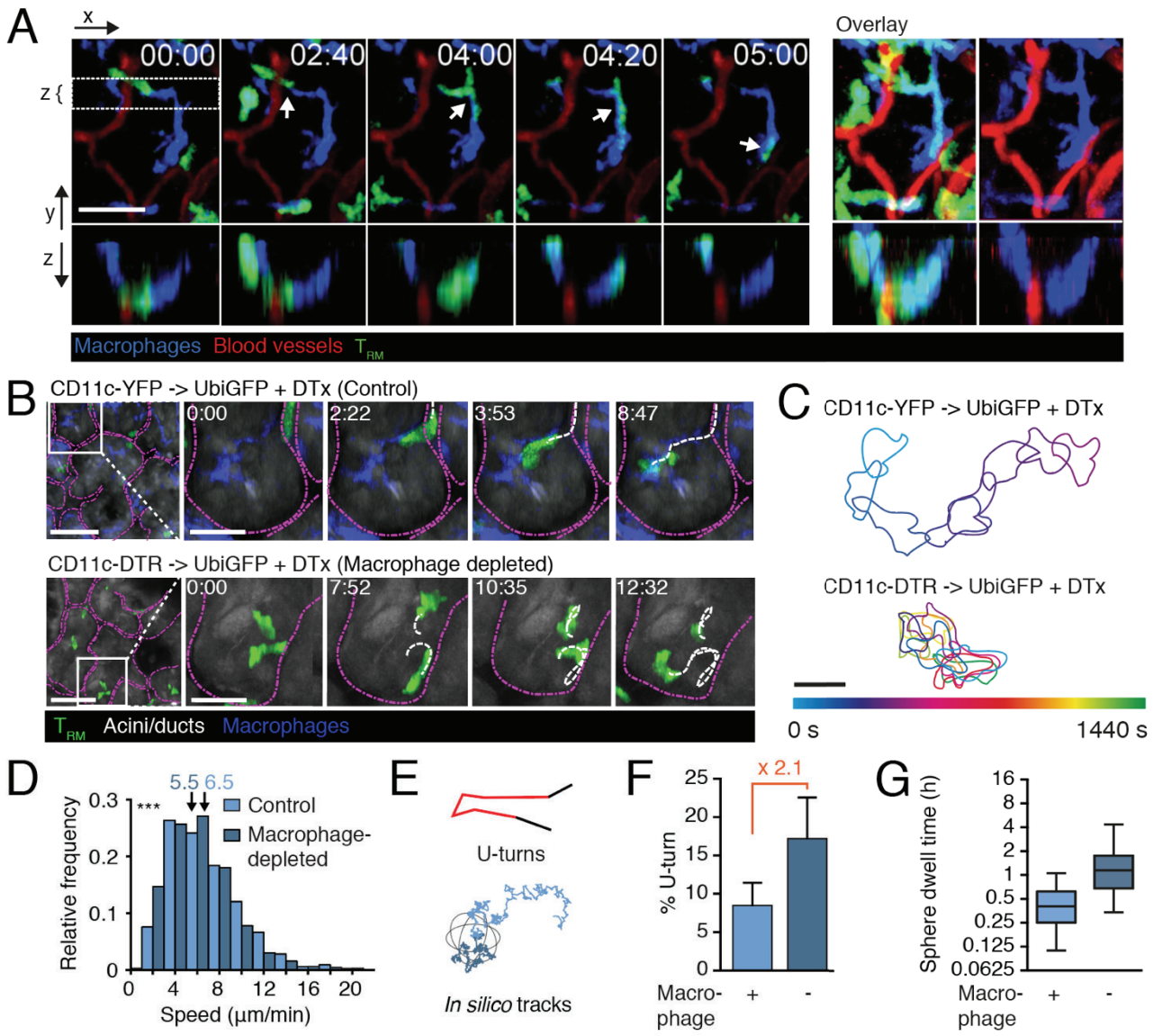**Fig. 8. T_{RM} insert protrusions for cell displacement in absence of external chemoattractants and friction. A.** Experimental layout. Arrows indicate protrusion direction. **B.** Image sequences of T_{RM} within T_N clusters in presence of EDTA. Arrowheads show membrane protrusions; segmented line indicates cell track. Scale bar, 10 μm. Time in min:s. **C.** Graphical representation of T_{RM} inside T_N cluster (i) or dispersed (ii). **D.** T_{RM} track speeds according to their location. Numbers indicate percentage of tracks > 3 μm/min (boxed). Lines indicate median. **E.** Meandering index of T_{RM} tracks sorted according to their location. Lines indicate median. **F.** Image sequences of T_{RM} alone (top) and with 7 μm polystyrene beads (bottom) in presence of EDTA. Arrowheads show membrane protrusions. Segmented line indicates cell track. Scale bar, 10 μm, time in min:s. **G.** T_{RM} track speeds according to their association with or without beads. Numbers indicate percentage of tracks > 3 μm/min (boxed). Lines indicate median. **H.** Meandering index of T_{RM} tracks sorted according to their location. **I and J.** Proposed model of tissue macrophage-assisted T_{RM} immune surveillance in SMG. **I.** Confined SMG T_{RM} display distinct migration modes. *Ex vivo* confined SMG T_{RM} respond to chemoattractants and adhesion

1202 molecules, while displaying autonomous motility through friction. Furthermore, SMG T_{RM} adapt to

1203 environmental topology to insert protrusions and move between adjacent structures that are not tightly

1204 connected. **J.** By associating with protrusion-forming tissue macrophages, which lack extensive contacts with

1205 surrounding tissue cells, T_{RM} exploit their distinct motility modes for organ surveillance. Arrows indicate

1206 protrusion direction. E, epithelium; M, tissue macrophage. Lines indicate median. Data in D, E, G and H are

1207 pooled from 4 - 5 independent experiments. Statistical analysis was performed with Mann-Whitney test. ***,

1208 p < 0.001.

A GFP+ OT-I (1 x 10^4) i.v.
LCMV-OVA (10^5 pfu) i.p.
IF/2PM
Day -1 0 6 15 ≥ 30

SMG
LG
pop LN

B PLN SMG Merge
GFP+ OT-I SMA EpCAM

C T_PLN-M HEV
0:00 0:40 1:20 2:00

D T_RM Blood vessels
0:00 1:40 2:00 3:40

E T_RM Blood vessels
0:00 2:00 3:40 4:40

F T_PLN-M T_RM
0 s 620 s

G Shape factor — Relative frequency — T_PLN-M / T_RM

H Speed (μm/min) 6.8 11.8 — T_PLN-M / T_RM

I Arrest coefficient (%) — T_PLN-M / T_RM

J T_PLN-M: - OVA_257-264 39.2, + OVA_257-264 2.5
T_RM: - OVA_257-264 15.3, + OVA_257-264 0.4
Mean displacement (μm) vs Time (min^1/2)

K % IFNγ+ OT-I T cells, OVA_257-264 - +, PLN SMG

L Speed (μm/min) T_PLN-M T_RM ***

M Meandering index T_PLN-M T_RM *

N Speed (μm/min) + OVA_257-264 + gp_33-41 ***

O Speed (μm/min) T_RM

1209 Figure 1

53

A SMG

E-cadherin Macrophages T$_{RM}$

B % of T$_{RM}$ adjacent to macrophages/FOV

C LG

T$_{RM}$ Macrophages E-cadherin

D

Macrophages T$_{RM}$

E

F

T$_{RM}$ CXCR3$^{-/-}$ T$_{RM}$ Macrophages

G CD3 CD68

H CD3 CD68

Figure 2

A

00:00 02:40 04:00 04:20 05:00 Overlay

Macrophages Blood vessels T$_{RM}$

B

CD11c-YFP -> UbiGFP + DTx (Control)

0:00 2:22 3:53 8:47

CD11c-DTR -> UbiGFP + DTx (Macrophage depleted)

0:00 7:52 10:35 12:32

T$_{RM}$ Acini/ducts Macrophages

C

CD11c-YFP -> UbiGFP + DTx

CD11c-DTR -> UbiGFP + DTx

0 s 1440 s

D

5.5 6.5
***
Control
Macrophage-
depleted

Relative frequency

Speed (µm/min)

E

U-turns

In silico tracks

F

x 2.1

% U-turn

Macro-
phage   +   −

G

Sphere dwell time (h)

Macro-
phage   +   −

1211 Figure 3

55

A CD11c-YFP -> UbiGFP

x →

Masked acinus

0:00   0:00   2:00   6:20

z{   y   z

Acini/ducts   Macrophages T_{RM}

B CD11c-YFP x mT/mG

x →

Masked acinus

0:00   0:00   2:30   5:00

z{   y   z

Membranes   Macrophages T_{RM}

C
□ - Macrophage
■ + Macrophage

% of all transitions

D
Transitions per h

*

Macro-phage   +   −

E
Transitions per 1000 μm

*

Macro-phage   +   −

F

BM chimera CD11c-YFP or
CD11c-DTR with OT-I T_{RM}

Anti-αL/α4
mAbs

Confocal
sections of
injection
site

4 h

CXCL10 + Qdots$_{655}$

G

95 ± 22 225 ± 66 118 ± 34 101 ± 10

T_{RM} per cm^2

*

> 500 μm < 500 μm > 500 μm < 500 μm

CD11c-YFP   CD11c-DTR

+ DTx

Figure 4

1212

56

A DAPI Macrophages pTyr

B Protrusions
pTyr+
pTyr-
Total=46

C Overlay with Calcein
CD11c-YFP+ SMG section
Super-resolution shadow imaging (SUSHI) with hyper-osmolar challenge

D ECS (Calcein)
Inverted LUT
E
E
BV

E CD11c-YFP

F 300 mOsm/L
350 mOsm/L

G CD11c-YFP
Overlay   300 mOsm/L   350 mOsm/L
Example 1
Example 2
Example 3

H
Gap size (nm)
300   350
mOsm/L
***

I z-projection
EpCAM   Laminin   Macrophages

J
Basement membrane   Macrophages

1213   Figure 5

Figure 6

1214

1215

1216   Figure 7

59

Figure 8

1217

1218

60

Supplementary Figures

Figure S1. Systemic viral infection leads to memory CD8⁺ T cell populations in secondary lymphoid organs and SMG for comparative analysis. A. Experimental setup to study CD8⁺ T cell behavior and phenotype in SMG and secondary lymphoid organs. GFP⁺ OT-I CD8⁺ T cells were i.v. injected on day -1. Recipient mice were infected with LCMV-OVA on day 0 and analyzed for viral titer determination and flow cytometry on indicated days. **B.** Viral titer determination on days 3 and 5 p.i. with LCMV-OVA. Each dot represents one mouse, lines indicate median. **C.** Representative flow cytometry analysis of adoptively transferred GFP⁺ OT-I T cells after gating on FSC/SSC-H lymphocyte CD45⁺ singlets. Numbers indicate percentages. **D.** Total and OT-I T cell numbers in spleen PLN and SMG at indicated days p.i. with LCMV-OVA. Each dot represents one mouse, lines indicate median. **E.** Surface marker expression on transferred OT-I T cells at indicated time points. Dotted lines are isotype or FMO (for CD69) controls. **F.** Quantification of cell surface marker expression. Plotted are mean ± SD. Data in B are from 2 independent experiments with a total of 5-10 mice per time point. Data in F are pooled from 3-4 independent experiments with a total of 6 to 15 mice per time point, except CD69 data showing 1 of 2 independent experiments with 3 mice/time point.

Figure S2. Characterization of CD11c-YFP⁺ macrophages. A. Flow cytometry of SMG from CD11c-YFP mice. The green line represents the FMO control. Numbers indicate percentage using FMO as control. **B.** Immunofluorescent SMG section of CD11c-YFP⁺ signal and Iba-1. Iba-1⁺ CD11c-YFP⁻ cells are marked by white arrows. Scale bar, 50 μm. **C.** Immunofluorescent SMG section showing tissue macrophages close to EpCAM⁺ secretory epithelium (arrow) and SMA⁺ interstitial vasculature (arrowhead). Scale bar, 40 μm. **D.** Immunofluorescent SMG section showing macrophages and epithelial cells in acini and ducts (identified by luminal ZO-1 labelling). Yellow dashed lines indicate outlines of acini and ducts. Scale bar, 10 μm.

Figure S3. LG T$_{RM}$ motility parameters after macrophage depletion. A. Frequency distribution of T$_{RM}$ speeds in DTx-treated CD11c-YFP or CD11c-DTR bone marrow chimera (control and macrophage-depleted, respectively). Arrows indicate median (μm/min). **B.** Percent of tracks making U-turn. Bars indicate 95% confidence intervals. Data are pooled from 2-3 independent experiments with 5 mice each and analyzed by

1246   unpaired t-test (A). ***, $p < 0.001$. **C.** In silico dwell times for T_{RM} tracks in 80 µm-spheres based on measured

1247   track parameters.

1248

1249   **Figure S4. Tissue macrophage-mediated efferocytosis during viral infection of SMG. A.** Experimental layout

1250   for local infection of the SMG via retrograde WD injection. **B.** Immunofluorescent SMG sections at 48 h post

1251   WD injection with MCMV-OVAmCherry. Infection is restricted to the SMG. Scale bar, 50 µm. **C.** T_{RM} and tissue

1252   macrophages cluster around infected cells. Scale bar, 50 µm. **D**. Macrophage engulfing infected cell (arrow).

1253   Scale bar, 20 µm. **E.** Quantification of mCherry$^+$ foci per FOV in SMG sections 48h after MCMV-OVAmCherry

1254   infection in the presence or absence of macrophages and T_{RM}. **F.** Intensity of mCherry signal per spot in the

1255   presence or absence of T_{RM} in macrophage-depleted SMG. Data in E were analyzed with Kruskal-Wallis and

1256   Dunn's multiple comparisons test and in F with Student's unpaired t-test. ***, $p < 0.001$.

1257

1258   **Figure S5. Analysis of integrins and T_{PLN-M} blockade *in vivo*. A.** Expression of integrins on memory phase OT-

1259   I T cells in spleen, PLN and SMG. **B.** mAb saturation testing. Immediately after 2PM recording, PLN and SMG

1260   single cells suspensions were either incubated with the same blocking mAb, followed by a Cy5-labeled Ab

1261   against the first mAb (1st and 2nd Ab), or just with the Cy5-labeled 2^{ndary} Ab (2nd Ab only) and analyzed by flow

1262   cytometry. **C.** Frequency distribution of T_{PLN-M} speeds after integrin blockade. Arrows indicate median

1263   (µm/min). **D.** Confocal image of $\beta1$ staining of SMG section. Arrowhead indicates macrophage - T_{RM} interface.

1264   Scale bar, 4 µm **E.** Confocal image of E-cadherin staining of SMG section. Scale bar, 5 µm. **F.** Frequency

1265   distribution of T_{PLN-M} speeds after PTx treatment. Arrows indicate median (µm/min). Data in C and F are

1266   pooled from 2-5 independent experiments with a total of 2-7 mice and analyzed with unpaired Student's t-

1267   test. ***, $p < 0.001$.

1268 **Supplementary movie Legends**

1269 **Movie S1. Intravital imaging of T$_{PLN-M}$ migration in PLN.** T$_{PLN-M}$ (green) display amoeboid shapes while

1270 migrating in the T cell zone of PLN close to HEV (red). Scale bar, 20 μm and 10 μm (zoom). Time in min:s.

1271

1272 **Movie S2. Intravital imaging of T$_{RM}$ migration in SMG.** T$_{RM}$ (green) migrate in the SMG between blood vessels

1273 (red). T$_{RM}$ frequently display multiple leading edges or shape changes. Scale bar, 20 μm and 10 μm (zoom).

1274 Time in min:s.

1275

1276 **Movie S3. Confocal image of SMG T$_{RM}$ colocalization with tissue macrophages.** T$_{RM}$ (green) are in close

1277 proximity to tissue macrophages (blue). EpCAM-1 (white) depicts epithelial cells. Close up shows a T$_{RM}$ in a

1278 duct, identified by ZO-1 staining (red).

1279

1280 **Movie S4. Intravital imaging of SMG T$_{RM}$ migration along tissue resident macrophages.** Time series (left)

1281 and time accumulated overlay (right), including xy and xz projections. T$_{RM}$ (green) frequently move along thin

1282 protrusions of CD11c$^+$ tissue resident macrophages (blue). Blood vessels are shown in red. Major ticks 50 μm

1283 and 20 μm (zoom). Time in min:s.

1284

1285 **Movie S5. Intravital imaging of T$_{RM}$ migration in the absence of macrophages.** T$_{RM}$ (green) show migration

1286 in the absence of macrophages but are mostly confined to individual acini and ducts. Stroma is white, and

1287 outlines of acini and ducts are indicated in magenta. Time in min:s.

1288

1289 **Movie S6. Intravital imaging of T$_{RM}$ migrate along a macrophage to enter acini.** T$_{RM}$ (green) migrating along

1290 a macrophage (blue) to enter an acinus. Stroma is white, and outlines of acini and ducts are indicated in

1291 magenta. T$_{RM}$ entering the acinus is indicated with an arrowhead. Second half of the Supplementary Video

1292 shows the image sequence in 3D. For better visibility, one acinus has been masked manually. Time in min:s.

1293

Movie S7. Intravital imaging of T$_{RM}$ migration in the absence of macrophages after 5 days of DTx treatment. T$_{RM}$ (green) show migration in the absence of macrophages but are confined to individual acini and ducts (white). Time in min:s.

Movie S8. Time-lapse SUSHI of SMG section during hyperosmotic challenge. Extracellular space (black) at time point "0:00" depicts image before starting at "0:20" with gradual increase of osmolarity to 350 mOsm/L. During challenge, ECS increases, in particular in interstitium. Scale bar, 10 µm. Time in h:min.

Movie S9. Confocal image of tissue macrophage protrusions and basement membranes in SMG. T$_{RM}$ (green) are in close proximity to tissue macrophages (blue). EpCAM-1 (white) depicts epithelial cells. Close up shows a tissue macrophage protruding between basement membranes labeled by laminin (magenta).

Movie S10. Intravital imaging of T$_{RM}$ migration and association with macrophages after blocking of αL, α4 and αE integrins. T$_{RM}$ (green) move rapidly and associate with macrophages (blue) 16 h after treatment with integrin-blocking mAbs. Blood vessels are shown in red. Scale bar, 20 µm and 10 µm (zoom). Time in min:s.

Movie S11. Intravital imaging of T$_{RM}$ migration and association with macrophages after treatment with PTx. T$_{RM}$ (green) move rapidly and associate with macrophages (blue) 3 h after treatment with PTx. Scale bar, 20 µm and 10 µm (zoom). Time in min:s.

Movie S12. Time-lapse imaging of T$_N$ migration under *in vitro* confinement on CCL21/ICAM-1-coated plates. T$_N$ (red) show efficient migration on CCL21/ICAM-1-coated glass slides under agarose. Scale bar, 50 µm. Time in min:s.

Movie S13. Time-lapse imaging of T$_{RM}$ migration under *in vitro* confinement on CXL10 + CXCL12/ICAM-1-coated plates. T$_{RM}$ (green) show efficient migration on chemokine/ICAM-1-coated glass slides under agarose. Scale bar, 20 µm. Time in min:s.

1321

1322 **Movie S14. Time-lapse imaging of T_{RM} and T_N migration under *in vitro* confinement on HSA-coated plates.**

1323 T_{RM} (green) but not T_N (red) show efficient migration on HSA-coated glass slides under agarose. Scale bar, 50

1324 µm. Time in min:s.

1325

1326 **Movie S15. Time-lapse imaging of T_{RM} and T_{PLN-M} migration under *in vitro* confinement.** T_{RM} (left) but not

1327 T_{PLN-M} (right) show efficient migration on HSA-coated glass slides under agarose. Major ticks 20 µm. Time in

1328 min:s.

1329

1330 **Movie S16. High temporal resolution analysis of T_{RM} motility under *in vitro* confinement.** T_{RM} migration on

1331 HSA-coated glass slides under agarose. Scale bar, 10 µm. Time in min:s.

1332

1333 **Movie S17. Time-lapse imaging of T_{RM} and tissue macrophages under *in vitro* confinement.** T_{RM} (green)

1334 detaching from SMG macrophages (blue) under agarose. Scale bar, 50 µm. Time in min:s.

1335

1336 **Movie S18. Time-lapse imaging of SMG and epidermal T_{RM} under *in vitro* confinement.** SMG T_{RM} (green)

1337 and epidermal T_{RM} (red) under agarose. Scale bar, 20 µm. Time in min:s.

1338

1339 **Movie S19. Time-lapse imaging of T_{RM} migration in presence of EDTA.** Left panel. T_{RM} form protrusions but

1340 do not translocate under agarose in the presence of EDTA. Right panel. T_{RM} migration is restored within

1341 clusters of T_N (red), which serve as scaffold for T_{RM} motility. Major ticks 5 µm. Time in min:s.
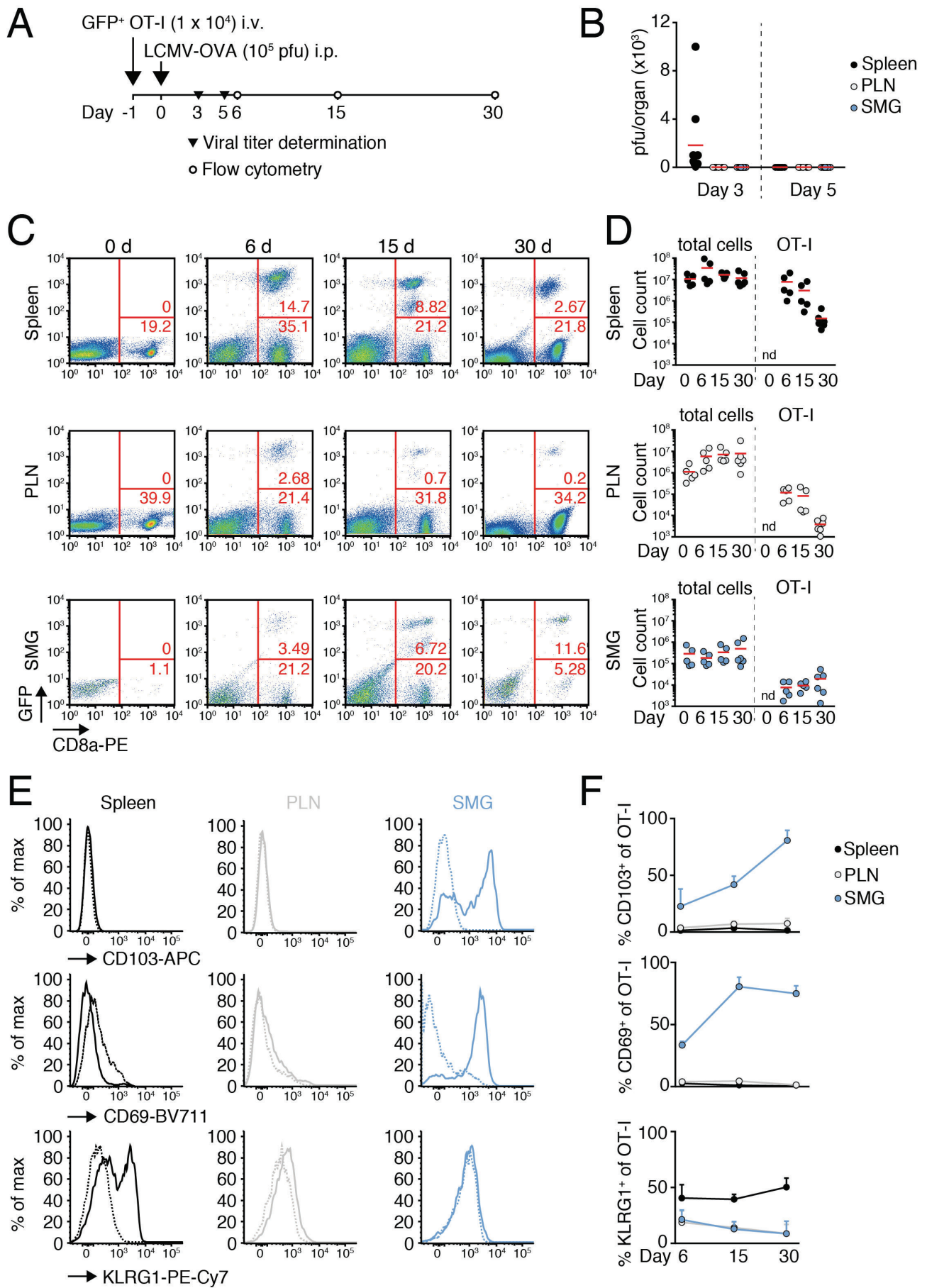
1342

1343 **Movie S20. Time-lapse imaging of T_{RM} migration inside T_N cluster in presence of EDTA.** T_{RM} (green) show

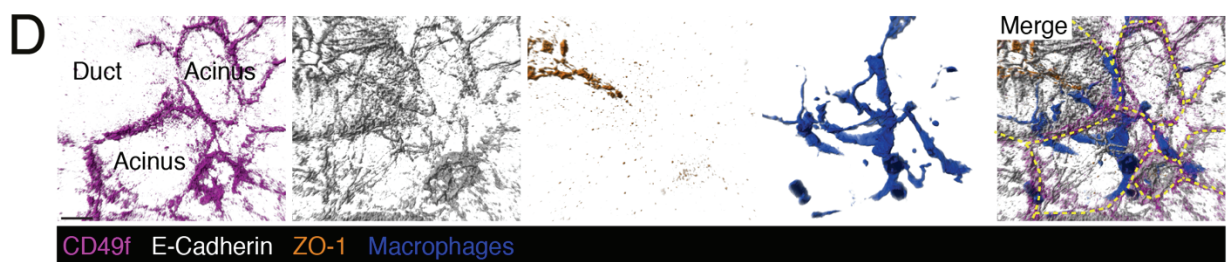1344 motility while traversing a cluster of T_N in presence of EDTA. Nuclei are labelled with Hoechst (blue). Major
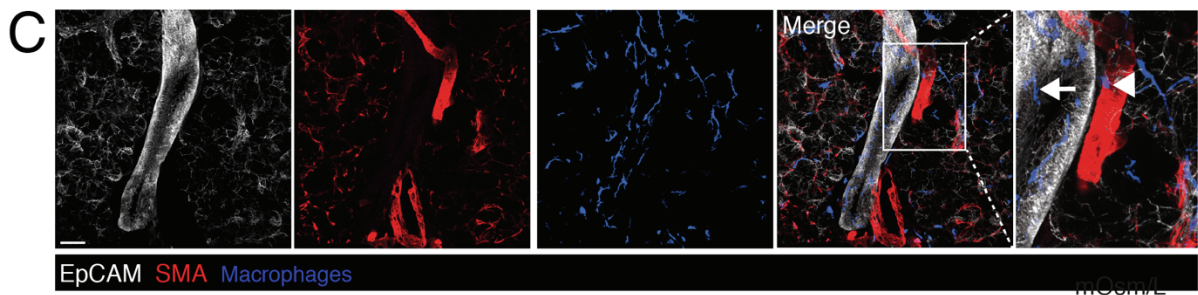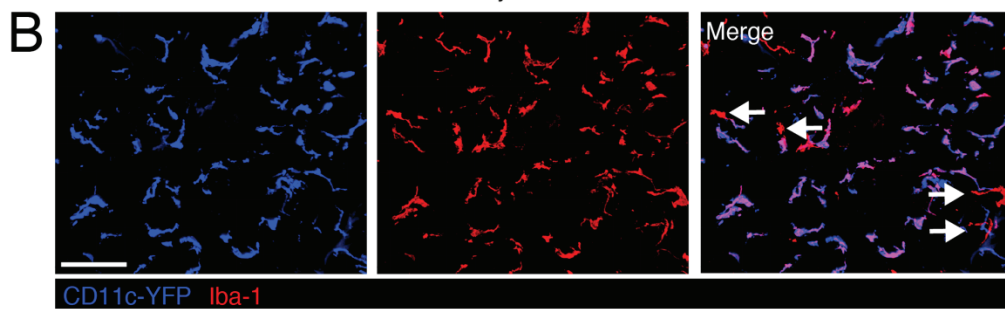
1345 ticks 5 µm. Time in min:s.

1346

1347   **Movie S21. Time-lapse imaging of T_{RM} migration along polystyrene beads in presence of EDTA.** T_{RM} (green)

1348   show motility while migrating in contact with polystyrene beads in presence of EDTA. Scale bar, 20 μm. Time

1349   in min:s.

Figure S1

A

Live cells → Singlets →

B

CD11c-YFP Iba-1

C

EpCAM SMA Macrophages

mOsm/L

D

Duct Acinus

Acinus

Merge

CD49f E-Cadherin ZO-1 Macrophages
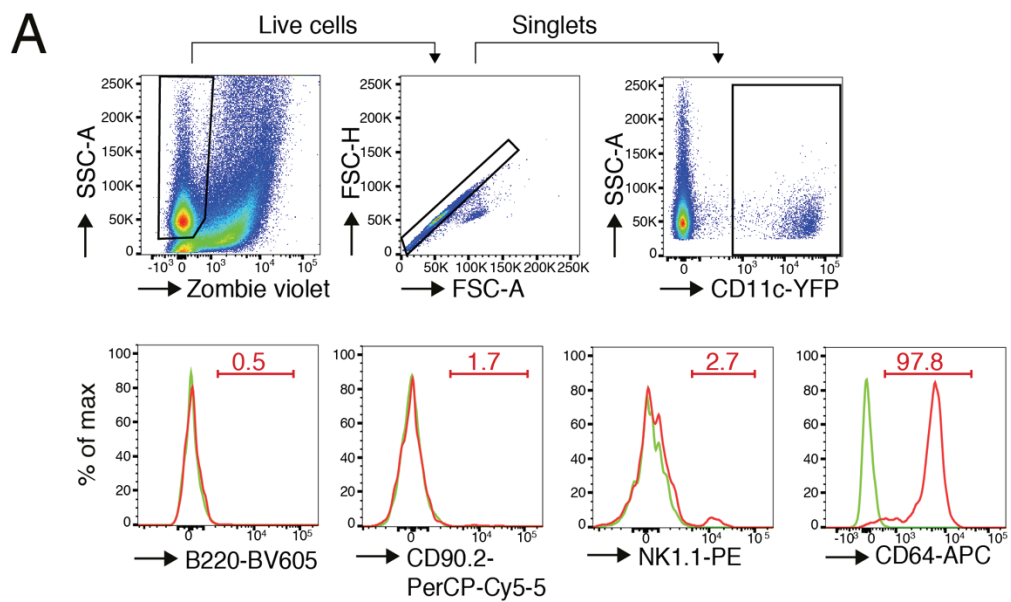
1351   Figure S2

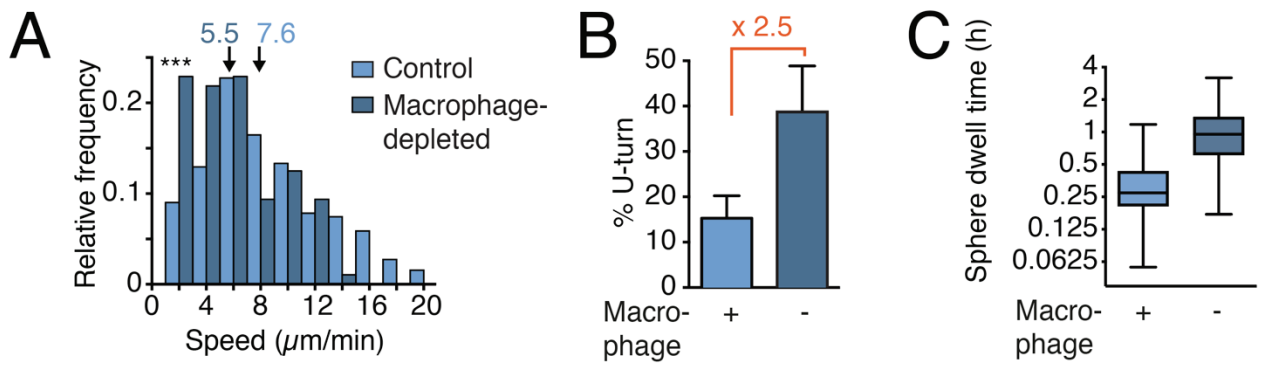1352   Figure S3

A

LCMV-OVA i.p.
GFP+ OT-I i.v.

CD11c-YFP or
CD11c-DTR

4 weeks → + DTx → 24 h → WD infection with MCMV-OVA → 48 h → Confocal analysis of SMG

B

Sublingual gland

Submandibular gland

OT-I MCMV-OVA

C

OT-I MCMV-OVA Macrophages Laminin

D

OT-I MCMV-OVA Macrophages

E

mCherry+ spots/FOV

2800
2400
2000
1600
1200
800
400
0

*** ***

T_{RM} + + −
Macrophages + − −

F

mCherry+ intenstiy/ spots (a.u.)

300

200

100

0

***

T_{RM} + −

1353   Figure S4

A

Spleen | PLN | SMG (alpha1-PE, alpha2-PE, alpha4-PE, alphaL-PE)

Spleen | PLN | SMG (alphaV-PE, beta1-PE, beta2-PE, beta7-biotin+SA-PE)

B

PLN — 99.5% | 1.05%
SMG — 27.0% | 2.95%

CD45-PerCP → GFP

CD45$^+$ | CD45$^+$ GFP$^+$

% of max → alphaE + anti-rat Cy5

1st + 2nd Ab
2nd Ab only

C

T$_{PLN-M}$
*** 8.8 11.7

Relative frequency vs Speed (μm/min)

WT
αItg

D

Macrophage T$_{RM}$
β1 integrin

E

Macrophage T$_{RM}$
Merge
E-cadherin

F

T$_{PLN-M}$
*** 8.6 11.3

Relative frequency vs Speed (μm/min)

PTx$_{mut}$
PTx

1354 Figure S5

1355