1 **Microbial uptake in oral mucosa-draining lymph nodes leads to rapid release of**

2 **cytotoxic CD8^{+} T cells lacking a gut-homing phenotype**

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28 **Running title**

29 T cell priming in oral mucosa-draining lymph nodes.

30

31 **Summary**

32 *Listeria monocytogenes* ingestion leads to priming of cytotoxic T cells in oral mucosa-

33 draining mandibular lymph nodes, which contribute to early systemic host protection.

34 **Abstract**

35 The gastrointestinal (GI) tract constitutes an essential barrier against ingested microbes,

36 including potential pathogens. While immune reactions are well-studied in the lower GI tract,

37 it remains unclear how adaptive immune responses are initiated during microbial challenge

38 of the oral mucosa (OM), the primary site of microbial encounter in the upper GI tract. Here,

39 we identify mandibular lymph nodes (mandLN) as sentinel lymphoid organs that intercept

40 ingested *Listeria monocytogenes* (Lm). Oral Lm uptake led to local activation and release of

41 antigen-specific $CD8^+$ T cells that constituted the majority of the early circulating effector T

42 cell (T_{EFF}) pool. MandLN-primed T_{EFF} disseminated to lymphoid organs, lung and OM, and

43 contributed substantially to rapid elimination of target cells. In contrast to $CD8^+$ T_{EFF}

44 generated in mesenteric lymph nodes (MLN) during intragastric infection, mandLN-primed

45 T_{EFF} lacked a gut-seeking phenotype, which correlated with low expression of enzymes

46 required for gut homing imprinting by mandLN stromal and dendritic cells. Accordingly,

47 mandLN-primed T_{EFF} decreased Lm burden in spleen but not MLN after intestinal infection.

48 Our findings extend the concept of regional specialization of immune responses along the

49 length of the GI tract, with $CD8^+$ T_{EFF} generated in the upper GI tract displaying homing

50 profiles that differ from those imprinted by lymphoid tissue of the lower GI tract.

51

Introduction

The digestive, or gastrointestinal (GI), tract constitutes the major external surface of the human body and comprises the upper GI tract with oral cavity, pharynx and esophagus, and the lower GI tract with stomach, small and large intestine and rectum. The GI tract needs to offset permissive uptake and digestion of nutrients and water with protection against microbes, including potential pathogens. Accordingly, GI immune responses range from tolerance against commensals and food antigens to reactivity against ingested pathogens. Thus far, innate and adaptive immune responses have been most extensively studied in the lower GI tract *(1-6)*. Gut-associated lymphoid tissues (GALT) of the small intestine such as Peyer's patches (PP), together with gut lymph nodes (gLN), form complementary inductive sites for intestinal immune reactions *(7)*. M cells embedded in the epithelium overlying GALT sample luminal antigen (Ag), while gLN including mesenteric LN (MLN) screen intestinal lymph to intercept pathogens, which have breached the epithelial barrier. Both MLN and PP promote the generation of $CD4^+$ and $CD8^+$ T effector cells (T_{EFF}) expressing the signature gut-homing receptors $\alpha_4\beta_7$ and CCR9 *(8)*. Induction of a gut-seeking phenotype in activated lymphocytes relies on retinoic acid (RA) synthesis by retinal aldehyde dehydrogenase family 1 (Aldh1) family members expressed by $CD103^+$ migratory dendritic cells (DC) and stromal cells of intestinal Ag-sampling lymphoid tissue *(9-11)*. Recent findings have further refined our understanding of gut immunity by identifying compartmentalized tolerogenic and inflammatory $CD4^+$ T cell immune responses in individual gLN, which drain lymph from proximal versus distal segments of the intestinal GI tract *(12, 13)*. Thus, the intestinal tract is characterized by regional specialization of adaptive immune responses according to the local microenvironment *(6)*.

The upper GI tract, in particular the mucosa of the oral cavity, also contains an abundant microbiota and acts as the first site of contact with dietary Ag and ingested microbes, including potential pathogens *(14, 15)*. The oral barrier has recently gained attention for its complex immune network characterized by abundant $CD4^+$ T cells and numerous macrophage subsets *(14-16)*. As example, periodontitis, which represents the most common

4

80   inflammatory disease in humans, is triggered by extracellular bacteria *(15)*. This chronic

81   condition imprints Th17 CD4$^+$ T cells with gut-homing capacity, which have recently been

82   linked to inflammatory disorders of the lower GI tract *(17)*. In addition to chronic infection,

83   recent reports have identified the oral cavity as a prominent site of airborne viral replication,

84   including influenza virus and SARS-CoV-2 *(18, 19)*. Similarly, oral ingestion is the most

85   common route of entry for pathogens including *Trypanosoma cruzi* and *Listeria*

86   *monocytogenes* (Lm) causing Chagas disease and listeriosis, respectively *(20)*

87   (https://www.who.int/news-room/fact-sheets/detail/listeriosis). Listeriosis is one of the most

88   serious foodborne diseases and caused by eating contaminated food with high numbers of

89   Lm.

90   Yet, despite the global rise of orally transmitted diseases, the initiation of adaptive immune

91   responses against microbes sampled from oral mucosa (OM) has not been well

92   characterized, in particular for cytotoxic CD8$^+$ T cell responses that occur in response to

93   intracellular pathogens. To the best of our knowledge, no comprehensive attempt has been

94   made to map OM-draining LN, although in a rodent periodontitis model, antibacterial

95   responses are detectable in mandibular and accessory mandibular LN (here collectively

96   abbreviated as mandLN; also sometimes referred to as superficial cervical LN) *(21-23)*. Our

97   current understanding of the local immune responses upon oral pathogen exposure is limited

98   since most oral immunization models in small rodents bypass the oral cavity by employing

99   intragastric (i.g.) gavage. In models where pathogens are administered intraorally (i.o.), the

100 analysis of immune responses often excludes lymphoid tissue of the neck and head region

101 *(24, 25)*. Thus, the precise priming location, magnitude and kinetics of oral CD8$^+$ T cell

102 responses remain unaddressed to date. Similarly, it remains unclear whether the ensuing

103 T$_{EFF}$ phenotype and dissemination pattern are comparable to the ones observed during

104 intestinal infections and whether local oral cavity-triggered immune reactions impact on

105 immune responses at distal sites *(14)*.

106 Here, we mimicked foodborne listeriosis by Lm administration into the oral cavity of

107 immunocompetent mice or by uptake of Lm-contaminated food. We detected early priming

108   and proliferation of Ag-specific CD8$^+$ T cells in mandLN that preceded activation kinetics in

109   spleen, MLN and PP. MandLN-primed CD8$^+$ T$_{EFF}$ constituted the majority of early circulating

110   T$_{EFF}$ and disseminated to secondary lymphoid organs (SLO), lung and OM, and contributed

111   to systemic host protection. In contrast to CD8$^+$ T$_{EFF}$ primed in MLN after i.g. Lm gavage,

112   mandLN CD8$^+$ T$_{EFF}$ did not display a gut-homing phenotype, correlating with low expression

113   of genes imprinting gut homing in the mandLN microenvironment. Our observations expand

114   the emerging concept of a compartmentalized host immune response along the length of the

115   entire GI tract according to the Ag sampling location and identify OM-draining mandLN as a

116   first-line "immunological firewall" after ingestion of microbial pathogens.

Results

Ingested Lm is intercepted in mandLN and leads to local LN hyperplasia

To examine drainage of OM lymph in C57BL/6 mice, we injected a lymph tracer into gingiva. Within ≤ 5 min after tracer injection, we observed its rapid accumulation in draining mandLN (**Fig. 1A**), suggesting a connecting lymphatic network between oral cavity and mandLN. Based on histological analysis, the gingiva adjacent to teeth was reported to contain numerous lymphatic vessels, which are located more superficially than in most other mucosal tissues *(21, 26)*. To obtain an overview of the intact oral lymphatic network, we carefully exposed the mandibular gingiva and draining mandLN in a Prox1-GFP reporter mouse, which allows the identification of lymphatic vessels *(27)* (**Fig. S1A**). We confirmed the presence of an extensive gingival lymphatic network around the mandibular incisors (**Fig. 1B, C**) connected to mandLN (**Fig. 1D**).

Next, we investigated the relevance of lymphatic drainage in an oral ingestion model. Lm is a foodborne gram-positive bacterium, which infects macrophages and hepatocytes in its target organs spleen and liver and is cleared by cytotoxic $CD8^+$ T_{EFF}. Lm expressing the model antigen ovalbumin (Lm-OVA) *(28)* was directly administered into the oral cavity of C57BL/6 mice. In the first 8 days post i.o. infection, we isolated mandLN, spleen, MLN and PP of infected mice and determined Lm-OVA spread by colony forming unit (CFU) analysis. We compared these values to CFU counts after i.v. administration of Lm-OVA, the most commonly used route for Lm infection in experimental immunology (**Fig. 1E**). We observed that mandLN contained the highest Lm-OVA CFU per organ on d 1 following i.o. infection, whereas spleen contained the highest CFU following systemic administration (**Fig. 1F**). On d 3 after i.o. infection, Lm-OVA became also detectable in spleen (**Fig. 1F**). In contrast, Lm-OVA CFU counts in MLN and PP remained low or undetectable in the first week after i.o. infection (**Fig. 1F**). This is in line with the notion that > 90% of ingested bacteria are killed by gastric acids *(29, 30)*. Residual Lm in the intestinal lumen may have been degraded by digestive enzymes, or failed to cross the mucus layer of the intestinal epithelium or their tight junctions, or become cleared by peristaltic contraction and mucus secretion. Together, these

145 factors likely contribute to the delayed spread of Lm to PP and MLN in our model of oral

146 ingestion.

147 To explore whether high bacterial load correlates with increased lymphocyte numbers in

148 reactive LN, we determined lymphoid organ cellularity following i.v. and i.o. Lm-OVA

149 infection. We observed a rapid onset of mandLN hyperplasia following i.o. infection,

150 consistent with local bacterial sampling at early time points (**Fig. 1G**). In contrast, i.v. Lm-

151 OVA infection did not cause mandLN hyperplasia at any time point analyzed, whereas

152 splenocyte numbers became significantly increased on d 8 p.i. (**Fig. 1G**). In our oral ingestion

153 model, MLN and PP cellularity remained unchanged after Lm infection, consistent with

154 limited CFU detection in the first week p.i. at these sites (**Fig. 1G**).

155 Next, we examined the mechanism underlying microbial sampling of OM by mandLN.

156 Expression of a mutant internalin InlA^m in Lm increases its binding affinity to mouse E-

157 cadherin by four orders of magnitude *(31)*. As a result, i.g. gavage of Lm-InlA^m leads to a

158 higher *in vivo* virulence as compared to non-murinized InlA Lm strains *(32)*. To examine

159 whether the interplay of InlA and E-cadherin facilitates early Lm accumulation in mandLN, we

160 compared CFU counts of Lm-OVA and Lm-InlA^m after i.o. infection. Lm-InlA^m CFU counts in

161 mandLN were significantly increased at 24 h p.i. as compared to Lm-OVA, suggesting that

162 increased adhesion to E-cadherin$^+$ OM epithelium might increase transport to mandLN (**Fig.

163 1H**). As a caveat, this finding may have been influenced by the higher virulence of the Lm

164 strain carrying the InlA mutation as compared to the Lm-OVA strain *(33)*. To further

165 investigate the mechanism underlying the rapid appearance of ingested Lm in mandLN, we

166 administered i.o. Lm-OVA in CCR7$^{-/-}$ recipients, which lack DC trafficking from peripheral

167 tissues to sentinel LN *(34, 35)*. Bacterial loads in CCR7$^{-/-}$ mandLN on d 1 p.i were

168 comparable to WT values, suggesting that active DC migration to mandLN was not a limiting

169 factor for initial Lm accumulation in draining LN (**Fig. 1I**).

170 These observations prompted us to examine a passive transport mechanism for ingested Lm

171 via oral cavity-draining lymphatic vessels. We administered GFP-expressing Lm (Lm-GFP)

172 onto the gingiva of mice prepared for intravital imaging to directly explore its retention in

173   draining mandLN. To this end, we adapted a model originally developed for submandibular

174   salivary gland surgery *(36)* to expose the adjacent mandLN (**Fig. S1B**). Lm-GFP

175   accumulated in the subcapsular sinus (SCS) of mandLN within 120 min post oral deposition,

176   as assessed by two-photon microscopy (2PM)-based intravital imaging of surgically prepared

177   mice (**Fig. 1J**). To assess whether the GFP signal reflects extracellular versus internalized

178   Lm, we treated mandLN single cell suspensions at d 1 post i.o. infection with Lm-OVA or Lm-

179   InlA^m with antibiotics to inhibit growth of extracellular Lm {Jones2015}. These data suggested

180   that most recovered Lm colonies were of extracellular origin (not shown). Taken together, our

181   data suggest that oral uptake of Lm leads to its drainage to mandLN, at least in part via

182   afferent lymphatics of the OM.

183

184   *Ag-specific CD8$^+$ T cells form clusters and dynamically interact with CD11c$^+$ cells in mandLN*

185   *following i.o. Lm infection*

186   To assess the relevance of Lm capture in mandLN on CD8$^+$ T cell activation, we transferred

187   GFP-, dsRed- or tdTom-expressing OT-I TCR tg T cells, which recognize the OVA$_{257-264}$

188   peptide in the context of H-2K^b *(37)*, together with fluorescently labeled polyclonal CD8$^+$ T

189   cells into CD11c-YFP mice. In these recipients, CD11c$^+$ cells including antigen-presenting

190   DC express YFP *(38)*. One day later, we i.o. infected mice with Lm-OVA and isolated

191   mandLN at 1, 2 and 3 d p.i. for histological analysis (**Fig. 2A**). At 1 d p.i., we observed

192   occasional large clusters of OT-I but not polyclonal CD8$^+$ T cells around CD11c$^+$ cells in both

193   mandibular and accessory mandibular LN, suggesting early Ag-driven interactions (**Fig. 2B**,

194   left panel). These clusters became smaller on d 2 and 3 post i.o. infection (**Fig. 2B**, middle

195   and right panel). As changes in dynamic T cell behavior act as sensitive indicator for priming

196   and precede detectable expression of activation markers *(39, 40)*, we performed 2PM of

197   mandLN in C57BL/6 or CD11c-YFP recipients containing OT-I T cells and polyclonal control

198   CD8$^+$ T cells during the first 72 h post i.o. Lm-OVA infection. To benchmark physiological T

199   cell behavior in steady state, we analyzed naïve OT-I motility parameters in mandLN of

200   uninfected mice. OT-I T cells displayed high speeds (15.4 $\pm$ 5.1 µm/min; mean $\pm$ SD), low

201   arrest coefficients (median of 1.6% of track segments < 5 µm/min; **Fig. 2D, E**) and a

202   corrected track straightness of 8.3 ± 2.7 (median $\pm$ SD, corresponding to a non-corrected

203   meandering index of 0.61 ± 0.21) *(41)* (**Video S1**). These values are comparable to T cell

204   motility parameters observed in non-inflamed skin-draining LN *(42-44)*, and serve as

205   reference for inflammation-induced changes of dynamic T cell behavior. At d 1 post i.o. Lm

206   infection, polyclonal CD8^{+} T cells displayed a random motility pattern comparable to T cell

207   migration in non-inflamed mandLN, albeit with slightly decreased speeds (12.0 ± 4.5 µm/min)

208   and increased arrest coefficients (**Fig. 2C-E**). This finding is in line with mild motility changes

209   displayed by non-cognate T cells in reactive LN *(43)*. In contrast, many antigen-specific OT-I

210   T cells were found to cluster around CD11c^{+} APC on d 1 and 2 p.i. (**Fig. 2C; Video S2**), and

211   most OT-I T cells, in particular in clusters, displayed decreased speeds and high arrest

212   coefficients (**Fig. 2D, E**). Such a behavior is consistent with interaction dynamics driven by

213   high cognate pMHC levels displayed on APC *(40, 45)*. Starting on d 2 post i.o. Lm infection,

214   OT-I T cell speeds and arrest coefficients began to recover, suggesting decreasing Lm-

215   derived Ag presentation (**Fig. 2D, E**). On d 3 post i.o. Lm infection, OT-I cells had dispersed

216   and showed speeds and arrest coefficients largely comparable to polyclonal CD8^{+} T cells

217   (**Fig. 2D, E; video S3**). The analysis of the corrected track straightness followed a similar

218   pattern, with OT-I T cells showing transient confinement on d 1 and 2 p.i., which became less

219   apparent on d 3 p.i. (**Fig. 2F**). Taken together, our confocal and dynamic imaging data are

220   consistent with fast and efficient antigen processing in OM-draining mandLN, leading to

221   cognate T cell – APC interactions within the first 2 d post i.o. infection.

222

223   *Oral infection-induced CD8^{+} T cell activation in mandLN is mediated by resident and*

224   *migratory DC*

225   Imaging of oral-cavity-draining mandLN suggested local OT-I T cell priming by CD11c^{+}

226   antigen-presenting DC. We therefore compared the activation status of CD103^{-} DC, which

227   mostly comprise LN resident cells, and migratory CD103^{+} DC in resting and i.o. challenged

228   mandLN on d 1 post i.o. Lm-OVA infection (**Fig. 3A**). Both populations showed higher and/or

229   more frequent expression of CD80 and CD86 as compared to DC isolated from non-infected

230   mandLN, in line with an activated phenotype (**Fig. 3B, C**). We obtained similar results when

231   we analyzed CD80 and CD86 activation markers gating on resident MHCII^{intermediate} CD11c^{high}

232   versus migratory MHCII^{high} CD11c^{intermediate} DC (**Fig. S2A-C**). While d 1 i.o. Lm-OVA-infected

233   LN showed only a minor expansion in total CD11c$^+$ MHCII$^+$ DC counts (from 15.3 ± 3.4 to

234   17.1 ± 3.8 x 10^4 cells/LN; mean $\pm$ SD), the percentage and absolute cell numbers of CCR7-

235   expressing CD103$^+$ and CD103$^-$ cDC1 was significantly increased (from 3.9 ± 1.6 to 7.1 ± 2.0

236   x 10^3 and 1.1 ± 0.2 to 2.4 ± 1.5 x 10^3 cells/LN, respectively; **Fig. S2D**). To further examine

237   whether resident and/or migratory DC were able to activate CD8$^+$ T cells, we sorted MHCII$^+$

238   CD11c$^+$ CD103$^-$ and CD103$^+$ DC from d 1 i.o. Lm-OVA-infected mandLN. Isolated DC

239   subsets were separately co-cultured *ex vivo* with Cell Tracer Violet (CTV)-loaded OT-I T cells

240   expressing Nur77-GFP as sensitive readouts for proliferation and TCR signaling, respectively

241   *(46)* (**Fig. 3D**). Both DC subsets induced Nur77-GFP, CD69 and CD25 expression in OT-I T

242   cells, as well as proliferation within 48 h (**Fig. 3E, F**). Thus, while initial Lm accumulation in

243   mandLN occurs in the absence of migratory DC (**Fig. 1I**), these cells contribute to local CD8$^+$

244   T cell activation in our experimental model.

245

246   *Oral Lm ingestion-induced mandLN T$_{EFF}$ generation precedes CD8$^+$ T cell activation in*

247   *spleen and MLN*

248   We compared our *in vitro* observations with *in vivo* induction of early activation markers

249   CD69 and CD25 on OT-I T cells following i.o. Lm-OVA infection (**Fig. 4A**; for gating strategy

250   see **Fig. S2E**). To exclude potential cross-contamination by interorgan cell trafficking after

251   Lm infection, we performed these experiments in the presence of FTY720, which sequesters

252   T cells in lymphoid tissue. On d 1 post i.o. Lm infection, mandLN OT-I T cells displayed

253   increased levels of CD69, followed by augmented CD25 expression starting on d 2 p.i. (**Fig.**

254   **4B, C**). At this time point, OT-I T cells in spleen and MLN showed a delayed and less

255 pronounced increase in these markers compared to mandLN OT-T cells (**Fig. 4B, C**).

256 Consistent with increased CD25 expression, more than half (58 ± 14.7%; median ± SD) of

257 mandLN OT-I T cells had undergone cell proliferation at d 3 post i.o. infection, whereas only

258 12 ± 5.3% and 5 ± 14% of spleen and MLN OT-I T cells had proliferated, respectively (**Fig.**

259 **4D, E**). We observed the same trend on d 4 post i.o. infection (**Fig. 4E**). As a result, OT-I T

260 cell numbers increased almost tenfold in mandLN on d 3 post i.o. infection, whereas i.v.

261 infection induced a delayed and less pronounced expansion of OT-I T cells (**Fig. 4F**).

262 We then characterized general activation markers on T_{EFF} generated following i.o. and i.v. Lm

263 uptake. Irrespective of the route of infection, mandLN T_{EFF} were CD44^{high} CD62L$^{low\ or\ high}$ on d

264 5 p.i., whereas spleen T_{EFF} were predominantly CD44^{high} CD62L^{low} (**Fig. 4G, H**). In the

265 memory phase (d 30 p.i.), most OT-I T cells showed a CD44^{high} CD62L^{high} central memory-

266 like phenotype while we detected a minor population of CD44^{high} CD62L^{low} effector memory-

267 like cells in spleen (**Fig. 4H**), again independent of the route of infection. We further

268 examined OT-I differentiation into CD127^{-} KLRG-1^{+} short-lived effector cells (SLEC) and

269 CD127^{+} KLRG-1^{-} memory precursor effector cells (MPEC) after i.o. *versus* i.v. Lm infection

270 (**Fig. 4I**). In both routes of infection, we observed comparable SLEC and MPEC proportions

271 on d 5 p.i. (**Fig. 4H**). At d 30 p.i., KLRG1^{-} CD127^{+} central memory-like T cells prevailed in

272 mandLN and spleen after either infection route (**Fig. 4H**). In sum, our data show that T cell

273 interactions with resident and migratory CD11c^{+} antigen-presenting cells result in T_{EFF}

274 generation in OM-draining mandLN, preceding their activation in other SLO. The similar

275 phenotype of effector and memory T cells irrespective of the route of infection likely reflects

276 the Lm spread by d 3 to 5 p.i. (**Fig. 1F**) and subsequent mixing of activated T cell

277 populations.

278

279 *CD8^{+} T cell activation in mandLN occurs over a wide range of Lm inocula and ingestion of*

280 *Lm-contaminated food*

281 Similar to most published oral infection models, we initially used a large inoculum to induce

282 an adaptive immune response. To test the impact of initial bacterial load on local OT-I T cell

283   activation, we titrated the Lm inoculum and measured OT-I T cell activation and proliferation

284   on d 3 post i.o. infection in the presence of FTY720 (**Fig. S3A**). We observed a dose-

285   dependent effect on total mandLN cellularity and percent $CD69^+$ endogenous $CD8^+$ T cells,

286   which became less pronounced with decreasing Lm inoculum (**Fig. S3G-I**). This was

287   reflected by a Lm inoculum-dependent activation marker expression and proliferation in OT-I

288   T cells (**Fig. S3B**). Notably, we observed increased expression of CD69 and CD25, as well

289   as proliferation with an inoculum of only 10^6 Lm (**Fig. S3C-F**). Next, we examined whether

290   OT-I activation is also observed after ingestion of foodborne Lm-OVA. To restrict lymphocyte

291   circulation, we performed these assays in the presence of FTY720 (**Fig. S3J**). Our data

292   confirmed an increase in cellularity and $CD69^-$ and $CD25^+$ OT-I numbers in mandLN at d 3

293   p.i. (**Fig. S3K-N**). Thus, sufficient antigenic material is collected by sentinel mandLN across

294   four orders of magnitude of initial Lm inoculum and following consumption of contaminated

295   food to trigger local $CD8^+$ T_{EFF} generation.

296

297   *MandLN-primed T_{EFF} represent the majority of the early systemic cytotoxic T cell pool*

298   To quantify to which extent mandLN OT-I cells contribute to the early circulating $CD8^+$ T_{EFF}

299   pool, we performed i.o. infections in the absence or presence of FTY720 (**Fig. 5A**). FTY720

300   treatment led to a 4.4-fold increase in mandLN OT-I T_{EFF} on d 4 post i.o. Lm infection,

301   corresponding to an increase from 33 ± 3 to 144 ± 30 x 10^4 cells/LN (mean $\pm$ SEM). This

302   increase was mirrored by a concomitant 67% reduction in OT-I T_{EFF} isolated from spleen

303   (from 126 ± 39 to 41 ± 7 x 10^4 cells in the absence and presence of FTY720, respectively),

304   suggesting that more than 70% of egressed mandLN T_{EFF} relocated to spleen (**Fig. 5B**). On

305   day 5 p.i., we observed a 5-fold increase in mandLN OT-I T_{EFF} (from 26 ± 8 to 127 ± 18 x 10^4

306   cells/LN) in presence of FTY720 (**Fig. 5B**). This was accompanied by a decrease in splenic

307   OT-I T_{EFF} numbers after FTY720 treatment (499 ± 165 and 209 ± 54 x 10^4 cells/spleen in

308   absence and presence of FTY720, respectively; **Fig. 5B**) although this did not reach

309   statistical significance. The partial rescue of spleen OT-I numbers on d 5 likely reflects Lm

310   dissemination to spleen by d 3 p.i. and onset of OT-I cell division by d 4 p.i. (**Fig. 1F and Fig.**

311   **4E**). In contrast, MLN OT-I T cell numbers on d 4 or 5 p.i. were not significantly affected by

312   FTY720 treatment (**Fig. 5B**).

313   To directly examine the relocation of mandLN T_{EFF} to other lymphoid organs, we adoptively

314   transferred OT-I T cells expressing the photoconvertible protein Kikume Green-Red (KikGR)

315   *(47)* one d prior to i.o. Lm-OVA infection. On day 3 p.i., we photoconverted exposed mandLN

316   and isolated mandLN, spleen, MLN, inguinal LN and PP 14 h later for flow cytometry-based

317   detection of activated (CD44^{high}) KikRed$^+$ photoconverted OT-I T cells (**Fig. 5C, D**). As

318   predicted, we found most CD44^{high} KikRed$^+$ cells in mandLN, reflecting ongoing cell activation

319   (**Fig. 5E**). We recovered the second highest absolute number of photoconverted T_{EFF} in

320   spleen ($1.8 \pm 0.4 \times 10^4$ cells/organ), several times higher than in MLN ($0.6 \pm 0.1 \times 10^4$

321   cells/organ) and other lymphoid organs. Taken together, these data provide direct evidence

322   that CD8$^+$ T cell priming in mandLN leads to a rapid release of T_{EFF} into the circulation, with a

323   substantial fraction localizing to spleen.

324   We examined to which extent these early mandLN T_{EFF} contribute to systemic host protection

325   through target cell elimination. To this end, we transferred OT-I T cells into mice that had

326   received an anti-CD62L (L-selectin) mAb 4 h earlier, which selectively blocks T cell entry into

327   peripheral LN (PLN) but only partially affects homing to MLN and PP, while T cell homing to

328   spleen is not impaired (**Fig. 5F**). We confirmed that anti-CD62L mAb treatment resulted in >

329   8-fold decrease in mandLN cellularity that was maintained until d 4 post blockade, while

330   inducing a trend to increased spleen cell numbers and an intermediate reduction on MLN cell

331   numbers (2.2-fold reduction; **Fig. 5G**). In these anti-CD62L mAb-treated recipients, OT-I T

332   cell homing to and priming in mandLN was therefore severely impaired, without affecting

333   CD8$^+$ T cell activation in spleen. On d 3 post i.o. Lm-OVA infection, we transferred a 1:1 mix

334   of fluorescently labeled control and OVA$_{257-264}$-pulsed B cells as surrogate target cells and

335   analyzed their ratio in the spleen 12 h post transfer (**Fig. 5H**). Both cell populations were

336   equally recovered from spleens of recipient animals that had not received OT-I T cell and

337   Lm-OVA (ratio OVA$_{257-264}$-pulsed to unpulsed B cells: 0.97 ± 0.05; mean $\pm$ SD; **Fig. 5I**). In

338   control mice that had received OT-I T cells and i.o. Lm-OVA without anti-CD62L mAb

339   treatment, most $OVA_{257-264}$-pulsed B cells were eliminated (ratio $OVA_{257-264}$-pulsed compared

340   to unpulsed B cells: 0.04 ± 0.05; **Fig. 5I**). In contrast, in anti-CD62L mAb-treated recipients,

341   significantly more $OVA_{257-264}$-pulsed B cells were recovered (ratio $OVA_{257-264}$-pulsed

342   compared to unpulsed B cells: 0.59 ± 0.17; **Fig. 5H and I**). This data correlated with strongly

343   reduced OT-I T cell numbers in spleens of anti-CD62L-mAb-treated recipients as compared

344   to control recipients (**Fig. 5J**), which contrasts with the high spleen cellularity in this setting

345   (**Fig. 5G**). Taken together, our data uncover a substantial contribution of T_{EFF} primed in

346   draining mandLN for early containment of an intracellular pathogen, which is reflected by a

347   more than tenfold increase of target cell killing.

348

349   *mandLN T_{EFF} priming does not induce expression of signature gut homing receptors*

350   Recent reports have identified a link between chronic inflammation of the oral cavity and the

351   induction of Th17 $CD4^+$ T cells in the draining mandLN that migrate to the intestine and

352   trigger colitis *(17, 48)*. We therefore set out to assess whether T_{EFF} generated in OM-draining

353   mandLN similarly gained the capacity to home and protect against infections of the lower GI

354   tract. To this end, we compared the chemokine and adhesion receptor expression, as well as

355   effector cytokine profile between i.o.-primed mandLN $CD8^+$ T_{EFF}, spleen T_{EFF} primed after i.v.

356   (systemic) and MLN T_{EFF} generated by i.g. infection. In addition, we performed subcutaneous

357   (s.c.) Lm infections to compare the OT-I T_{EFF} phenotype after priming in oral cavity-draining

358   mandLN versus skin-draining popliteal LN (popLN) (**Fig. 6A**). Flow cytometry analysis on d 5

359   following i.o., i.v., i.g. and s.c. Lm-OVA infections revealed that irrespective of the route of

360   infection, OT-I T_{EFF} increased expression of the adhesion receptors α_L (CD11a), α_4 (CD49d)

361   and β_1 (CD29) integrins as compared to endogenous $CD8^+$ T cells (**Fig. 6B**). While mandLN-

362   primed OT-I showed highest CD11a levels, MLN- and to a lesser extent spleen-primed T_{EFF}

363   expressed high levels of α_4 and β_7 integrins (**Fig. 6B**). In contrast, the skin homing receptor

364 cutaneous lymphocyte antigen (CLA) was only induced in popLN T_{EFF}, whereas $\alpha1$ integrin

365 expression was only modestly induced by any route of infection (**Fig. S4A**).

366 Next, we examined expression of the activation-induced chemokine receptors CXCR3,

367 CX3CR1 and CXCR6. Irrespective of the route of infection, OT-I T_{EFF} increased CXCR3

368 surface levels (**Fig. 6C**). Furthermore, OT-I T_{EFF} increased CX3CR1 and CXCR6 levels (**Fig.**

369 **6C**), in particular spleen-primed T_{EFF}, which may reflect the high Lm burden in this organ after

370 i.v. infection (**Fig. 1F**). In contrast, we did not find evidence for elevated surface expression

371 of CCR4 and CCR6 in any subset, whereas we detected a minor population of CCR10$^+$ MLN

372 T_{EFF} (**Fig. S4**). We further compared effector protein levels in T_{EFF} activated via distinct

373 infection routes. Spleen-primed OT-I T_{EFF} showed the highest proportion of Granzyme B$^+$

374 cells (**Fig. 6D**), while mandLN- and popLN-primed T_{EFF} expressed increased levels of TNFα,

375 IFN-γ, and in case of mandLN T_{EFF} also IL-2 (**Fig. 6D**).

376 To explore in more detail on the gut homing capacity of mandLN T_{EFF}, we examined

377 expression the MAdCAM-1 receptor $\alpha_4\beta_7$ using direct labeling of the heterodimer. In line with

378 published observations *(8, 25)*, we confirmed increased $\alpha_4\beta_7$ levels on MLN- but not

379 mandLN-primed OT-I T_{EFF} (**Fig. 6E**). Furthermore, while the gut homing chemokine receptor

380 CCR9 was induced in MLN-primed T_{EFF} (29.8 $\pm$ 6.8% mean $\pm$ SEM from 2 independent

381 experiments with n = 8 mice), mandLN-primed OT-I did not increase CCR9 expression (**Fig.**

382 **6E**). We confirmed the lack of a gut-homing phenotype imprinting on CD8$^+$ T cells after

383 ingestion of Lm-contaminated food (**Fig. S4B**). These data demonstrate that in an acute

384 infection, mandLN-primed T_{EFF} do not increase signature chemokine and adhesion receptors

385 associated with homing to intestine or skin, but expressed high cytokine levels.

386

387 *mandLN T_{EFF} home preferentially to SLO, lung and OM but not small intestine*

388 We tested how local imprinting affected the dissemination pattern of T_{EFF} generated at

389 distinct lymphoid priming organs. We isolated congenically marked mandLN- and spleen-

390 primed T_{EFF} isolated from FTY720-treated donor mice at 4 d after infection by the i.o. and i.v.

route with Lm-OVA and co-transferred them into i.o. Lm-infected mice (**Fig. 6F**). We chose

the i.o. route of infection for recipients in this model, because we wanted to address the

homing capacity of T_{EFF} populations to the OM. As an internal control for homing to the

intestine, we also co-transferred MLN-primed T_{EFF} generated by i.g. Lm-OVA infection (**Fig.**

S5A). At d 4 post transfer (d 8 p.i.), we injected fluorescently labeled CD45 mAb prior to

mouse culling to distinguish circulating from parenchymal cells and isolated spleen, mandLN,

MLN, PP, liver, lung, submandibular salivary glands (SMG), OM and small intestine (SI) of

recipient mice (for gating strategy see **Fig. S5B**). Owing to the low recovery rate of MLN-

primed T_{EFF} from donor mice, we could transfer only limited numbers of cells and did not

detect cells of this effector population in all organs. Therefore, we analyzed mandLN- versus

spleen-primed OT-I T_{EFF} homing separately from MLN- versus spleen-primed T_{EFF} homing.

When corrected for the input, we recovered on d 8 p.i. more parenchymal mandLN-primed

OT-I as compared to spleen-primed OT-I in most recipient organs examined (**Fig. S5C**).

While this could indicate an increased tissue infiltration capacity of mandLN-primed T_{EFF}, it

might reflect the earlier initiation of the contraction phase in spleen-primed T_{EFF} because of

the high pathogen burden associated with i.v. infections (**Fig. 1F**). In support of this, we

consistently observed lower CD62L and CD127 but increased KLRG1 expression on

recovered spleen-primed OT-I as compared to mandLN-primed OT-I T_{EFF} (**Fig. S5D, E**). We

therefore normalized the homing ratios of recovered parenchymal MLN- or mandLN-primed

OT-I *versus* spleen-primed OT-I T_{EFF} in the analyzed organs to the mean value of their ratio

in recipient spleens. As control, transferred MLN-primed OT-I T_{EFF} were 4.1 ± 0.9 more

frequent in SI as compared to transferred spleen-primed OT-I T_{EFF} after normalization to

recipient spleen mean ratios (**Fig. 6G**), in line with their high $\alpha_4\beta_7$ and CCR9 expression

levels. In addition, MLN-primed T_{EFF} recovery from OM and liver was strongly increased as

compared to spleen-primed T_{EFF} (x 6.1 ± 2.3 and x 8.0 ± 3.3, respectively), whereas it

remained comparable in mandLN, MLN, PP and lungs (x 1.1-1.9) (**Fig. 6G**).

417   Similarly, transferred mandLN-primed OT-I T_{EFF} were more frequent as compared to spleen-

418   primed OT-I T_{EFF} in mandLN (x 2.8 ± 0.6; mean ± SEM), MLN (x 2.4 ± 0.2), and in particular

419   in lungs (x 3.7 ± 0.4) and OM (x 4.2 ± 1.6) (**Fig. 6H, I**). In contrast, mandLN-primed T_{EFF} were

420   underrepresented as compared to spleen-primed T_{EFF} in PP (x 0.8 ± 0.1), liver (x 0.6 ± 1),

421   SMG (x 0.6 ± 0.1) and most prominently in SI (x 0.4 ± 0.1), reflecting low induction of $\alpha_4\beta_7$

422   and CCR9 expression in these cells (**Fig. 6H, I**). Taken together, mandLN- and MLN-primed

423   T_{EFF} generated during infection of the upper and lower GI tract differ markedly in their

424   dissemination pattern, with mandLN T_{EFF} being substantially impaired in SI homing.

425

426   *MandLN stromal cells and DC display low expression of RA-producing enzymes*

427   We further examined the mechanism underlying the low tropism of mandLN-primed T_{EFF} for

428   the lower GI tract. The lymphoid microenvironment including stromal cells and DC provides

429   critical cues for tissue-selective imprinting of effector homing potential. A hallmark of

430   intestinal mucosa-surveilling inductive lymphoid tissue is the RA-mediated induction of gut-

431   homing receptors on activated T cells, which promote their subsequent accumulation in

432   lamina propria, GALT and gLN *(8)*. To characterize the gene expression profile of stromal

433   cells, we performed a single cell RNA sequencing (scRNAseq) analysis of the TER-119^{-}

434   CD45^{-} stromal compartment of non-infected mandLN and compared it with stromal cells

435   isolated from resting MLN and as control, skin-draining inguinal, axillary and brachial PLN.

436   Unsupervised clustering of combined stromal scRNAseq data identified multiple Col1a1[+] and

437   Col1a2[+] fibroblast-like and Cdh5^{+} vascular cell populations (**Fig. 7A and Fig. S6**). While

438   scRNAseq data suggested comparable expression of the RA-producing enzyme Aldh1a1 in

439   fibroblast-like cells LN of all three sites, expression of Aldh1a2 and Aldh1a3 was higher in

440   MLN than in PLN or mandLN fibroblasts (**Fig. 7B, C**). Furthermore, the transcription factor

441   WT1, which drives expression of Aldh1a1 and Aldh1a2 *(49)*, was mainly expressed in MLN

442   fibroblast-like cells (**Fig. 7B, C**). To corroborate these data, we performed qPCR analysis for

443   RA-generating enzymes on sorted TER-119^{-} CD45^{-} stromal cells isolated from MLN, PLN

444   and mandLN. This analysis confirmed that mRNA levels of all three Aldh1a family members

445   were higher in MLN as compared to PLN and mandLN stromal cells (**Fig. 7D**). Since DC

446   contribute to gut-homing phenotype imprinting in activated T cells *(10)*, we isolated CD45$^+$

447   CD11c$^+$ MHC-II^{high} DC from MLN, PLN and mandLN and compared mRNA levels of Aldh1a1-

448   3 by qPCR. While Aldh1a1 and Aldh1a3 were expressed at low levels in all CD11c$^+$

449   populations, Aldh1a2 mRNA levels were highest in MLN CD11c$^+$ cells (**Fig. 7D**). Taken

450   together, the mandLN microenvironment shares with skin-draining PLN the lack of gut-

451   homing imprinting capacity. At the same time, scRNAseq data analysis uncovered increased

452   expression of genes involved in "lymphocyte and monocyte chemotaxis" and "cellular

453   responses to IL-1, IFN-γ and TNF" in *bona fide* mandLN CCL21a$^+$ T cell zone reticular cells

454   (TRC; cluster 7 in **Fig. 7A**) as compared to PLN TRC, whereas genes related to "collagen

455   fibril organization" and "negative regulation of DC differentiation" were decreased (**Fig. S7**).

456   In combination with increased expression of genes for "T cell costimulation" in mandLN TRC

457   *versus* MLN TRC (**Fig. S7**), these data support the presence of a pro-inflammatory mandLN

458   microenvironment that facilitates rapid generation of CD8$^+$ T$_{EFF}$ against oral pathogens.

459

460   *MandLN T$_{EFF}$ reduce Lm burden in spleen but not MLN and PP*

461   We set out to correlate these data with the protective capacity of mandLN-generated T$_{EFF}$ as

462   compared to those generated in spleen and MLN. We adoptively transferred OT-I CD8$^+$ T

463   cells and infected recipient mice separately by i.o., i.v. or i.g. Lm-OVA administration in the

464   presence of FTY720. On day 5 p.i., we isolated OT-I T$_{EFF}$ from mandLN, spleen and MLN,

465   respectively, and transferred equal numbers of effector cells separately into secondary

466   recipient mice, which were subsequently infected with Lm-OVA (**Fig. 8A**). We chose the i.g.

467   route of infection for secondary recipients to induce Lm spread to MLN and PP, as i.v. or i.o.

468   administration of Lm does not result in efficient MLN or intestinal infection *(50)*. This

469   approach thus allowed to address the degree of protection afforded by transferred T$_{EFF}$

470   against regional (intestinal) *versus* systemic Lm burden, with the caveat of the distinct

471   cytokine and GzmB expression profiles induced by distinct routes of priming (**Fig. 6D**).

472   Irrespective of the priming site, transferred OT-I T_{EFF} showed a comparable reduction of

473   bacterial burden in spleen on d 3 p.i. (**Fig. 8B**), while liver was not strongly infected in our

474   setting (not shown). In contrast, mandLN-primed OT-I T_{EFF} did not confer protection in MLN

475   or PP, similar to results obtained after transfer of splenic-primed OT-I T_{EFF} (**Fig. 8B**). In turn,

476   MLN-primed OT-I T_{EFF} caused a reduction in the percentage of MLN and PP containing

477   detectable Lm in secondary recipients (**Fig. 8B**), suggesting a close correlation of gut-

478   homing receptor expression and protection against intestinal Lm infections. This decrease

479   became significant for bacterial loads in MLN when compared to "no OT-I" or transfer of

480   "mandLN-" and "spleen-primed OT-I T_{EFF}" in side-by-side comparisons ($p < 0.05$, Mann-

481   Whitney). In sum, in our model OT-I T_{EFF} generated in OM-draining mandLN constitute a

482   large fraction of the early circulating T_{EFF} pool after pathogen ingestion. Our findings further

483   indicate that orally primed T_{EFF} are capable to reduce Lm burden in spleen and conceivably

484   other SLO, lung and OM, but do not contribute to substantial protection of the lower GI tract

485   and associated intestinal lymphoid tissue (**Fig. 8C**).

486

Discussion

Antimicrobial immune responses have been extensively examined in the gut, leading to the discovery of site-specific imprinting of the host immune response along the length of the intestinal GI tract. In contrast, there is a lack of information on the initiation of adaptive immune responses after microbial challenge of the OM, the primary site of pathogen encounter of the upper GI tract. Furthermore, it has remained unknown how orally triggered T cell activation affects immunity at distal sites. Here, we performed a comprehensive analysis of the kinetics, magnitude and phenotype of CD8$^+$ T cell responses after acute oral exposure to an intracellular pathogen. We also examined the contribution of orally primed CD8$^+$ T cells to the early circulating T$_{EFF}$ pool, their dissemination patterns and protective capacity. Our data reveal that after ingestion of *Listeria monocytogenes*, mandLN serve as OM-sentinel lymphoid organs. Our data further show that the mandLN microenvironment supports rapid generation of cytotoxic T$_{EFF}$ with a markedly distinct homing profile from the one imprinted by intestinal lymphoid tissue, further expanding the concept of regional specialization of adaptive immunity along the GI tract.

The mucosa lining the oral cavity is the first site of contact with ingested microbes before their passage to the esophagus, stomach and intestinal tract. The presence of lymphatic vessels in the OM suggests a continuous surveillance of regional lymph by sentinel LN *(26, 51)*. The sequestration of orally administered Lm in mandLN observed here confirms that lymphatic drainage leads to continuous sampling of oral microbes at this anatomical localization, similar to observations made after oral delivery of *Trypanosoma cruzi (52, 53)*. It remains unclear how Lm entry into oral lymphatic vessels is regulated. In the oral cavity, the gingival sulcus, which is the space between the gingiva and teeth, is a particularly vulnerable site exposed to trauma caused by mastication and biting. Its epithelium is non-keratinized and transitions to the junctional epithelium that binds directly to teeth *(14, 15)*. Conceivably, lymphatic vessels below the crevicular epithelium lining the gingival sinus may participate in collecting microbe-containing tissue fluids for transport to mandLN. In addition, the vascular-rich sublingual mucosa has absorptive properties, which is clinically relevant to systemically

515 administer drugs or vaccines *(15)*. Oral microbiota might be collected here for lymphatic

516 transport to regional LN, a process which may be facilitated by the lack of a thick mucus

517 layer as is present in intestinal mucosa. In this context, mice lack tonsils, and lumen-

518 sampling M cells are restricted to nasopharynx-associated lymphoid tissue (NALT) of the

519 nasal cavity *(54)*. Our data further suggest a role for migratory DC in transporting processed

520 microbial Ag from the OM to mandLN. It has been reported that infection with foodborne Lm

521 leads to the appearance of endogenous T_{EFF} in MLN and PP one week post Lm infection

522 *(25)*. We expand these observations here by showing that mandLN harbor activated $CD8^+$ T

523 cells within the first 3 d following exposure to a foodborne intracellular pathogen, highlighting

524 the role of mandLN as first line immunological firewall of the upper GI tract.

525 One of the hallmarks of cellular immune responses is the imprinting of tissue-specific homing

526 molecules during T cell activation. In skin-draining LN, sunlight-generated vitamin D3 triggers

527 expression of CCR4, CCR10 and P- and E-selectin ligands, while GALT and gLN process

528 dietary carotenes and retinol to imprint a gut-homing $\alpha_4\beta_7^+$ $CCR9^+$ phenotype *(9, 55)*. This

529 remarkable "division of labor" ensures an optimal use of resources by directing T cell

530 responses to the anatomical site of pathogen entry. Most of this paradigm has been

531 established by analyzing lymphocyte activation in lymphoid tissue of the lower GI tract. This

532 raises the question why mandLN differ from those sites, even though both drain lymph from

533 microbe-rich mucosal tissue. One explanation is that the induction of RA-generating

534 enzymes in migratory $CD103^+$ DC for gut-homing phenotype imprinting in T cells requires RA

535 generated by epithelial cells of the small intestine *(11, 56, 57)*. Similarly, we observed low

536 expression of RA-producing enzymes in stromal cells of mandLN, suggesting that RA is not

537 present in sufficiently high concentration in oral cavity-draining mandLN to direct substantial

538 T_{EFF} trafficking towards gut. In line with this, mandLN do not only drain lymph from the OM

539 but also of the anterior eye chamber, NALT and cerebrospinal fluid of the central nervous

540 system (CNS) *(22, 54, 58, 59)* (**Fig. 8C**). This may exert an influence on the expression of

541 genes that direct homing patterns in dendritic and stromal cells, e.g. to minimize loss of $CD8^+$

542 T_{EFF} to intestinal sites during CNS inflammation. In support of our findings, i.n. Lm infection

543   leading to Ag presentation in NALT does not confer intestinal immunity *(25)*. In a reverse

544   conclusion, our data, in combination with previous studies *(13)*, suggest that induction of gut-

545   homing molecules is most efficient in lymphoid tissue of the small intestine. This locally

546   defined imprinting restricts massive infiltration of gut mucosal tissue with orally primed $CD8^+$

547   T cells reacting to microbial Ag of the upper GI tract, whose microbiomes do not strongly

548   overlap *(60)*. Thus, our data suggest the presence of a "staggered" immune response where

549   acute oral microbial exposure will not induce pre-emptive immune responses in the lower GI

550   tract. Instead, intestinal immune responses are only triggered when microbes pass the

551   stomach barrier and reach the gut.

552   While mandLN stromal cells and DC resemble more their skin-draining LN than gut-draining

553   MLN counterparts, we also uncovered a distinct baseline gene expression signature of skin-

554   draining versus mandLN stroma (**Fig. S7**), which potentially reflects a higher proinflammatory

555   microenvironment in the latter owing to continuous oral microbiome exposure. In this context,

556   mandLN priming does not induce expression of hallmark receptors involved in skin homing,

557   such as CLA or CCR4, presumably reflecting limited drainage of dermal tissue. Instead,

558     $CD8^+$ T_{EFF} primed in mandLN showed efficient homing to other SLO including mandLN and

559   spleen, as well as OM and lung. Although we have focused here on their contribution to early

560   target cell killing in spleen, it is highly probable that these cells contribute to elimination of

561   spreading intracellular infections in all target organs including the respiratory tract. This may

562   be of particular importance for host protection given the close anatomical proximity of the

563   upper airways and the oral cavity, which is exploited by airborne viruses to spread to lungs.

564   As a result, early interception of orally encountered microbes will conceivably contribute to

565   the generation of an early T cell response, which in recent clinical studies has been shown to

566   correlate with efficient immunity and rapid clearance in airborne virus infections *(61, 62)*.

567   An open question is to which extent mandLN priming contributes to the generation of

568   systemic and OM $CD8^+$ memory cells. After adoptive transfer of mandLN OT-I T_{EFF} into

569   infection-matched recipients, we recovered memory T cells in spleen but not in MLN or PP

570   (not shown). Furthermore, the requirements for entry into the oral cavity are not well defined

571   to date. Our finding that mandLN-primed T_{EFF} home efficiently to OM despite poor gut homing

572   receptor expression suggests that $\alpha_4\beta_7$ and CCR9 are dispensable for this process. Of note,

573   2PM imaging and confocal imaging identified memory T cells in the OM at > 30 d post i.o. Lm

574   infection (**Video S4**; **Fig. S8**), suggesting that mandLN may contribute to local memory

575   generation of their surveilled barrier tissue.

576   PP and gLN constitute a complementary surveillance system of the lower GI tract, with PP

577   containing gut lumen-sampling M-cells and gLN draining lymphatic vessels originating in

578   lamina propria. Our data suggest that together with M-cell-containing MALT including tonsils

579   (in humans) and NALT, mandLN form a comparable "dual surveillance" system for the

580   oropharyngeal section of the upper GI tract (**Fig. 8C**). The role for mandLN in this process

581   has thus far remained largely overlooked, since most immunologists use i.v. infection of Lm

582   as a robust systemic infection model or bypass the oral cavity by i.g. infection. Taken

583   together, our study adds to the increasingly acknowledged site-specific imprinting of host

584   immune responses along the length of the GI tract, consistent with an instructional role for

585   the Ag sampling location *(6, 13)*.

Material and methods

Mice

Male and female C57BL/6JRj (Janvier, Le Genest-Saint-Isle, France), CCR7[tm1Rfor] (CCR7^{-/-}) *(63)*, Tg(Itgax-Venus)1Mnz "CD11c-YFP" *(38)* and Tg(Prox1-EGFP)KY221Gsat "Prox1-GFP" *(27)* mice were used for imaging, as recipients for T cell adoptive transfer or for Lm infection. Polyclonal T cells were isolated from C57BL/6 or C57BL/6-Tg(CAG-EGFP)1Osb/J "GFP" donors *(64)*. Tg(TcraTcrb)1100Mjb "OT-I" TCR mice *(37)* backcrossed on a GFP[+], dsRed[+] *(65)* or tdTom[+] *(66, 67)* background were described before *(68)*. Mice expressing the photoconvertible protein Kikume-Green-Red (KikGR) *(47)* were backcrossed to the OT-I strain. In some experiments, CD45.1, CD90.1 and CD45.1/.2 x CD90.1/.2-expressing OT-I were used. Mice were kept at the central animal facilities of the University of Fribourg. All experimental procedures are approved by the Cantonal Committees for Animal Experimentation and conducted according to federal guidelines (2018_22_FR, 2018_24_FR, 2018_27_FR).

T cell purification and adoptive transfer

Spleens and LN were dissociated using 70 μm cells strainers, and CD8^{+} T cells were negatively isolated using the EasySep[TM] Mouse CD8^{+} T cell (Stem Cell Technologies) or MojoSort[TM] Isolation Kits (BioLegend) according to the manufacturer's protocol. Purity of isolated CD8^{+} T cells was > 90%. For 2PM imaging, polyclonal CD8^{+} T cells were labelled with 20 μM CellTracker[TM] Blue (CMAC; 7-amino-4-chloromethylcoumarin; Invitrogen) for 20-30 min at 37° C. For proliferation assays, OT-I T cells were labelled with 5 μM CellTrace[TM] Violet (Invitrogen) at 37° C for 20-30 min.

Bacterial infection and CFU quantification

Lm strain 10403s expressing ovalbumin (Lm-OVA) *(28)* or GFP (Lm-GFP) *(69)* and *Lm* strain EGD-e, carrying a recombinant InlA with S192N and Y369S mutations (Lm-InlA[m]) *(32)* were kindly provided by Dr. Doron Merkler (University of Geneva, Switzerland), Neal M. Alto

614 (University of Texas, Southwestern Medical Center, US) and Colin Hill (University College

615 Cork, Ireland), respectively. Bacteria from glycerol stocks were grown in Brain heart infusion

616 (BHI) medium until mid-log phase was reached. Recipient mice were deprived of food and

617 water for 4 h and 15-30 min before and after infection, respectively. Infections performed by

618 pippeting Lm into the mouth over 1-5 min as described *(70)* (i.o.; 1 x 10^6 – 2 x 10^9 CFU), by

619 intragastric gavage (i.g.; 2 x 10^9 CFU), or by i.v. (5 x 10^3 CFU) or s.c. (1 x 10^4 CFU) injection.

620 Bacterial suspensions were serially diluted and plated on BHI agar plates to verify the actual

621 number of CFU in the inoculum. For foodborne infection, mice were food- and water-deprived

622 for 4 h prior to infection. Bacteria suspension containing Lm-OVA (2 x 10^9 CFU) was pipetted

623 over small pieces of the mouse chow (0.25-0.5 cm^3) and mice were kept individually in cages

624 with minimal bedding for 1-2 h until complete food consumption *(71)*.

625 For CFU quantification, mandLN, MLN, PP and spleen were aseptically harvested,

626 homogenized in 70 µm cell strainers and lysed in sterile water. Alternatively, organs were

627 homogenized using MagNALyser tubes Green Beads (Roche) containing sterile PBS (5000

628 rpm for 1 min). Serial dilutions of homogenates were plated on BHI agar, and colonies were

629 counted after 24 h of incubation at 37°C. For PP, BHI agar plates contained 200 µg/mL

630 streptomycin to prevent gut bacteria contamination, since the 10403s strain is strepromycin

631 resistant. CFU was adjusted according to the dilutions and calculated per number of LN or

632 PP collected. For protection experiments, spleen, liver, PP and MLN were lysed in 2 mL

633 tubes containing one steal bead and 1 mL PBS + 0.1% Tween20 (Sigma-Aldrich, St. Louis,

634 USA), using a QiaTissueLyzer (Qiagen) at 25 Hz, 3 min. Serial dilutions were plated on BHI

635 agar, and colonies were counted after 48 h of incubation at 30°C. For PP, BHI agar plates

636 contained 200 µg/mL streptomycin. CFU was adjusted according to the dilutions and

637 calculated per g of organ.

638

639 *FTY720 treatment*

640 Lymphocyte egress from lymphoid tissue was blocked by treating mice intraperitoneally with

641 2 mg/kg FTY720 (Sigma), a sphingosine-1-phosphate receptor 1 (S1PR1) inhibitor, starting

642 at d 1 p.i. with repeated doses after 48 h.

643

644 *Flow cytometry*

645 LN, PP and spleen were harvested at the indicated time points and single cell suspensions

646 were obtained by passing organs through 70 µm cell strainers. Red blood cell lysis was

647 performed on splenocytes. Fc receptors were blocked with purified anti-CD16/CD32 mAb

648 (2.4G2) in FACS buffer (PBS with 2% FCS, 2 mM EDTA and 0.05% NaN_3) for 10 min. Cell

649 surface staining was performed on ice for 30 min in the dark, followed by wash in FACS

650 buffer. Zombie Aqua™, Zombie Violet™ or Zombie Red™ (BioLegend) were used to

651 discriminate live and dead cells. After staining, cells were fixed for 20 min with 4% PFA,

652 washed and resuspended in PBS. Acquisition was performed using LSR Fortessa (BD

653 Bioscience), LSR II (BD Bioscience) or Attune NxT Flow cytometer (Thermo Fisher) and

654 analyzed using FlowJo (TreeStar) software.

655 For cytokine staining, cells were restimulated *in vitro* with 1 µM SIINFEKL (EMC

656 Microcollections) in the presence of 5 µg/mL brefeldin A (BioLegend) for 5 h at 37° C, 5%

657 CO_2. Surface staining was performed in the presence of 5 µg/mL brefeldin A, followed by

658 fixation with 4% PFA for 20 min on ice. For intracellular staining, cells were permeabilized

659 with 1x PermWash solution (BD Biosciences). Staining for CCR8, CX3CR1, CXCR6, CCR4,

660 CCR7, CCR10 and CCR6 was performed for 1 h at 37°C.

Antibody/streptavidin	Fluorochrome	Clone	Concentration (µg/mL)	Company	Reference number
CCR10	PE	248918	1:20 dilution	R&D	FAB2815P
CCR8 (CD198)	BV421	SA214G2	2	BioLegend	150305
CD103	APC	2 E7	2	BioLegend	121414
CD103	BV421	2E7.	2	Biolegend	121422
CD11a (integrin αL)	BV605	M17/4	1	BD Biosciences	740347
CD11b	PE-Cy7	M1/70	0.4	BioLegend	101215/101216

CD11c	BV605	N418	2	BioLegend	117334
CD127	APC	A7R34	2	BioLegend	135012
CD186 (CXCR6)	BV711	SA051D1	2	BioLegend	151111
CD19	biotinylated	6D5	5	BioLegend	115503
CD194 (CCR4)	biotinylated	2G12	10	BioLegend	131216
CD196 (CCR6)	PE-Cy7	29-2L17	2	BioLegend	129816
CD197 (CCR7)	PerCP/Cy5.5	4B12	5	BioLegend	120105
CD199 (CCR9)	PE-Cy7	CW1.2	1	BioLegend	128711
CD25	APC	PC61	1-2	Biolegend	102012
CD29 (integrin β1)	AF488	HMβ1-1	2.5	BioLegend	102211
CD3	PerCP/Cy5.5	17A2	2	BioLegend	100217
CD44	biotinylated	IM7	2.5	BD Biosciences	553132
CD44	PerCP	IM7	2	BioLegend	103036
CD44	PerCP-Cy5.5		2	BioLegend	103032
CD44	BV605	IM7	1-2	BioLegend	103047
CD45	PE-Cy5	30-F11	2.5µg/mouse i.v.	Biolegend	103110
CD45.1	PE	A20	1	BioLegend	110708
CD45.1	BV785	A20	1	Biolegend	110743
CD45.1	FITC	A20	2.5	Biolegend	110706
CD45.2	AF488	A20	2.5	BioLegend	110718
CD45.2	BUV395	104	1	BD Biosciences	564616
CD45R (B220)	AF647	RA3-6B2	1	BioLegend	103229
CD49a (integrin α1)	BUV496	Ha31/8	4	BD Biosciences	741111
CD49d (integrin α4)	PE/Cy7	R1-2	2	BioLegend	103617
CD62L	BV421	MEL-14	0.5	BioLegend	104436
CD62L	AF488	MEL-14	2.5	BioLegend	104420
CD69	BV711	H1.2F3	1	BioLegend	104537
CD69	PE	H1.2F3	1	BioLegend	104508
CD69	APC-Cy7	H1.2F3	2	Biolegend	104526
CD80	FITC	16-10A1	2.5	BioLegend	104705
CD86	APC-Fire 750	GL-1	1	BioLegend	105045
CD8α	APC/Fire 750	53-6.7	1	BioLegend	100766
CD8α	BV711	53-6.7	0.5	Biolegend	100748
CD90.1	BV711	OX-7	0.1	Biolegend	202539
CD90.2	Pacific blue	53-2.1	0.25	Biolegend	140306

CLA (cutaneous lymphocyte antigen)	AF647	HECA-452	1	BioLegend	321310
CX3CR1	BV650	SA011F11	2	Biolegend	149033
CXCR3 (CD183)	BV510	CXCR3-173	2	BioLegend	126527
CXCR3 (CD183)	BV605	CXCR3-173	2-4	BioLegend	126523
Granzyme B	AF647	GB11	2	BioLegend	515405
Granzyme B	FITC	GB11	2	BioLegend	515403
I-A/I-E (MHCII)	BV421	M5/114.15.2	0.5	BioLegend	107632
I-A/I-E (MHCII)	APC-Cy7	M5/114.15.2	0.2	Biolegend	107628
IFN-γ	BV711	XMG1.2	1	BioLegend	505835/6
IL-2	PE	JES6-5H4	2	BioLegend	503807
KLRG1	PE-Cy7	2F1	1	BioLegend	138415
LPAM-1 (Itga4b7)	APC	DATK32	2	eBioscience	17-5887-82
Ly6C	AF700	HK1.4	0.6	Biolegend	128024
Ly6G	biotinylated	1A8	2.5	BioLegend	127603
Ly-6G/C	biotinylated	Gr-1	2.5	BioLegend	108403
NK1.1	PE	PK136	1	BioLegend	108707
NK1.1	biotinylated	PK136	2.5	BioLegend	108703
Rabbit IgG	AF568	polyclonal	5	Thermo Fisher	A11011
RFP	purified	polyclonal	2	Abcam	ab62341
SIRPalpha/CD172a	AF488	P84	2	Biolegend	144023
Streptavidin	BV711	_	0.5	BioLegend	405241
Streptavidin	PE	_	2	BioLegend	405203
Streptavidin	BUV737	_	1	BD Biosciences	564293
Streptavidin	AF488	_	2	Thermo Fisher	S11223
TCRβ	biotinylated	H57-597	2.5	BioLegend	109203
TNF-α	BV421	MP6-XT22	1	BioLegend	506328
XCR1	BV785	ZET	2	Biolegend	148225
β7	biotinylated	FIB504	1.25	BioLegend	321209

661 **Table 1.**

662 For flow cytometry analysis of DC, LN were cut in small fragments and digested in type IV

663 collagenase (1 mg/mL, Worthington) and DNase I (100 U/mL; Roche) in 24-well plates under

664 rotation at 20 rpm for 15-20 min at 37°C. Tissue fragments were dissociated by pipetting

665 using a Pasteur pipette and incubated for additional 15 min. In case tissue fragments were

666 not completely dissociated by pipetting, samples were incubated for additional 10 min. For

667 sorting of DC and stromal cells, we used collagenase P (0.5 mg/mL; Roche), Dispase II (0.5

668 mg/mL; Roche) and DNase I (100 U/mL; Roche) for digestion in a 5 mL round bottom tube.

669

670 *Intravital imaging of mandLN and gingiva*

671 Mice were anesthetized by i.p. injection of 8-10 µL/g ketamine (20 mg/mL) and xylazine (1

672 mg/mL) and after 15 min, 30 µL i.p. of acepromazine (2.5 mg/mL). Anesthesia was

673 supplemented when needed with ketamine/xylazine (half dose of xylazine). The fur was

674 removed from the operating area using an electric razor followed by hair removal cream.

675 Mice were placed on a customized stage used for submandibular salivary gland (SMG)

676 imaging *(36)*. Neck and teeth were fixed to reduce shifting. To expose mandLN, a 0.5-1 cm

677 incision was made along the neck region in the skin above the left lobe of the SMG. Under

678 the stereomicroscope, the connective tissue between left and right lobes of the SMG, and

679 between the left lobe and the adjacent skin was disrupted to set the left lobe free. The

680 mandLN and surrounding blood and lymph vasculature were kept untouched and moist with

681 saline during the surgical procedure. The left lobe of the SMG was pulled on top of a

682 coverslip on a holder and glued using veterinary adhesive (Vetbond, M3). A ring of grease

683 (Glisseal N, VWR) was made to create space to be filled with saline and keep tissue moist.

684 The SMG was fixed in a position to expose the mandLN and for removal of fat tissue. A

685 coverslip was placed on top, slightly touching the LN without impairing blood supply. To

686 maintain tissue at 35-38°C, a heating ring was placed on top as described for other LN

687 surgeries *(72)*.

688 To expose the gingiva, a 0.5 cm incision was made in the lower lip using an electric

689 cauterizer. Sutures were tied to both sides and fixed into the surface of the surgical stage to

690 stretch the area and expose the inferior teeth. Viscotears (Alcon) was used to keep tissue

691 moist and a holder with a coverslip and heating ring was placed on top. For two-photon

692 microscopy (2PM) imaging of Lm accumulation in mandLN, we surgically prepared mice to

693 expose gingiva and mandLN as described above and added 4-8 x 10^6 CFU Lm-GFP in 10 µL

694 onto the gingiva. 2PM was performed with an Olympus BX50WI microscope equipped with

695 20X Olympus (NA 0.95) or 25X Nikon (NA 1.10) objectives and a TrimScope 2PM system

696 controlled by ImSpector software (LaVisionBiotec). Prior to recording, Alexa Fluor 633- or

697 Alexa Fluor 488 conjugated MECA-79 (10 µg/mouse) was injected i.v. to label high

698 endothelial venules (HEV). For excitation, a Ti:sapphire laser (Mai Tai HP) was tuned to 780

699 or 840 nm. For 4-dimensional analysis of cell migration, 11 to 16 x-y sections with z-spacing

700 of 4 µm were acquired every 20 s for 20-30 min with a field of view of 150-350 x 150-350 µm.

701 Emitted light and second harmonic generation (SHG) signals were detected through 447/55

702 nm, 525/50 nm, 593/40 nm and 655/40 nm bandpass filters with non-scanned detectors

703 when using C57BL/6 recipient mice. For CD11c-YFP[+] recipient mice, 447/55 nm, 513/20 nm,

704 543/39 nm and 624/30 nm were used as bandpass filters. Second harmonic generation or

705 HEV signals were used as anatomical reference channel for real-time offset correction to

706 minimize tissue shift *(73)*. Sequences of image stacks were transformed into volume-

707 rendered four-dimensional videos with using Imaris software (Bitplane), which was also used

708 for semi-automated tracking of cell motility in three dimensions. Cell centroid data was used

709 to calculate key parameters of cell motility using Matlab (R2019b, MathWorks). Speed was

710 defined as total track length divided by total track duration in µm/min. The arrest coefficient

711 was derived from the percentage of time a cell is migrating below a motility threshold speed

712 of 5 µm/min. Meandering index was calculated by dividing displacement by track length.

713 Since the meandering index is influenced by track duration, we calculated the corrected track

714 straightness defined as meandering index multiplied by the square root of cell track duration

715 *(41)*.

716

717 *Stereomicroscope imaging*

718 MandLN and gingiva were exposed and pictures were taken using a Leica MZ16 FA

719 stereomicroscope equipped with color high resolution camera (Leica). Images were

720 processed using Adobe Photoshop CS6.

721

722 *Immunofluorescence*

723   Mice were anesthetized with i.p. injection of ketamine and xylazine and perfused with cold

724   2% PFA. MandLN were harvested and fixed overnight in 4% PFA, and dehydrated in 30%

725   sucrose o.n. prior to embedding in TissueTek O.C.T. compound (Sakura) for cryostat

726   sectioning. Slides with 10 μm-thick cryosections were mounted with ProLong™ Gold Antifade

727   Mountant (Molecular Probes). Fluorescence microscopy was performed using Leica SP5

728   confocal microscope with 20X (NA 0.7) and 63X (NA 1.3) Leica objectives. Images were

729   processed using Adobe Photoshop CS6 and Imaris 8.4.1 (Bitplane). Brightness and contrast

730   were adjusted for each image individually.

731   Gingival tissues anterior to the incisors teeth, buccal mucosa, palate and tongue were

732   harvested. Tissues were fixed in 4% PFA for 4-6 h under agitation at 4° C and embedded in

733   TissueTek O.C.T. compound (Sakura) for cryostat sectioning. Slides with 6 μm-thick

734   cryosections were fixed with 4% PFA for 15 min at RT. After three washing steps, PFA was

735   quenched by adding 0.1 M Glycine for 15 min. Sections were permeabilized for 15-30 min

736   with PBS + 0.2% Triton-X 100 and washed three times. Samples were incubated with

737   blocking solution (PBS with 2% BSA and 10% donkey serum) for 45-60 min at RT. Primary

738   purified antibody anti-RFP (Abcam; ab62341) diluted in blocking solution was added and

739   kept overnight at 4° C. After three washing steps, sections were incubated with secondary

740   AF568 donkey anti-rabbit Ab (Invitrogen A11011) diluted in blocking solution for 5 h at RT.

741   After three more washing steps, slides were mounted with ProLong™ Gold Antifade

742   Mountant (Molecular Probes). Fluorescence microscopy was performed using the Stellaris 8

743   confocal microscope with 63X objective (Leica). Images were processed using Adobe

744   Photoshop CS6 and Imaris 8.4.1 (Bitplane).

745

746   *Photoconversion*

747   C57BL/6 mice received i.v. injections of 7 x 10^6 naïve KikGR OT-I cells before i.o. infection of

748   Lm-OVA (2 x 10^9 CFU) on the following day. At day 3 p.i., the fur of anaesthetized mice was

749   removed in the neck area using an electric razor followed by hair removal cream. A sagittal

750   incision of approximately 1-2 cm length was made at the height of SMG and tissue around

the mandLN was gently removed. MandLN were photoconverted with a spot lamp system

(BlueWave 200, Dymax) using a 433/24 filter (AHF) with 260 mW/m^2 for 3 min. The same

procedure was performed on the contralateral side. The incision was closed using veterinary

adhesive (Vetbond, M3) and mice were allowed to rest. After 14 h, organs were harvested for

flow cytometry.

Adoptive transfer of mandLN-, spleen- and MLN-primed OT-I T$_{EFF}$

CD45.1, CD90.1 or CD45.1/.2 x CD90.1/.2 OT-I T$_{EFF}$ were obtained at d 4 p.i. from mandLN

(i.o.), spleen (i.v.) and MLN (i.g.) of FTY720-treated C57BL/6 mice by magnetic bead sorting

of CD8$^+$ T cells in medium containing 25 µg/mL of anti-ARTC2 Nanobody (Biolegend).

MandLN-, spleen- and MLN-primed OT-I T$_{EFF}$ (0.7 – 7 x 10^5 each/mouse) were co-

transferred into d 4 i.o.-infected age-and sex-matched recipients. At d 8 (4 d after OT-I T$_{EFF}$

transfer), mice received CD45 i.v. 3-5 min before euthanasia to label leukocytes in blood.

OM, lung, liver, SMG, small intestine, PP, mandLN, MLN and spleen were harvested for flow

cytometry. Cell frequencies were normalized to the input.

Lymphocyte isolation from non-lymphoid tissues

OM (buccal mucosa, maxillary and mandibular molar areas with surrounding gingiva, palate

and tongue) was dissected and enzymatically digested with 1 mL of 0.5 mg/mL Liberase DL

(Roche) for 20 min followed by addition 1 mL of 0.5 mg/mL Liberase TL (Roche) for another

20 min at 37° C under rotation. Digestion was stopped by addition of 1 mM EDTA. Gingival

tissues were removed from each block of tissue, using fine surgical forceps. Digested tissues

were filtered through a 70-µm cell strainer. Isolated cells were passed through a density

gradient with 40 and 70% Percoll (Cytiva) for 20 min at 700 g. The interphase containing

lymphocytes was collected, washed, and resuspended in cell culture media containing

DNase I before further analysis *(16, 74)*.

For small intestine, small intestinal intraepithelial lymphocytes (IEL) and lamina propria

lymphocytes (LPL) were isolated as previously described with minor modifications *(75)*. The

779   small intestine was opened longitudinally and feces removed by rinsing the tissue with

780   HBSS/5% FCS. After removing PP, IEL were isolated by incubating the small intestinal tissue

781   for 18 min in 30 mL HBSS/2 mM dithiothreitol/0.5 mM EDTA/5% FBS at 37° C under rotation.

782   IEL were enriched using 40 and 70% Percoll (Cytiva) for 20 min at 700 g. IEL were collected

783   from the interphase and kept in HBSS/5% FCS containing DNase I on ice. Tissue pieces

784   were digested in 30 mL HBSS/5% FBS/1.3 mM $CaCl_2$/0.5 mM $MgCl_2$/0.6 mM

785    $MgSO_4$/Collagenase IV (1mg/mL, Sigma-Aldrich)/ DNase I (20 µg/mL, Roche) for 20 min at

786   37° C under rotation. LPL single-cell suspension was pelleted, resuspended in HBSS/5%

787   FCS/DNase I and stored on ice for further processing. Enriched IEL were pooled with LPL as

788   small intestine lymphocytes.

789   Liver was cut and dissociated using a 70-µm cell strainer (adding 10 mL ice cold RPMI four

790   times). Samples were resuspended in 10 mL RPMI containing 2 mg collagenase IV (Sigma-

791   Aldrich) and 6.25 µg DNase (Roche). Tissue homogenates were vortexed and incubated at

792   37°C for 40 min (vortexing every 10 min). After digestion, 25 mL RPMI was added, samples

793   were vortexed and centrifuged at 20 g for 3 min to allow only the connective tissue to pellet.

794   Supernatant was transferred to a new tube and centrifuged at 540 g for 5 min. The pellet was

795   resuspended in 4 mL 40% Percoll (Cytiva) and centrifuged at 1000 g for 20 min. The

796   leukocyte pellet was lysed with RBC lysis buffer, washed and resuspended in RPMI/5% FCS.

797   Lung tissue was cut in small fragments and digested in 5 mL of RPMI/5% FCS/Collagenase

798   type IV (1 mg/mL; Sigma-Aldrich)/Dispase II (0.5 mg/mL; Roche)/DNase I (1 kU/mL; Roche)

799   for 45 min at 37°C under rotation. Tissue fragments were dissociated by pipetting every 15

800   min, first using a 5 mL serological pipette, followed by a P1000 microtiter pipette at the

801   second and last rounds of pipetting. After complete dissociation of tissue fragments,

802   digestion was stopped by adding 5 mL of 5 mM EDTA. Cells were resuspended in 7 mL 40%

803   Percoll (Cytiva), with 2.5 mL 70% Percoll laid underneath, and spun down for 20 min at 1000

804   g. Cells at the interphase were collected, washed and resuspend in 1 mL of RPMI/5% FCS.

805   SMG was cut in small pieces and transferred to an Eppendorf tube containing 1mL of

806   digestion mix (RPMI/5% FCS/ collagenase II (2 kU/mL; Gibco)/DNase I (1 kU/mL; Roche).

807   Samples were incubated for 30 min at 37°C under rotation with vortexing every 5 min.

808   Remaining tissue was passed through a 70 um cell strainer and washed with 15 mL of PBS/5

809   mM EDTA. Pellet was resuspended in CMR (RPMI supplemented with 10% FCS/10 mM

810   HEPES/2 mM L-Glu/10 mM Na-pyruvate/50 µM β-ME/NEAA and PenStrep).

811

812   *B cell killing assay*

813   C57BL/6 mice treated with or without 200 µg Mel-14 (BioXCell; injected i.p. 4 h before cell

814   transfer) received i.v. 3×10^6 OT-I cells. The following day, mice were i.o. infected with 2 x

815   10^9 CFU of Lm-OVA. B cells were isolated from sex-matched C57BL/6 donor mice using

816   MojoSort^{TM} Mouse Pan B Cell Isolation kit II (BioLegend) and pulsed or not with 100 nM

817   OVA$_{257\text{-}264}$ (EMC Microcollections). Pulsed and unpulsed B cells were stained with 1 and 7.5

818   µM CFDA SE Cell Tracker Kit (Molecular Probes), respectively, and 5×10^6 cells of each

819   were injected i.v. (1:1 ratio) into d 3-infected recipients. After 12 h, lymphoid organs were

820   harvested for flow cytometry analysis.

821

822   *Single cell gene expression and data analysis*

823   Single cell gene expression of TER119$^-$ CD45$^-$ stromal cells was measured using the 10x

824   Chromium system, with the Next GEM Single Cell 3' Reagent Kit v3.1 (10x Genomics,

825   Pleasanton, CA, USA). GEM generation and barcoding, reverse transcription, cDNA

826   amplification and 3' Gene Expression library generation steps were all performed according

827   to the manufacturer's user guide. Specifically, the designated volume of each cell suspension

828   (800 -1200 cells/µL) and nuclease-free water was used for a targeted cell recovery of 10'000

829   cells according to the Cell Suspension Volume Calculator Table of the abovementioned user

830   guide. GEM generation was followed by a GEM-reverse transcription incubation, a clean-up

831   step and 15 cycles of cDNA amplification. The quality and quantity of the cDNA was

832   assessed using fluorometry and capillary electrophoresis, respectively. The barcoded cDNA

833   libraries were pooled and sequenced paired-end and single indexed on an Illumina NovaSeq

834   6000 sequencer using a S2 flowcell (100 cycles). The read setup was as follows: read 1: 28

835   cycles, i7 index: 8 cycles, i5: 0 cycles and read 2: 91 cycles. An average of 740,045,087

836   reads/library were obtained, which corresponds to an average of 74,091 reads/cell.

837   Mapping and counting of the UMIs for the samples from mandLN, MLN and PLN were

838   performed using Cellranger (version 3.0.2, 10x Genomics) with the reference genome

839   GRCm38.93 from Ensembl to build the necessary index files. Subsequent analysis was

840   performed in R (version 4.0.2) *(76)*. The Scater package (version 1.14) *(77)* was used to

841   assess the proportion of ribosomal and mitochondrial genes as well as the number of

842   detected genes. Cells were considered as outliers and filtered out if the value of the

843   proportion of expressed mitochondrial genes or the number detected genes deviated more

844   than three median absolute deviations from the median across all cells. After quality control,

845   the sample from mandLN retained 4434 cells, the sample from MLN retained 10058 cells and

846   the sample from PLN retained 7750 cells. Normalization between samples was done with the

847   deconvolution method of Lun et al. *(78)* using the package Scran (version 1.14) *(79)*.

848   Samples were integrated with the FindIntegrationAnchors function of the package Seurat

849   (version 3.1) based on the first 20 principal components (PCs) *(80)*. Graph-based clustering

850   was done with the FindNeighbors and FindClusters functions of the Seurat package using

851   the first 40 PCs from the dimensionality reduction step. The Clustree package (version 0.4)

852   *(81)* was used to determine the resolution resulting in clustering concurring with the

853   presumed cell types, which was 0.4. Clusters were manually annotated based on marker

854   genes that were identified with the FindMarkers function of Seurat. Following this, seven

855   clusters were removed from the analysis as they represented hematopoietic cells. The

856   remaining cells were re-clustered in an identical fashion but with a resolution of 0.3. The

857   dataset has been deposited on ENA (project ID: PRJEB40464).

858

859   *Quantitative PCR*

860   For sorting of DC and stromal cells, cell suspensions were stained with mAbs and propidium

861   iodide to discriminate between live *versus* dead cells. CD45^{+} MHC-II^{high} CD11c^{+} DCs and

862   CD45⁻ stromal cells were sorted using a FACSAria Fusion (BD). For qPCR, the total RNA

863   from 2.7-30 x 10^4 sorted DC and stromal cells was extracted using Trizol Reagent (Sigma-

864   Aldrich) and coprecipitant GlycoBlue™ (Invitrogen). Reverse transcriptase reactions were

865   performed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA)

866   according to the manufacturer's instructions. qPCR assays were performed on StepOnePlus

867   (Applied Biosystems, USA) using FastStart Universal SYBR Green Master (Rox) (Roche,

868   Switzerland). QuantiNova LNA-enhanced primers for *Aldh1a1*, *Aldh1a2*, *Aldh1a3* and as

869   endogenous housekeeping reference gene hypoxantine-guanine phosphoribosyltransferase

870   (HPRT) were purchased from Qiagen (Germany) (**Table 2**). qPCR reactions were performed

871   in duplicates in a total volume of 20 μL. The cycling conditions were: 95°C for 10 min,

872   followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. After amplification, dissociation

873   curves were performed to monitor primer specificity, revealing only one melting peak for each

874   amplified fragment. qPCR data were normalized to the housekeeping gene HPRT and the

875   relative changes in mRNA expression were calculated by $2^{-\Delta Ct}$.

Assay Name	Full name	Gene ID	Ref Seq	Catalogue #
MM_HPRT_2521490	hypoxanthine guanine phosphoribosyl transferase	15452	NM_013556	SBM1225379
MM_ALDH1A1_2045606	aldehyde dehydrogenase family 1, subfamily A1	11668	NM_001361506	SBM0801506
MM_ALDH1A2_1966774	aldehyde dehydrogenase family 1, subfamily A2	19378	NM_009022	SBM0722689
MM_ALDH1A3_2125665	aldehyde dehydrogenase family 1, subfamily A3p	56847	NM_053080	SBM0881559

876   **Table 2.**

877

878   *Statistical analysis*

879   Student's t-test, Mann-Whitney U-test, ANOVA, Kruskal-Wallis and Wilcoxon tests were used

880   to determine statistical significance as indicated (Prism, GraphPad). Significance threshold

881   was set at $p < 0.05$.

882

883   *Data availability statement*

884 <u>All datasets (beyond the data provided in the "RawDataFile" and the ENA repository) are</u>

885 <u>available through JVS.</u>

886

887 *Supplemental material*

888 Fig. S1. Depiction of surgery for gingiva and mandLN.

889 Fig. S2. Flow cytometry of DC and CD8$^+$ T cell subsets.

890 Fig. S3. CD8$^+$ T cell activation with distinct Lm inocula and food consumption.

891 Fig. S4. Expression of trafficking and effector markers on orally primed OT-I T cells.

892 Fig. S5. Adoptive transfer of T_{EFF} primed with distinct routes of infection.

893 Fig. S6. scRNAseq data on expression of selected genes of interests in SLO stromal cells.

894 Fig. S7. Differentially expressed genes in TRC of mandLN, MLN and PLN.

895 Fig. S8. Confocal images of memory CD8$^+$ T cells in OM.

896 Video S1. OT-I in uninfected mandLN.

897 Video S2. OT-I in mandLN on d 2 post i.o. Lm infection.

898 Video S3. OT-I in mandLN on d 3 post i.o. Lm infection.

899 Video S4. OT-I in gingiva in memory phase.

Author contribution

JBdA performed experiments with help from LMA, JA, DvW, SW, and JMM. JA, GvG and DF performed scRNAseq analysis under supervision of RB. XF and MI provided a script for cell tracking analysis. JBdA, CM and JVS designed experiments and wrote the manuscript with input from all coauthors.

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Figure legends

Figure 1. Lm uptake into oral cavity leads to rapid bacterial accumulation in mandLN.
A. Drainage of a tracer injected into the gingiva to mandLN (arrowheads). **B.** Overview of lymphatic vessel network in gingiva of Prox1-GFP reporter mouse. **C.** 2PM reconstruction of lymphatic network in gingiva surrounding mandibular incisors. Scale bar, 20 μm. **D.** Overview of lymphatic vessels between mandibular incisors and mandLN of Prox1-GFP reporter mouse. MM, masseter muscle; mandLN, mandibular lymph node; SLG, sublingual salivary gland; SMG, submandibular salivary gland. **E.** Experimental layout. **F.** Lm-OVA CFU per lymphoid organ after intraoral (i.o.) *versus* intravenous (i.v.) Lm infection. Lines depict median, and dotted line indicates limit of detection. **G.** Lymphoid organ cellularity during i.o. and i.v. Lm-OVA infection. **H.** CFU counts in mandLN after i.o. infection of Lm-OVA or Lm-InlA^m. **I.** Lm-OVA CFU counts in mandLN after i.o. infection of WT or CCR7$^{-/-}$ mice. **J.** Lm-GFP accumulation (arrowheads) at distinct depths from mandLN capsule after i.o. infection. SHG, second harmonic generation. Scale bar, 40 μm. Data in F and G are from 2-3 independent experiments with each 2-3 mice/time point. Data in G were analyzed using a Kruskal-Wallis test against "d 0". Data in H and I are pooled from 2-3 independent experiments and analyzed using a Mann-Whitney test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 2. Visualization of CD8$^+$ T cell dynamics in mandLN after oral Lm uptake. A. Experimental layout. **B.** Confocal images of mandLN sections after i.o. Lm-OVA administration. Scale bar, 200 (top) and 20 (bottom) μm. **C.** Representative 2PM images of infected mandLN of CD11c-YFP host containing OT-I cells and polyclonal T cells at d 2 p.i.. Left panel shows OT-I clusters around CD11$^+$ cells (dotted line), right panel shows overlaid tracks of OT-I (yellow) and polyclonal CD8$^+$ T cells (blue). Scale bar, 20 μm. **D-F.** Quantification of imaging data. Track speeds (D), arrest coefficient (E), and corrected track straightness (F). Lines in D and E depict median. Data in D were analyzed using ANOVA with Sidak's multiple comparison test, in E using Kruskal-Wallis with Dunn's multiple

1196 comparison test and in F using Mann-Whitney test. Data are representative (B, C) or pooled

1197 (D-F) from at least two independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

1198

1199 **Figure 3. MandLN DC trigger CD8$^+$ T cell activation. A.** Flow cytometry plot of CD103$^+$

1200 and CD103$^-$ DC. **B, C.** CD80 (B) and CD86 (C) expression and MFI on CD103$^+$ and CD103$^-$

1201 DC before and after Lm infection. **D.** Experimental set up for *in vitro* CD8$^+$ T cell activation by

1202 mandLN DC. **E, F.** Representative flow cytometry plots (E) and quantification (F) of Nur77-

1203 GFP, CD69 and CD25 expression, and proliferation. Data in B and C are from one of two

1204 independent experiments with 4 mice/group and analyzed using an unpaired t-test. Data in F

1205 are pooled from 2 independent experiments with 1-4 replicates each and analyzed with one-

1206 way ANOVA with Dunnett's multiple comparison test against "- DC" (no DC). *, p < 0.05; ***,

1207 p < 0.001.

1208

1209 **Figure 4. Oral Lm exposure triggers rapid CD8$^+$ T cell effector generation in mandLN.**

1210 **A.** Experimental layout. **B, C.** CD69 (B) and CD25 (C) expression in OT-I cells isolated from

1211 mandLN, spleen and MLN after i.o. Lm-OVA infection. **D.** Representative flow cytometry

1212 plots of OT-I T cell proliferation after i.o. Lm-OVA infection. Numbers indicate percent of

1213 proliferated cells. **E.** OT-I proliferation in mandLN, spleen and MLN after i.o. Lm infection. **F.**

1214 OT-I cell number in lymphoid organs after i.o. and i.v. infection. Numbers depict fold increase

1215 in median "d 3" OT-I cell numbers over "d 0". **G-I.** Examples of flow cytometry plots (G) and

1216 pie charts showing the proportion of OT-I populations based on CD44/CD62L (H) and

1217 KLRG1/CD127 (I) expression on d 5 and 30 following i.o. and i.v. infections. Lines in B, C, E,

1218 and F depict median. Data in B and C are pooled from 2-5 experiments per time point with 3

1219 mice per experiment and analyzed by ANOVA. Data in E are pooled from 2 independent

1220 experiments. Data in F, H and I are pooled from 2-3 independent experiments with each 2-3

1221 mice/group/time point and analyzed using a Kruskal-Wallis test against "d 0" (F). *, p < 0.05;

1222 **, p < 0.01; ***, p < 0.001.

1223

1224 **Figure 5. MandLN CD8$^+$ T$_{EFF}$ constitute the majority of the early circulating T$_{EFF}$ pool for**

1225 **host protection. A.** Experimental layout of egress blockade. **B.** OT-I cell numbers in

1226 mandLN, spleen and MLN on d 4 and 5 post i.o. Lm-OVA infection in presence or absence of

1227 FTY720. **C.** Experimental layout of photoconversion. **D.** Representative flow cytometry plots

1228 for photoconverted (KikGR-red) and non-photoconverted (KikGR-green) OT-I cells and their

1229 CD44 expression from mice in the presence or absence of Lm-OVA. Numbers depict

1230 percentages. **E.** Numbers of KikGR-red$^+$ CD44^{high} OT-I cells per lymphoid organ. **F.**

1231 Experimental layout of target cell killing. **G.** Cell number in lymphoid organs in the presence

1232 or absence of anti-CD62L treatment. **H.** Flow cytometry histograms for unpulsed (U) and

1233 OVA$_{257-264}$-pulsed (P) B cells in the presence or absence of anti-CD62L. **I.** Ratio of OVA$_{257-}$

1234 $_{264}$-pulsed and unpulsed B cells in the spleen. **J.** Numbers of OT-I cells in the spleen in the

1235 presence or absence of anti-CD62L. Data in B are pooled from 2 independent experiments

1236 with 3-4 mice each and analyzed using an unpaired t-test. Data in E are pooled from 2

1237 independent experiments with 2-3 mice each. Data in G, I and J are pooled from 2

1238 independent experiments with 3-5 mice/group for presence/absence of anti-CD62L mAb, and

1239 2 independent experiments each with 2 mice/group as controls. Data in G were analyzed

1240 with unpaired t test and Welch`s correction. Data in I and J were analyzed with Mann-

1241 Whitney test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

1242

1243 **Figure 6. Mand LN T$_{EFF}$ lack expression of signature gut trafficking receptors and**

1244 **home to SLO, lung and OM but not the small intestine. A.** Experimental layout of Lm-

1245 OVA infections. **B-E.** Flow cytometry plots and quantification for adhesion receptors α_L

1246 (CD11a), α_4 (CD49d), β_1 (CD29) and β_7 integrins (B), chemokine receptors CXCR3, CX3CR1

1247 and CXCR6 (C), effector proteins Granzyme B (GzmB), IFN-γ, TNF-α and IL-2 (D) and gut

1248 trafficking receptors $\alpha_4\beta_7$ and CCR9 (E). Black bar indicate gate, grey is FMO. **F.**

1249 Experimental layout of OT-I T$_{EFF}$ adoptive transfer. **G-I.** Ratio of MLN- to spleen-primed OT-I

1250 (G) and mandLN- to spleen-primed OT-I T$_{EFF}$ (H) recovered from each organ. Values are

1251 normalized to the T_{EFF} ratio in recipient spleens. nd, not detected. SMG, submandibular

1252 salivary gland; OM, oral mucosa; SI, small intestine. **I.** Normalized ratio of mandLN- to

1253 spleen-primed OT-I T_{EFF} in lung, OM and small intestine (from data in G). Data in B-D and G-

1254 I are pooled from 2 independent experiments with 4-5 mice each and analyzed using ANOVA

1255 and Tukey post-test. Data in E are representative of two independent experiments. Data in I

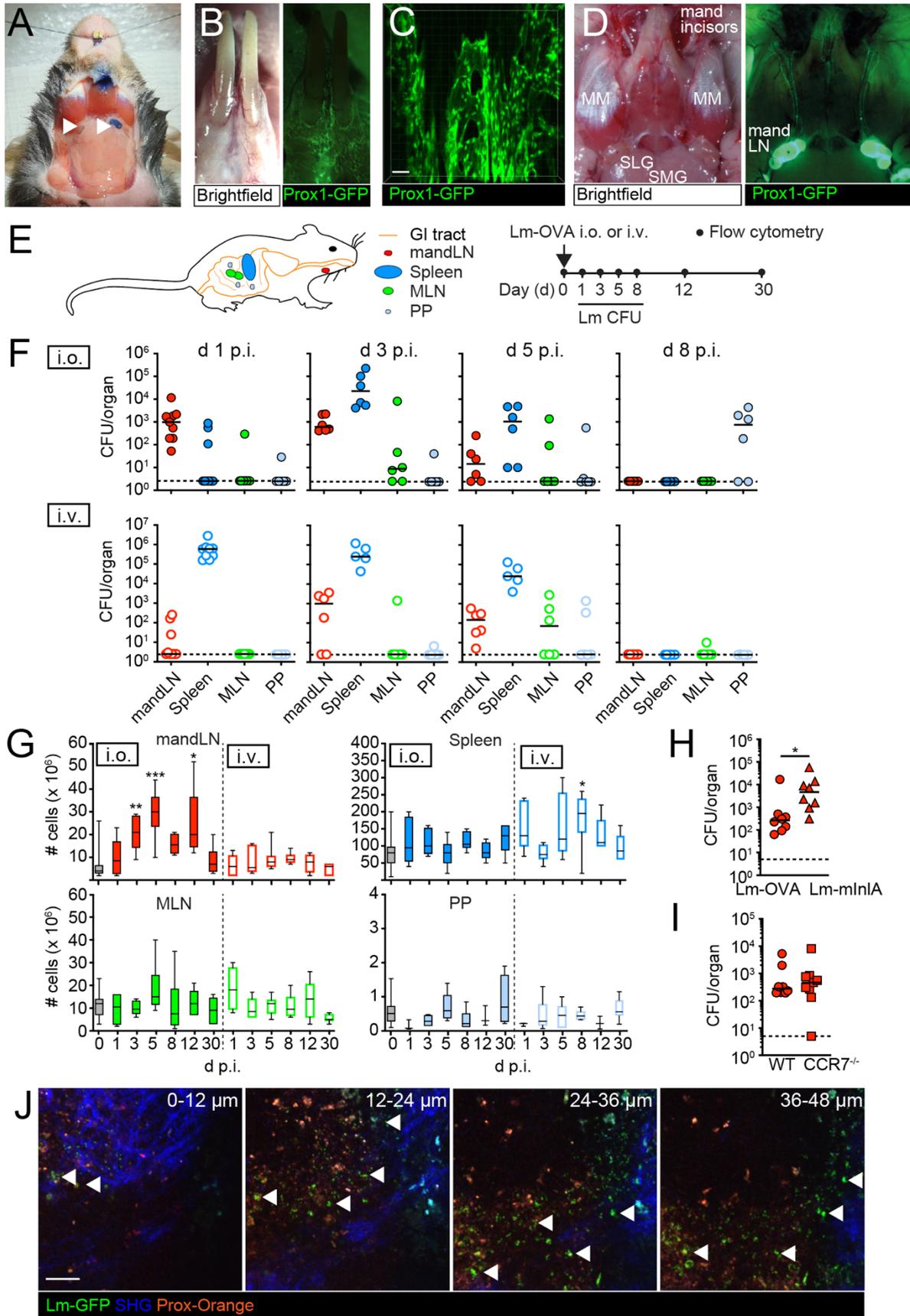1256 were analyzed using Wilcoxon test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

1257

1258 **Figure 7. Low expression of genes for gut-homing imprinting in mandLN stromal cells**

1259 **DC. A.** UMAP clustering of pooled TER119⁻ CD45⁻ stromal cells of MLN, PLN and mandLN

1260 based on scRNAseq data. Numbers indicate individual clusters. **B.** Expression of *Aldh1a1*,

1261 *Aldh1a2*, *Aldh1a3* and *WT1* in MLN (10058 cells), PLN (7750 cells) and mandLN stroma

1262 (4434 cells) based on scRNAseq data. **C.** Expression frequency of *Aldh1a1*, *Aldh1a2*,

1263 *Aldh1a3* and *WT1* in fibroblast-like cells based on data in B. **D.** *Aldh1a1*, *Aldh1a2* and

1264 *Aldh1a3* expression in TER-119⁻ CD45⁻ stromal cells and CD11c⁺ MHC-II⁺ DC isolated from

1265 of MLN, PLN and mandLN assessed by qPCR. Shown are the $2^{-\Delta CT}$ means of duplicates

1266 from two independent experiments (grey and white fill). Bars represent mean.

1267

1268 **Figure 8. MandLN-primed OT-I T_{EFF} reduce systemic Lm burden but do not protect**

1269 **MLN and PP after intragastric infection. A.** Experimental layout. **B.** Lm-OVA CFU in

1270 spleen, MLN and PP after adoptive transfer of spleen-, mandLN- and MLN-primed OT-I T_{EFF}.

1271 Numbers indicate percentage of organs with Lm-OVA CFU above limit of detection (dotted

1272 line). **C.** Graphical summary. Inception of oral pathogen in mandLN leads to a rapid release

1273 of protective CD8⁺ T_{EFF} that preferentially relocate to SLO, lung and OM. CSF, cerebrospinal

1274 fluid; AEC, anterior eye chamber. Created with BioRender.com. Data in B are pooled from 4

1275 independent experiments and analyzed using a Kruskal Wallis test against "no OT-I". ***, $p <$

1276 0.001.

Figure 1

A

OT-I +/−
polyclonal
CD8+ T cells   Lm-OVA i.o.

● Immunofluorescence &
  Intravital imaging

d -1 0 1 2 3

B

d 1 p.i.   d 2 p.i.   d 3 p.i.

OT-I CD11c polyclonal CD8+ T cells

C

Tracks at t = 20 min

OT-I CD11c polyclonal CD8+ T cells HEV

D

Speed (µm/min)

No Lm   1   2   3
        d p.i.

***
***
*
**
**

E

Arrest coefficient (%)

No Lm   1   2   3
        d p.i.

***
*
**
***
***
**

○ OT-I (no Lm)
○ OT-I
● OT-I (clustered)
● polyclonal CD8+

F

Relative frequency

d 1 p.i.   ***
d 2 p.i.   ***
d 3 p.i.   *

Corrected Track straightness (MI x track duration^{0.5})

□ OT-I (clustered/non-clustered)
■ polyclonal CD8+

Figure 2

1278

51
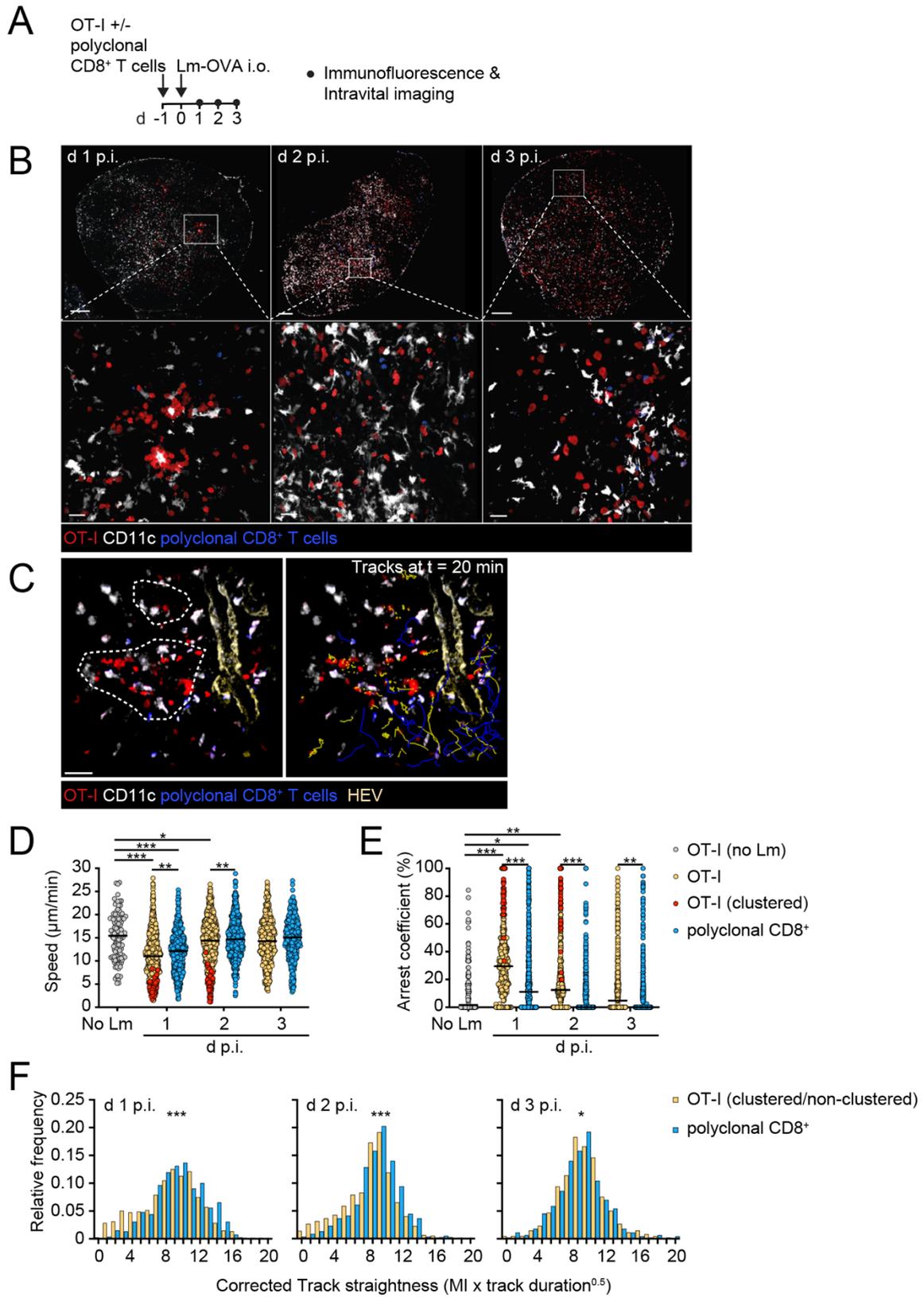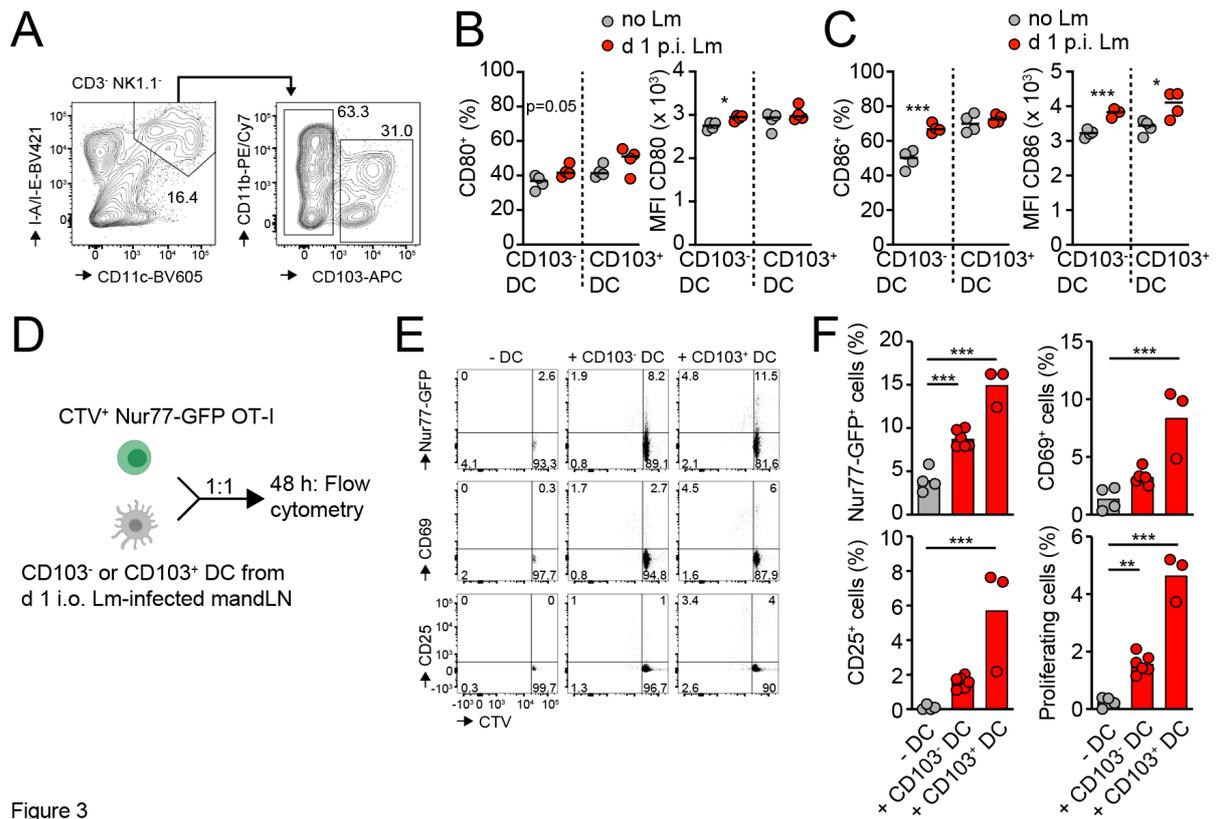
Figure 3

Figure 4

Figure 5

1281

54

Figure 6

Figure 7

1283

A

Lm-OVA i.o.,
OT-I   i.v. or i.g.   Isolation OT-I
                        - mandLN
                        - spleen
                        - MLN

d -1 0 1 3 5

FTY720

Lm-OVA
i.g.

d 3 p.i. CFU
spleen/MLN/PP

B

Spleen

100 94 73 69

CFU/g

no OT-I mandLN OT-I spleen OT-I MLN OT-I

MLN

83 94 87 31

no OT-I mandLN OT-I spleen OT-I MLN OT-I

PP

33 38 33 8

no OT-I mandLN OT-I spleen OT-I MLN OT-I

C

— GI tract
• mandLN
● Spleen
● MLN
○ PP

Intestine   Tooth

PP (luminal
sampling)   Gingiva

CSF
AEC
Skin

MLN,
other gLN   mandLN

Sampling of intestinal   Sampling of oral
mucosa lymph   mucosa lymph
(lower GI tract)   (upper GI tract)

Homeostasis
Inception of oral pathogen

SI   mandLN

SLO   — Lm
OM   ✳ CD11c+
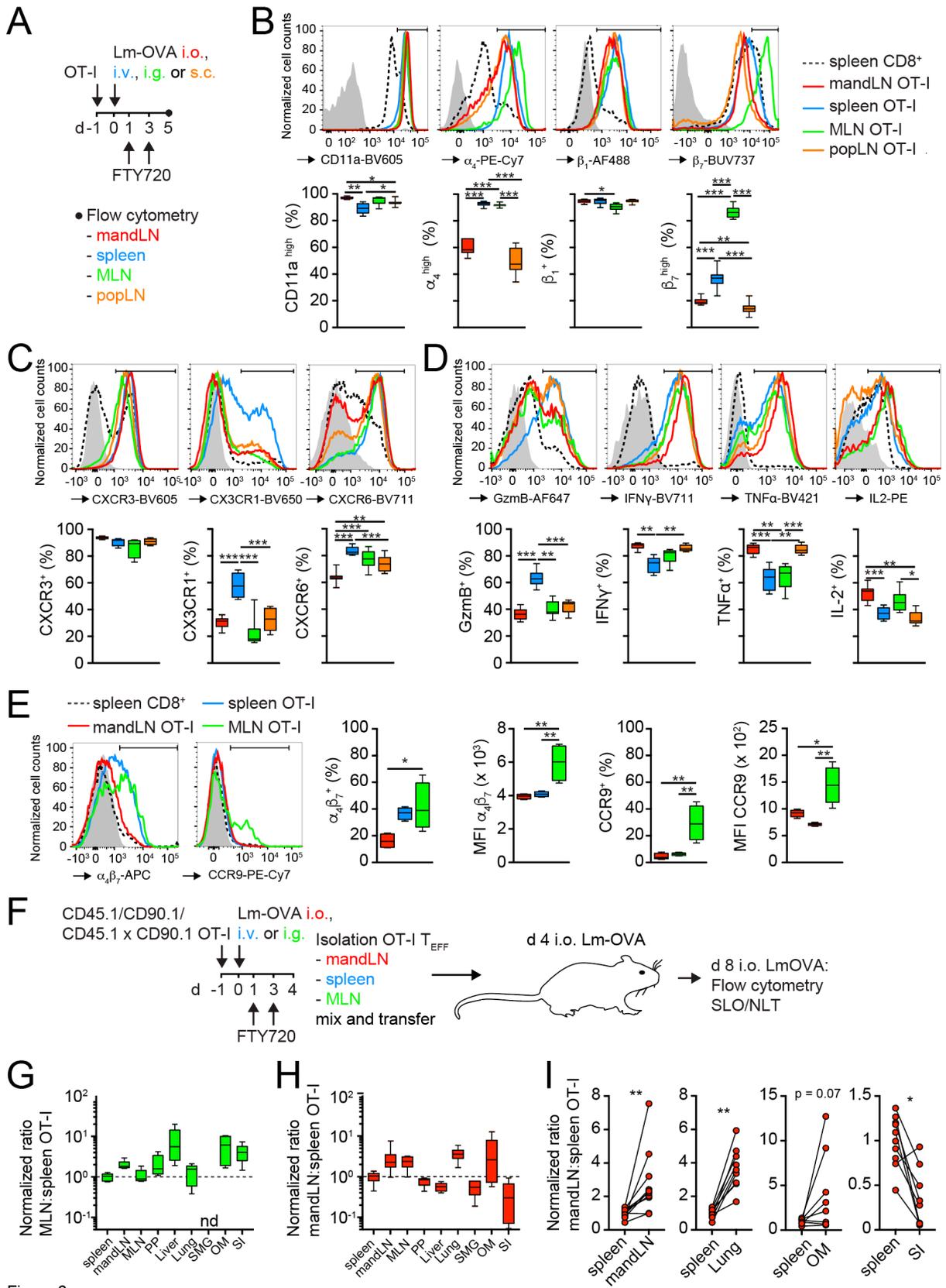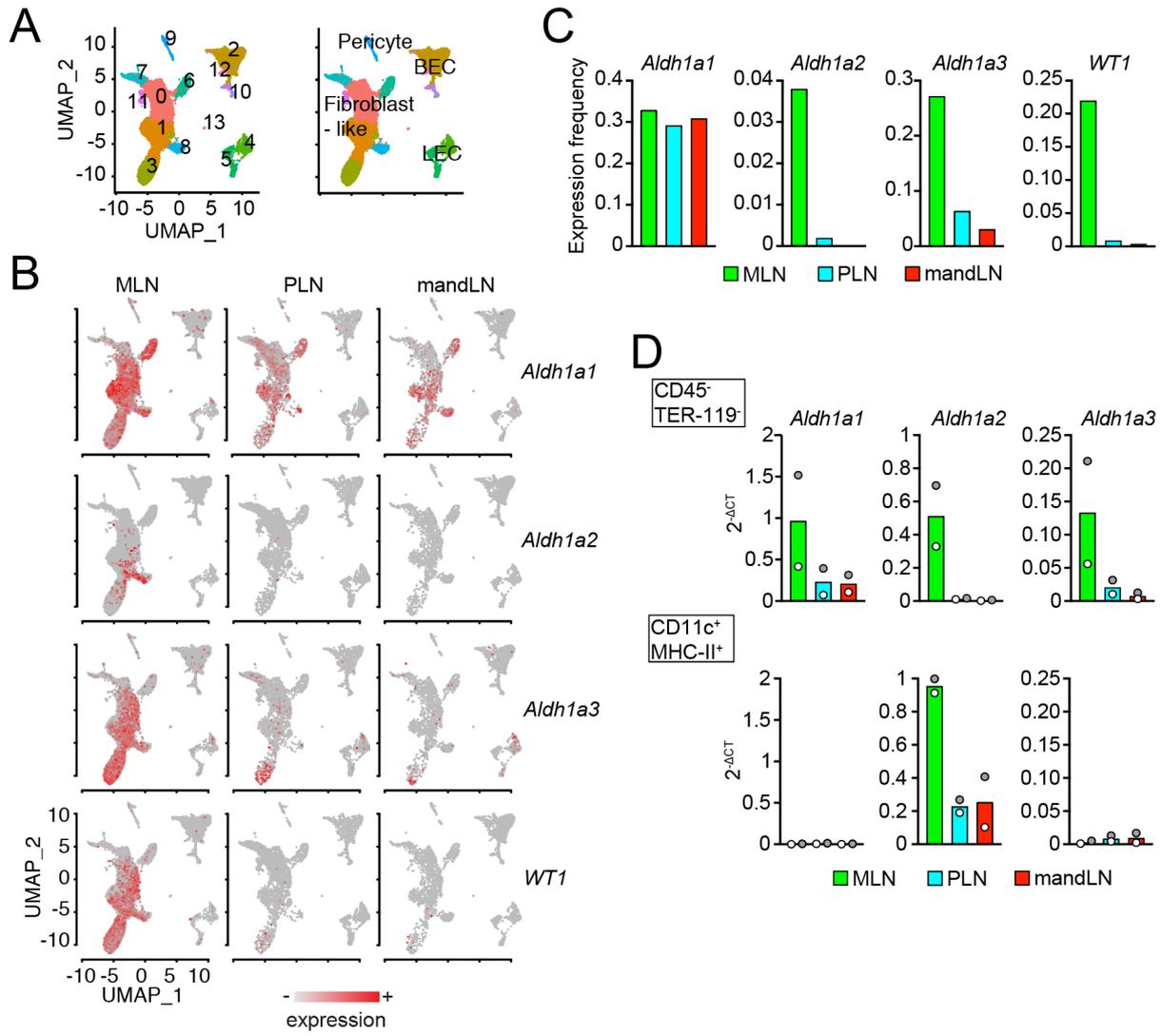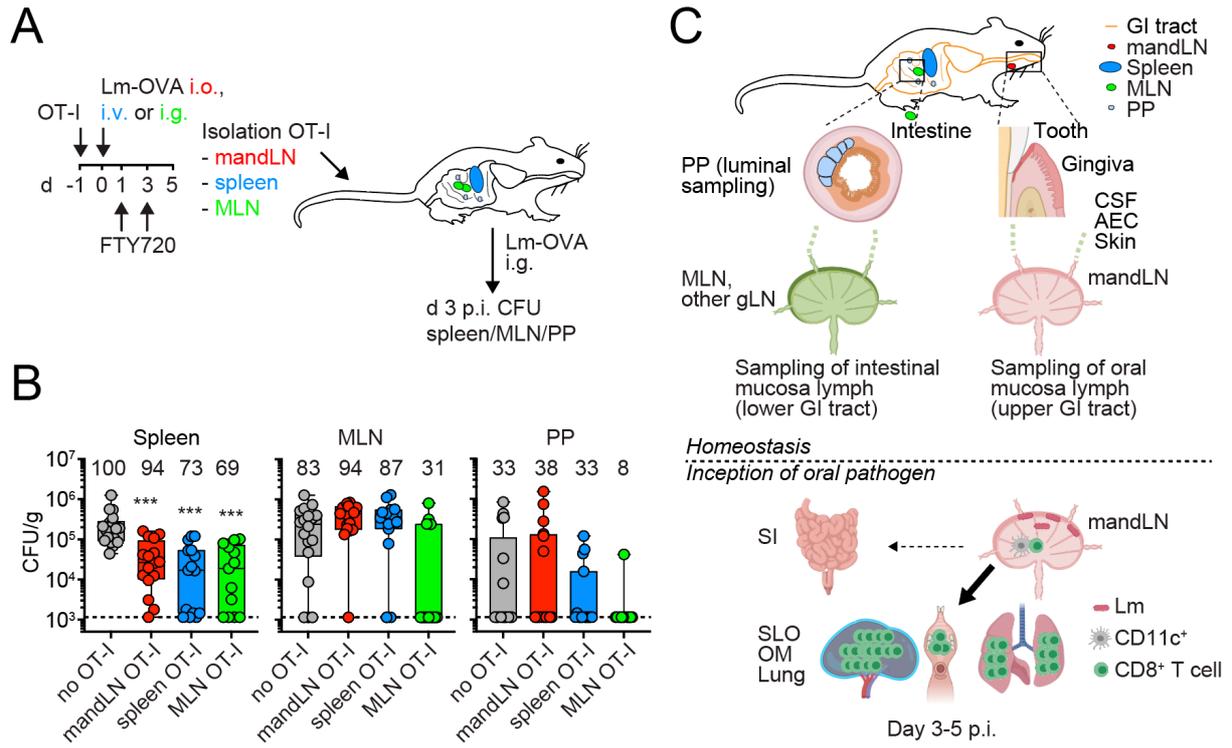Lung   ● CD8+ T cell

Day 3-5 p.i.

Figure 8

1284

57

Supplemental Figure legend

Figure S1. A, B. Surgical setup (left panels) and stereomicroscope images (right panels) of

gingiva (A) and mandLN (B). SMG, submandibular salivary gland.

Figure S2. Flow cytometry analysis of DC and CD8$^+$ T cells. A. Flow cytometry plots of

migratory (CD11c^{intermediate} MHCII^{high}) and resident (CD11c^{high} MHCII^{intermediate}) DC

classification and expression of CD80 and CD86. Grey shade, FMO. Numbers indicate

percent expressing cells. **B, C.** CD80 (B) and CD86 (C) expression and MFI on migratory

and resident DC. **D.** Gating strategy for CD103$^+$ and CD103$^-$ DCs and CCR7 expression on

cDC1 (XCR1$^+$ SIRPalpha$^-$) and cDC2 subsets (XCR1$^-$ SIRPalpha$^+$) isolated from mandLN.

Lineage markers for exclusion contain anti-NK1.1, TCRβ, CD19 and Ly6C mAbs. Numbers

indicate percentage in gate. **E.** Gating strategy for adoptively transferred OT-I T cells. Data in

B and C are from one of two independent experiments with 4 mice/group, while data in D are

pooled from 2 experiments with 4-6 mice, and analyzed using an unpaired t-test.

Figure S3. CD8$^+$ T cell activation in mandLN occurs over a wide range of Lm inocula

and consumption of Lm-contaminated food. A. Experimental layout for Lm-OVA titration.

B. Flow cytometry plots of CD69/CD25 expression and proliferation in mandLN OT-I T cells

on d 3 p.i. after titrating Lm-OVA inoculum. **C-F.** CD69 expression (C), MFI CD69 (D), CD25

expression (E) and proliferation (F) on mandLN OT-I T cells after titrating Lm-OVA inoculum.

Numbers in F depict percent proliferated $\pm$ SEM. **G.** MandLN cellularity on d 3 after i.o. Lm-

OVA infection. **H, I.** CD69 expression (H) and MFI CD69 (I) on endogenous mandLN CD8$^+$

cells after titrating Lm-OVA inoculum. **J.** Experimental layout for Lm-OVA infection with

ingested contaminated food. **K-N.** Total cellularity (K), number of OT-I (L), CD69 (M) and

CD25 (N) expression in mandLN. Lines in C-I and K-N depict median. Data in C-I are pooled

from 2 independent experiments with each 3 mice/condition and analyzed using a Kruskal-

Wallis test against "no Lm". Data in K-N are pooled from 2 independent experiments with

1312 each 3-5 mice and analyzed using a Mann-Whitney test. *, p < 0.05; **, p < 0.01; ***, p <

1313 0.001.

1314

1315 **Figure S4. <u>Expression of trafficking and effector markers on orally primed OT-I T cells.</u>**

1316 **A.** Flow cytometry plots and quantification for CLA, α_1, CCR6, CCR7, CCR8 and CCR10

1317 expression after i.o. Lm infection. Black bar indicate gate, grey shade; FMO. Data are pooled

1318 from 1-2 independent experiments with 2-4 mice each and analyzed using ANOVA and

1319 Tukey post-test. <u>**B.** Expression of adhesion receptors, chemokine receptors and effector</u>

1320 <u>proteins on mandLN OT-I T cells after ingestion of Lm-contaminated food. Stainings were</u>

1321 <u>performed as in Figure 6 and are from one experiment with n = 5 mice.</u> *, p < 0.05; **, p <

1322 0.01; ***, p < 0.001.

1323

1324 **Figure S5. Adoptive transfer of T$_{EFF}$ primed at distinct SLO. A.** Flow cytometry of OT-I

1325 T$_{EFF}$ populations in input. **B.** Gating strategy for transferred T$_{EFF}$ OT-I populations. **C.**

1326 Percentages of mandLN- versus spleen-primed OT-I in spleen, mandLN, MLN, PP, liver,

1327 lung, SMG (submandibular salivary gland), OM and SI after correction for input ratio. **D, E.**

1328 Flow cytometry plots and histograms for CD44 and CD62L (D), as well as KLRG1 and

1329 CD127 expression (E) in endogenous and recovered OT-I T$_{EFF}$ populations. Numbers in A, B

1330 and D indicate percentages. Data in C are pooled from 2 independent experiments.

1331

1332 **Figure S6. Expression of selected genes of interests in stromal cell clusters by**

1333 **scRNAseq.** *Col1a1*, collagen type I alpha 1 chain; *Col1a2*, collagen type I alpha 2 chain;

1334 *Pdpn*, podoplanin; *Cdh5*, VE-cadherin; *Chst4*, Carbohydrate Sulfotransferase 4; *Tnfsf11*,

1335 TNF Superfamily Member 11 (RANKL).

1336

1337 **Figure S7. Differentially expressed genes in T cell zone reticular cells (TRC) cells of**

1338 **mandLN, MLN and PLN.** Differentially expressed genes in CCL21a^{+} TRC (cluster 7) were

1339   analyzed for mandLN *versus* MLN (**A**) and mandLN *versus* PLN (**B**). Pathway enrichment is

1340   expressed as -log(P).

1341

1342   **Figure S8**. **Antigen-specific memory CD8$^+$ T cells in OM.** Confocal images of tdTom$^+$ OT-I

1343   T cells in buccal mucosa, gingiva, palate and tongue on d 72 post oral infection. Scale bar,

1344   40 µm (top) and 5 µm (bottom). Blue, nuclei (DAPI); red, tdTom amplified by anti-RFP

1345   staining.

Supplementary video legend

1347 **Video S1.** OT-I motility (red) in mandLN of CD11c-YFP (white) recipients in the absence of

1348 infection. Scale bar, 30 µm; time in min:s.

1349 **Video S2.** Polyclonal CD8$^+$ (blue) and OT-I T cell (red) motility in mandLN of CD11c-YFP

1350 (white) recipients on d 2 post i.o. Lm-OVA infection. HEV are marked in brown. Scale bar, 30

1351 µm; time in min:s.

1352 **Video S3.** Polyclonal CD8$^+$ (blue) and OT-I T cell (red) motility in mandLN of CD11c-YFP

1353 (white) recipients on d 3 post i.o. Lm-OVA infection. HEV are marked in brown. Scale bar, 30

1354 µm; time in min:s.

1355 **Video S4.** OT-I T cell (red) migration in gingiva in memory phase following i.o. Lm-OVA

1356 infection. SHG, second harmonic generation. Scale bar, 50 µm; time in min:s.

1357

Figure S1
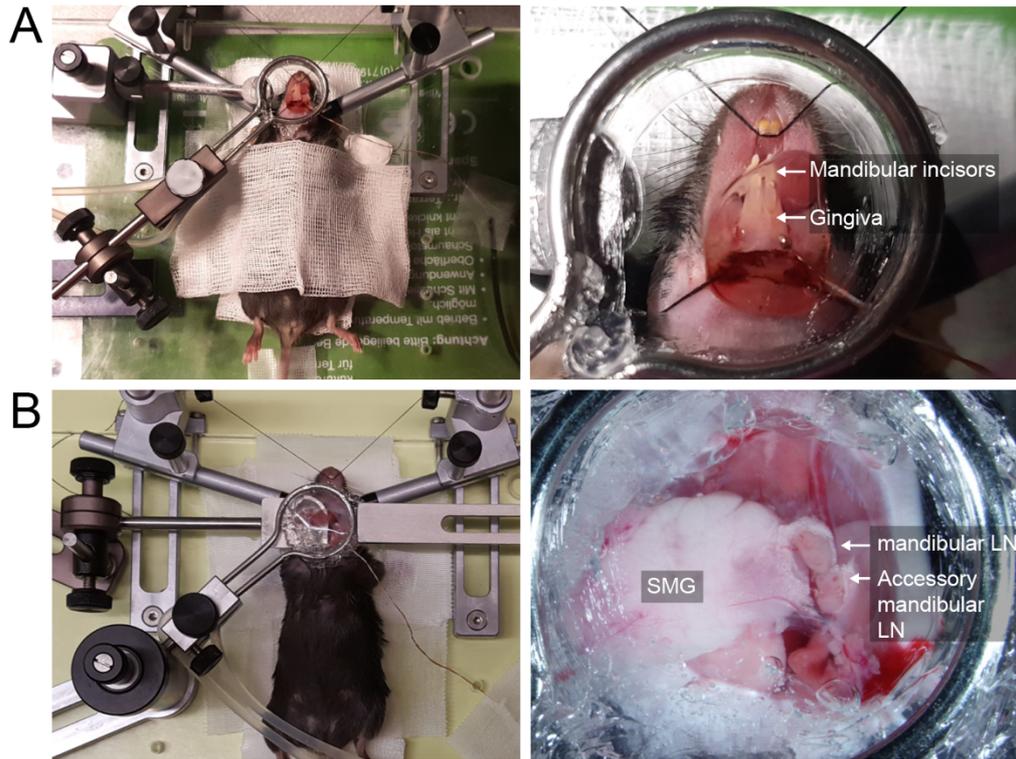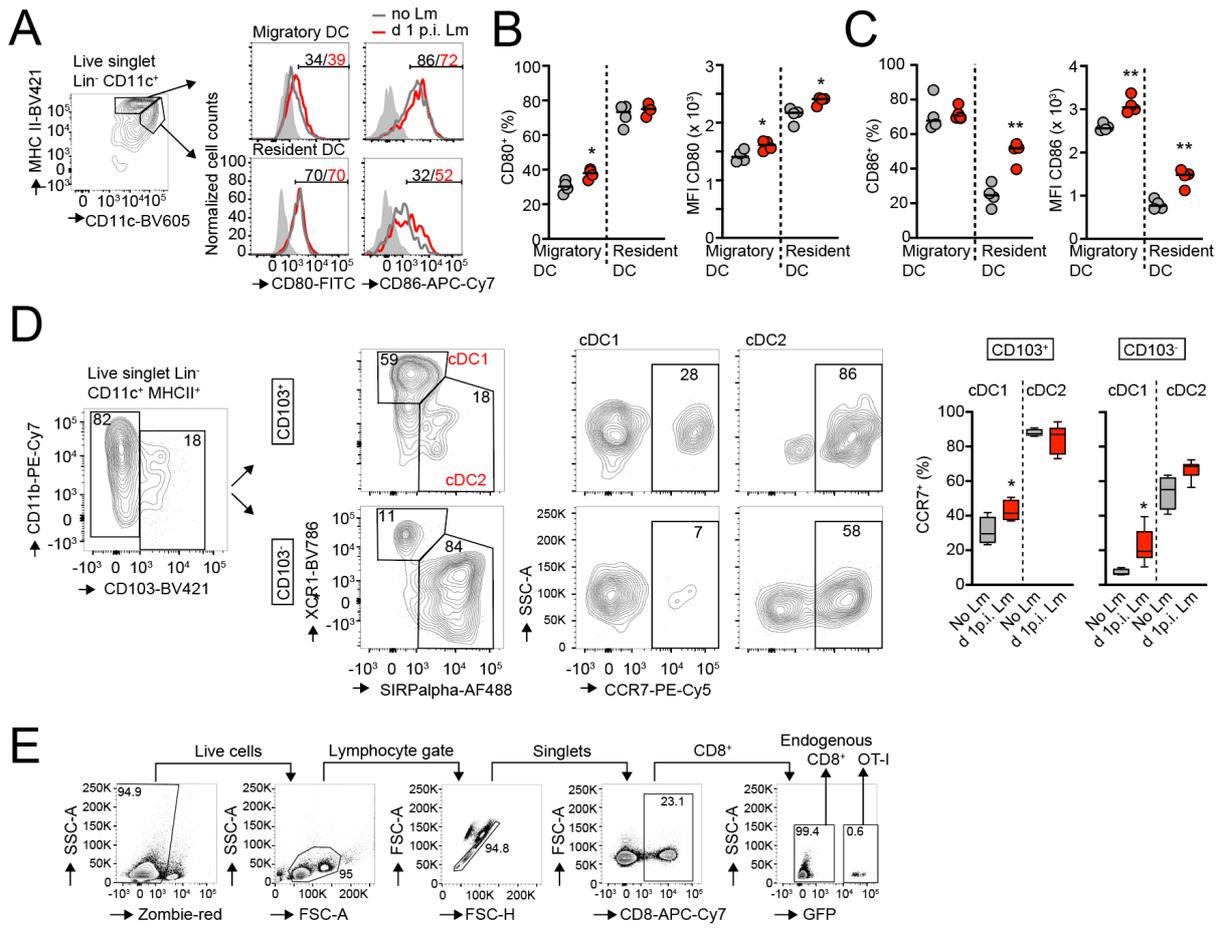
Figure S2
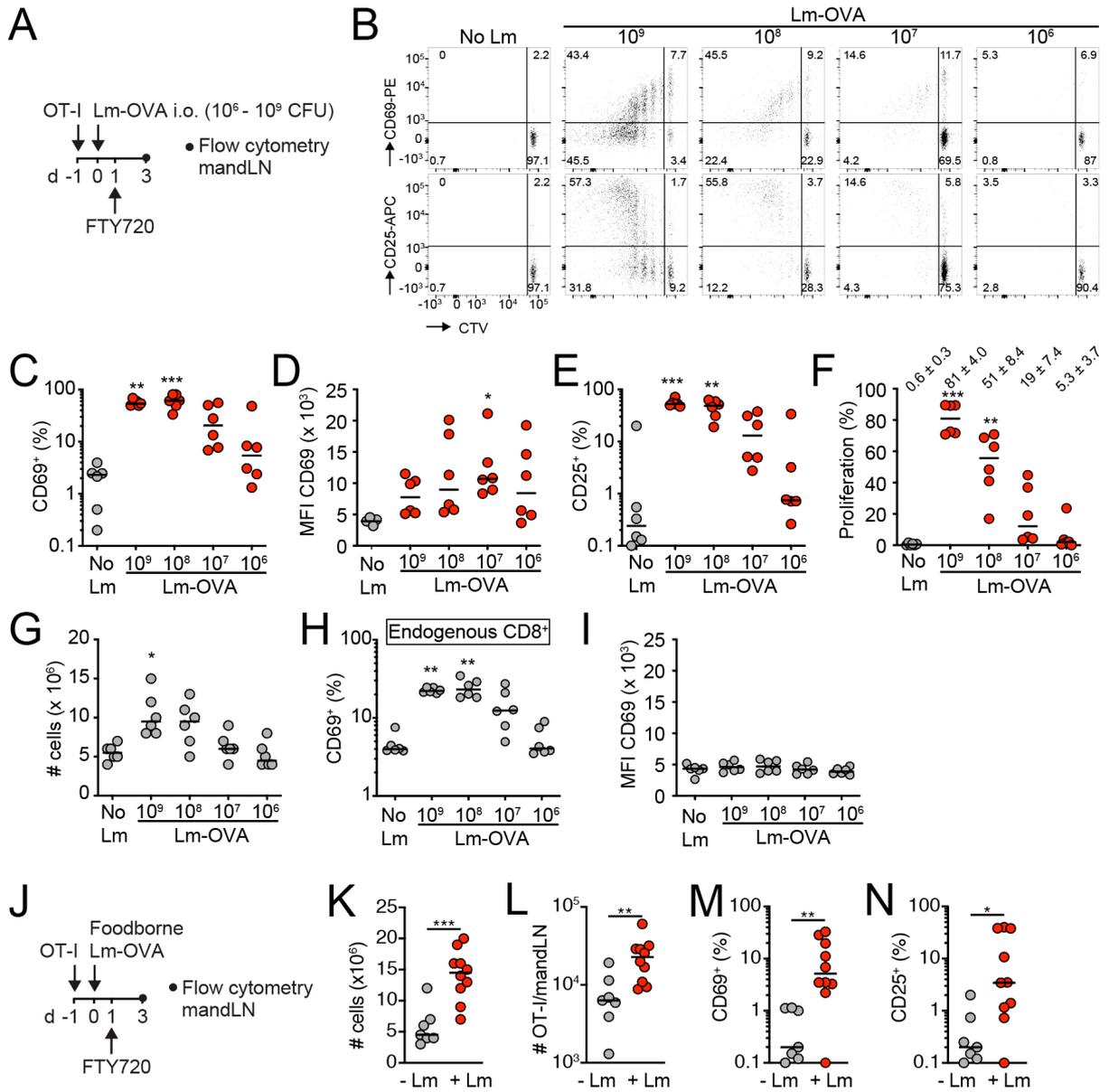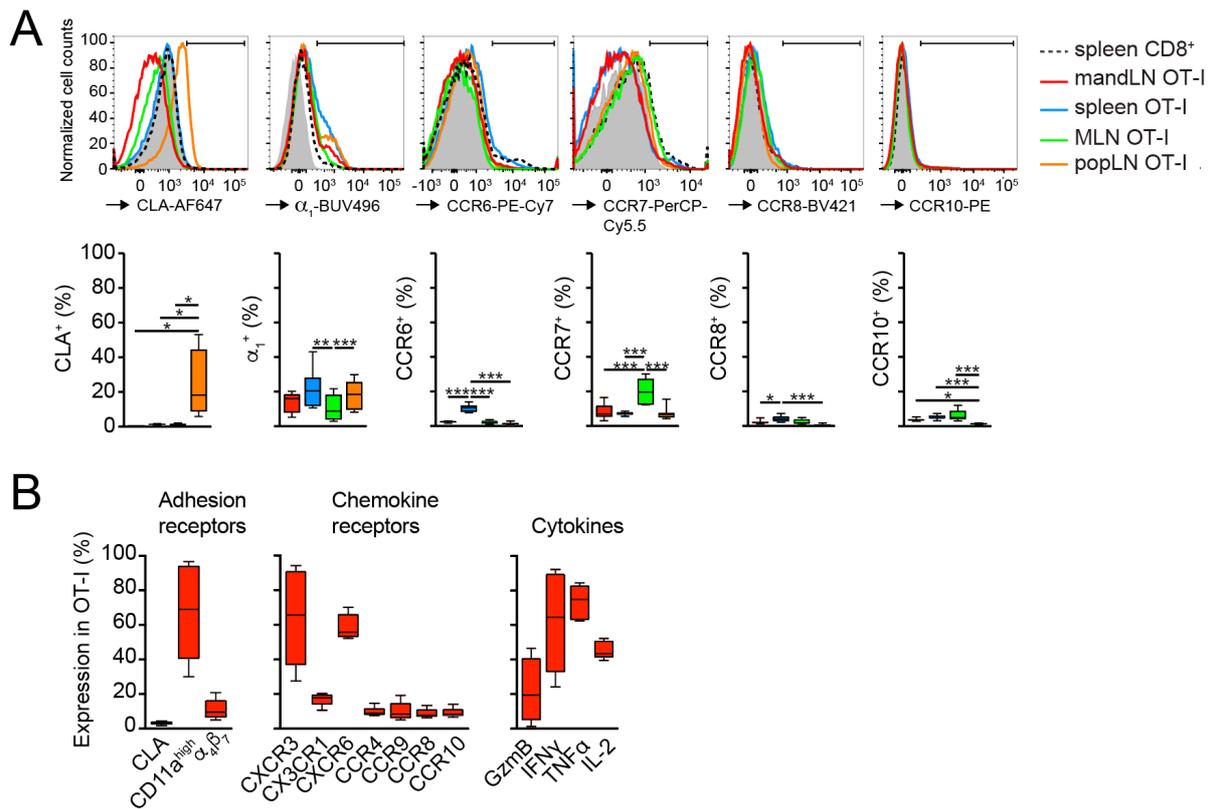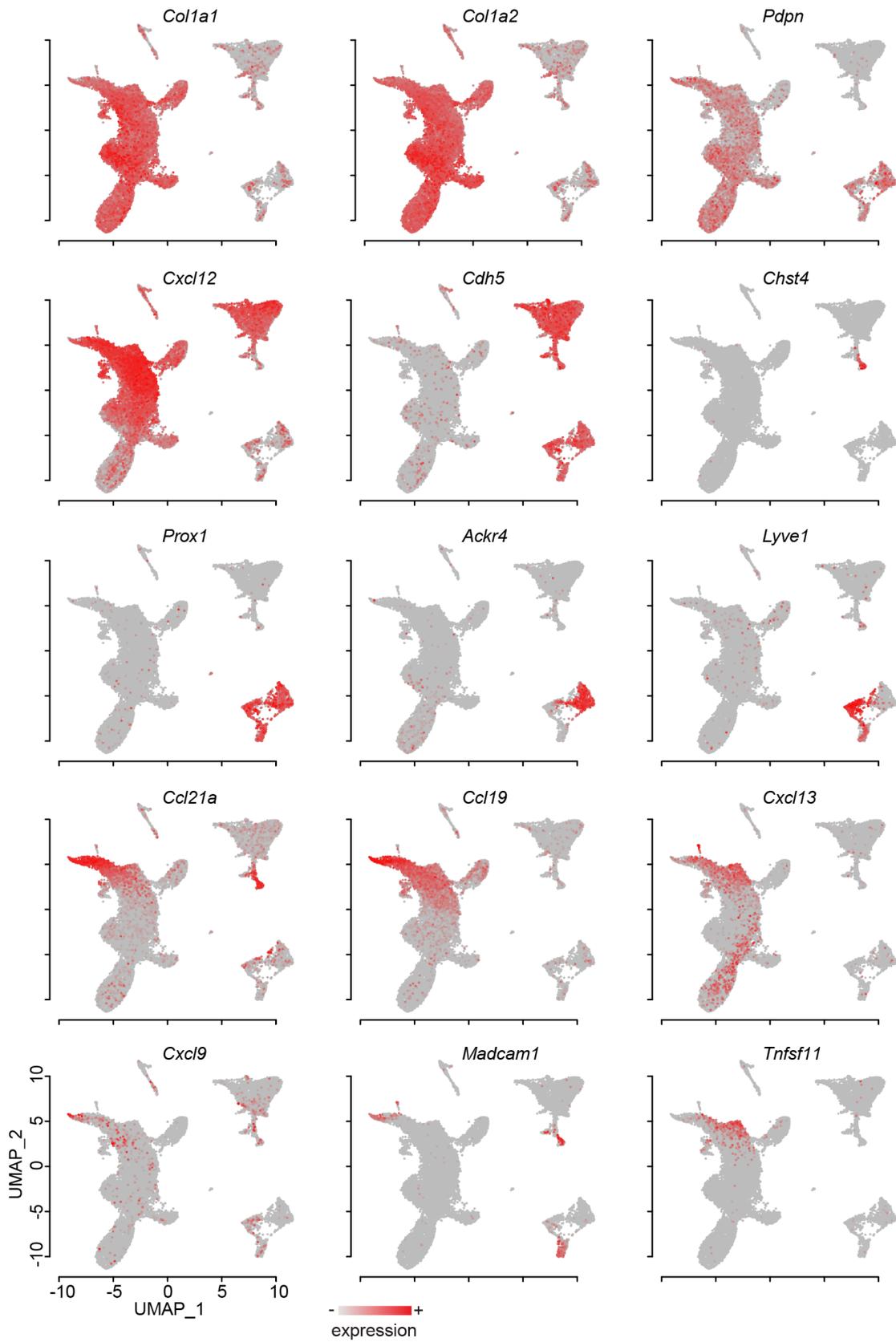
Figure S3

A

B

Figure S4

A OT-I input

B Live cells | Lymphocyte gate | Singlets | CD8⁺ | i.v. CD45-PE-Cy7⁻

C

D Endogenous CD8⁺ | mandLN OT-I | spleen OT-I | MLN OT-I

E

Figure S5

Figure S6

1363

A

Increased mandLN *versus* MLN

T cell costimulation
negative regulation of cysteine-type endopeptidase activity
negative regulation of chromosome condensation
subtelomeric heterochromatin assembly
positive regulation of ERK1 and ERK2 cascade
pericentric heterochromatin assembly
myoblast fusion involved in skeletal muscle regeneration
negative regulation of dendritic cell apoptotic process
myoblast differentiation
negative regulation of neuron apoptotic process

0 1 2 3 4 5 6
-log(P)

Decreased mandLN *versus* MLN

collagen fibril organization
negative regulation of osteoclast proliferation
vitamin E biosynthetic process
negative regulation of bone trabecula formation
negative regulation of bone mineralization involved in bone
positive regulation of transcription from RNA polymerase II
negative regulation of monocyte chemotaxis
oxygen metabolic process
negative regulation of lipoprotein lipase activity
positive regulation of peptidyl-tyrosine autophosphorylation

0 1 2 3 4
-log(P)

B

Increased mandLN *versus* PLN

lymphocyte chemotaxis
monocyte chemotaxis
chemokine-mediated signaling pathway
cellular response to interleukin-1
positive regulation of dendritic cell dendrite assembly
positive regulation of I-kappaB kinase/NF-kappaB signaling
cellular response to interferon-gamma
cellular response to tumor necrosis factor
negative regulation of dendritic cell apoptotic process
mature conventional dendritic cell differentiation

0 1 2 3 4 5
-log(P)

Decreased mandLN *versus* PLN

collagen fibril organization
response to cytokine
negative regulation of dendritic cell differentiation
negative regulation of endopeptidase activity
growth plate cartilage chondrocyte morphogenesis
extracellular matrix organization
positive regulation of protein folding
ribosomal large subunit assembly
collagen biosynthetic process
estrous cycle

0 2 4 6 8 10
-log(P)

Figure S7

1364

Figure S8

1365