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Macrophages induce long-term trapping of $\gamma\delta$ T cells with innate-like properties within secondary lymphoid organs in the steady state

By

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28 ABSTRACT

29 So far, peripheral T cells are mostly described to circulate between blood, secondary
30 lymphoid organs and lymph in the steady state. This nomadic existence would allow
31 them to accomplish their surveying task for both foreign antigens and survival signals.
32 Although it is now well established that $\gamma\delta$ T cells can be rapidly recruited to
33 inflammatory sites or in certain tumor microenvironments, the trafficking properties of
34 peripheral $\gamma\delta$ T cells have been poorly studied in the steady state. In the present paper,
35 we highlight the existence of resident $\gamma\delta$ T cells in the secondary lymphoid organs of
36 specific pathogen free mice. Indeed, using several experimental approaches such as the
37 injection of integrin-neutralizing antibodies which inhibit the entry of circulating
38 lymphocytes into lymph nodes and long-term parabiosis experiments, we have found
39 that, contrary to $\text{Ly}6\text{C}^{-/+} \text{CD}44^{\text{lo}}$ and $\text{Ly}-6\text{C}^{+} \text{CD}44^{\text{hi}}$ $\gamma\delta$ T cells, a significant proportion
40 of $\text{Ly}-6\text{C}^{-} \text{CD}44^{\text{hi}}$ $\gamma\delta$ T cells are trapped for long periods of time within lymph nodes and
41 the spleen in the steady state. Specific *in vivo* cell-depletion strategies have allowed us to
42 demonstrate that macrophages are the main actors involved in this long-term retention
43 of $\text{Ly}-6\text{C}^{-} \text{CD}44^{\text{hi}}$ $\gamma\delta$ T cells in secondary lymphoid organs.

INTRODUCTION

$\gamma\delta$ T cells are unique and distinct from other lymphocyte subsets, such as NK cells, B cells, and $\alpha\beta$ T cells, in that they combine adaptive features with rapid, innate-like responses that allow them to play an important role in all phases of an immune response. $\gamma\delta$ T cells are crucially involved in host immune defense against infections (1) but are also known to have a strong clinical association with various autoimmune diseases such as inflammatory bowel disease (2, 3), rheumatoid arthritis (or collagen-induced arthritis, the murine model of rheumatoid arthritis) (4, 5), and multiple sclerosis (or experimental autoimmune encephalomyelitis, the murine model of multiple sclerosis) (6, 7).

In addition, there is compelling evidence to indicate that $\gamma\delta$ T cells play an important role in immunity to cancer by sensing and reacting to cellular stress. This has been clearly demonstrated in murine models of spontaneous (8), chemically induced (9), transgenic (10), and transplantable tumors (11, 12). However, it seems that the activity of $\gamma\delta$ T cells in response to tumors can differ radically according to the tumor type or environment (13, 14). These data may actually reflect the high diversity of the $\gamma\delta$ T cell compartment.

In this regard, we have recently showed that within murine secondary lymphoid organs (SLOs), $\gamma\delta$ T cells can be subdivided into four subsets according to CD44 and Ly-6C expression. More precisely, we have characterized the functional heterogeneity of the peripheral $\gamma\delta$ T cell compartment that comprises Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells exhibiting innate-like features and naive and memory adaptive-like $\gamma\delta$ T cells (Ly6C^{-/+} CD44^{lo} $\gamma\delta$ T cells and Ly-6C⁺ CD44^{hi} $\gamma\delta$ T cells respectively; (15)).

This phenotypic and functional heterogeneity of the peripheral $\gamma\delta$ T-cell compartment may be also associated with differential T-cell trafficking dynamic properties. So far, peripheral T

67 cells are mostly described to circulate between blood, SLOs and lymph in the steady state.
68 This nomadic existence would allow them to accomplish their surveying task for both foreign
69 antigens and survival signals (16, 17). In line with this concept, Mandl et al., have showed in
70 a recent study that the mean transit time for CD4⁺ and CD8⁺ $\alpha\beta$ T cells in peripheral lymph
71 nodes (pLNs) were of 12 and 18 hours respectively (18). Concerning $\gamma\delta$ T cells, it is now well
72 established that $\gamma\delta$ T cells can be rapidly recruited to inflammatory sites or to certain tumor
73 microenvironments (19, 20). These $\gamma\delta$ T-cell infiltrations are notably orchestrated by
74 inflammatory cytokines such as IL-1 β or IL-23 or chemokine axes such as CCL2/CCR2 (12,
75 21, 22, 23). However, the T-cell trafficking dynamics of peripheral $\gamma\delta$ T-cell subsets in the
76 steady state have still been poorly studied.

77 Here, using several experimental approaches such as the injection of integrin-neutralizing
78 antibodies which inhibit the entry of circulating lymphocytes into lymph nodes (LNs) and
79 long-term parabiosis experiments, we have found that contrary to Ly-6C⁺ CD44^{hi} and CD44^{lo}
80 $\gamma\delta$ T-cell subsets which are circulating cells, an important proportion of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T
81 cells is residing within SLOs in the steady state. Moreover, specific *in vivo* cell-depletion
82 strategies have allowed us to demonstrate that macrophages are the main actors involved in
83 this long-term trapping of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells in SLOs in the steady state.

MATERIALS AND METHODS

Mice

C57BL/6 CD45.1 mice, C57BL/6 CD45.2 mice, C57BL/6 μ MT mice and C57BL/6 CD11c.DTR mice (24), were maintained in our own animal facilities, under specific pathogen-free conditions. C57BL/6 ROR γ t-GFP mice were initially obtained from Dr. Gerard Eberl, Institut Pasteur, France (25). 6- to 12-week-old mice were used for experiments. Animal housing, care and research were carried out in accordance with the guidelines of the French Veterinary Department. All procedures were approved by the French animal experimentation and ethics committee and validated by “Service Protection et Santé Animales, Environnement” with the numbers C-75-562 and A-75-1315. Sample sizes were chosen to assure reproducibility of the experiments and in accordance with the 3R of animal ethics regulation.

Cell suspensions

Peripheral lymph nodes (pooled cervical, axillary, brachial and inguinal lymph nodes; pLNs), mesenteric LNs (mLNs), cervical LNs (cLNs), and spleen were homogenized and passed through a nylon cell strainer (BD Falcon) in RPMI 1640 Glutamax (Gibco) supplemented with 10% fetal calf serum (FCS; Biochrom) for adoptive transfer and cell culture (LNs only), or in 5% FCS, 0.1% NaN₃ (Sigma-Aldrich) in phosphate saline buffer saline (PBS), for flow cytometry.

To verify the efficiency of DCs or macrophages *in vivo* depletion, LNs and Spleens were disrupted in RPMI medium containing 5% FCS, 1 mg Collagenase from Clostridium histolyticum type II and type IV (Sigma) and 0.5 mg DNase I (Sigma) and incubated for 20 min at 37 °C.

Fluorescence staining and flow cytometry

Cell suspensions were collected and dispensed into 96-well round-bottom microtiter plates (Greiner Bioscience; 6×10^6 cells/well). Surface staining was performed as previously described (26, 27). Briefly, cells were incubated on ice, for 15 minutes per step, with antibodies (Abs) in 5% FCS (Biochrom), 0.1% NaN_3 (Sigma-Aldrich) PBS. Each cell-staining reaction was preceded by a 15-minute incubation with a purified anti-mouse CD16/32 Ab (Fc γ RII/III block; 2.4G2) obtained from hybridoma supernatants. For determination of intracellular cytokine production, cells were stimulated with 0.5 $\mu\text{g/ml}$ PMA (Sigma-Aldrich), 0.5 $\mu\text{g/ml}$ ionomycin (Sigma-Aldrich), and 10 $\mu\text{g/ml}$ BrefeldinA (Sigma-Aldrich) for 2 hours at 37°C. Cells were then stained for surface markers, fixed in 2% paraformaldehyde in PBS, and permeabilized with 0.5% saponin, followed by labeling with specific cytokine Abs.

S1PR1 was detected with a rat monoclonal antibody (R&D Systems). Briefly, cells were first incubated for 90 min with the anti-S1PR1 rat mAb (50 $\mu\text{g/ml}$) in PBS containing 0.5% FCS, 1mM EDTA, 0.05% azide and 2% normal mouse serum. After washing, cells were then incubated with donkey biotinylated anti-rat IgG polyclonal Abs (Jackson ImmunoResearch) for 30 min in the above medium followed by streptavidin-APC (BioLegend).

Multicolor immunofluorescence was analyzed using BD LSR2 and BD Fortessa cytometers (BD Biosciences). List-mode data files were analyzed using Diva software (BD Biosciences). Data acquisition was performed on the Cochin Cytometry and Immunobiology (CIBIO) facility.

Parabiosis

Female host parabionts were generated with 8-12 week-old C57BL/6 CD45.1 mice and C57BL/6 CD45.2 mice. The percentage of host cells in a given T-cell subset corresponds to the percentage of CD45.1⁺ cells for the CD45.1 parabiont and the percentage of CD45.2⁺ cells for the CD45.2 parabiont.

136 **Blocking T-cell entry into LNs**

137 Purified anti-VLA-4 ($\alpha 4$) (clone PS/2) and anti-LFA-1 (αL) (clone M17/4) Abs were
138 obtained from Bio-XCell. For short-term treatment, mice were injected intraperitoneally one
139 time with 200 μ g of both Abs and then analyzed 48 hours later. For long-term treatment, mice
140 were injected intraperitoneally every 2 days during 6 days (4 injections) with 200 μ g of both
141 Abs and analyzed 24 hours later.

142 ***In vivo* cell depletions**

143 For dendritic cell depletion, C57BL/6 CD11c.DTR mice (referred as CD11c DOG mice) were
144 injected intraperitoneally every 2 days during 6 days (4 injections) with 250ng of Diphtheria
145 Toxin (DT).

146 The CSF1R inhibitor PLX3397 incorporated into rodent chow at 290 p.p.m. (delivering daily
147 doses of approximately 45 mg kg⁻¹) was provided along with control chow from Plexxikon
148 Inc. (Berkeley, CA). For macrophage *in vivo* depletion, C57BL/6 mice were fed with
149 PLX3397 or control chow during 7 days.

150 **FTY720 treatment**

151 Mice were injected intraperitoneally daily with FTY720 (Cayman Chemical) in saline at a
152 dose of ~1 μ g/g during 7 days.

153 **Immunofluorescence microscopy**

154 Inguinal, axillary, brachial and maxillary mouse LNs were initially fixed overnight in 0.1M
155 phosphate buffer containing 75mM L-Lysine, 10mM NaIO₄ and 1% PFA (PLP fixative
156 buffer). These organs were then embedded in 5% low-gelling-temperature agarose (type VII-
157 A; Sigma-Aldrich) prepared in PBS. 230- μ m LN-slices were cut with a vibratome (VT
158 1000S; Leica) in a bath of ice-cold PBS. Slices were then incubated at 37°C, for 20 minutes
159 per step, with Abs in PBS. Stained slices were photographed on an upright Leica stand with a
160 confocal spinning disk Yokogawa head with a 25x 0.95 Leica objective. Acquisitions were

161 made with Flash 4 LT HAMAMATSU camera. Analysis has been performed using Image J
162 software. Data acquisition was performed on the Cochin imaging photonic (IMAG'IC)
163 facility.

164 ***In vivo* LPS challenge**

165 C57BL/6 ROR γ t-GFP mice were injected intravenously with 100 μ g of LPS (from Escherichia
166 Coli 0111:B4; Sigma). Three hours after LPS challenge, cells recovered from pLNs, mLNs
167 and the spleen were incubated with 10 μ g/ml BrefeldinA (Sigma-Aldrich) for 2 hours at 37°C
168 for determination of intracellular cytokine production.

169 **Statistics**

170 Data are expressed as mean $\pm$ SEM, and the significance of differences between two series of
171 results was assessed using the student's paired or unpaired t test. Values of $p < 0.05$ were
172 considered significant. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

RESULTS

An important proportion of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes are trapped in LNs in the steady state

According to Ly-6C and CD44 expression, we have recently characterized the heterogeneity of the peripheral $\gamma\delta$ T cell compartment by showing that it comprises Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells exhibiting innate-like features and naive and memory adaptive-like $\gamma\delta$ T cells (Ly6C^{-/+} CD44^{lo} $\gamma\delta$ T cells and Ly-6C⁺ CD44^{hi} $\gamma\delta$ T cells respectively (15)). To go further, we have now decided to study the T-cell trafficking dynamics of these subsets. We first inhibited the entry of circulating lymphocytes into LNs through the injection of integrin-neutralizing antibodies (18, 28, 29). More precisely, C57BL/6 mice were injected i.p. with anti-LFA-1 (α L) and anti-VLA-4 (α 4) antibodies (Fig. 1A). We first verified that all $\gamma\delta$ T cells from peripheral LNs, mesenteric LNs and the spleen were expressing both LFA-1 and VLA-4 integrins (Fig. S1). The phenotype of $\gamma\delta$ T cells remaining within LNs was analyzed 48 hours after initiating the treatment (Fig. 1A). Interestingly, at that time-point, Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells were greatly enriched within the $\gamma\delta$ T cells remaining within peripheral LNs (pLNs; Fig. 1B-C). This resulted from the fact that the great majority of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells (~75%) remained trapped within pLNs whereas almost all the other $\gamma\delta$ T cells have egressed from them (Fig. 1D). Such a phenomenon could also be observed, to a lower extent, in mesenteric LNs (mLNs) as about 15% of the Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells were still present within this SLO 48 hours after the injection of integrin-neutralizing antibodies (Fig. 1C-D). Of note, in this model, we observed a strong increase in the absolute numbers of all the different $\gamma\delta$ T-cell subsets in the spleen (Fig. 1D). This phenomenon could result from the accumulation in the spleen of T lymphocytes egressing from LNs.

Narayan et al. (30) have previously showed that distinct cell types in the $\gamma\delta$ T-cell lineage

could be identified on the basis of their TCR repertoire usage. Therefore, we then analyzed the V γ -chain repertoire of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells remaining within pLNs and mLNs after the injection of integrin-neutralizing antibodies (Fig. 1E). Although we noticed a decreased proportion of V γ 1.1-expressing cells associated with an enrichment of V γ 2-expressing cells within pLNs following treatment, such a discrepancy was not observed within mLNs, indicating that V γ -chain usage does not allow to precisely identify LN-trapped $\gamma\delta$ T lymphocytes.

Consistent with the fact that Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes are the only $\gamma\delta$ T cells able to produce IL-17 in response to stimulation (15), we observed, after treatment, within both peripheral and mesenteric LNs, a clear and significant increase in the proportion of IL-17-producing $\gamma\delta$ T cells correlated with a diminished ability of the remaining $\gamma\delta$ T cells to produce IFN γ (Fig. 1F-G).

Because circulatory dynamics could differ between $\gamma\delta$ T-cell subsets (i.e. Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells could need more time to exit LNs than Ly-6C⁺ CD44^{hi} and CD44^{lo} $\gamma\delta$ T cells), we then decided to extend to 7 days the duration of the treatment blocking T-cell entry into LNs from the blood (Fig. 1H). In this setting, we still observed that whereas almost all Ly-6C⁺ CD44^{hi} and CD44^{lo} $\gamma\delta$ T cells have left LNs, the great majority of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells were still trapped in pLNs and to a lesser extent in mLNs (Fig. 1I). Taken together, our data suggest that contrary to the other $\gamma\delta$ T-cell subsets, Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes from LNs are in part non-circulating cells.

A significant proportion of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes is long-term SLO-resident cells in the steady state

To explain the results presented above, one may argue that Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells could enter into LNs in a LFA-1- and VLA-4-independent manner. To address this issue, we then

examined the cell exchange rate of the various $\gamma\delta$ T-cell subsets from SLOs (using CD45.1 and CD45.2 congenic markers) that occurred between parabiotic pairs (Fig. 2A). Four weeks following surgery, although both Ly-6C⁺ CD44^{hi} and CD44^{lo} $\gamma\delta$ T cells have achieved full chimerism in all studied SLOs, Ly-6C⁻ CD44^{hi} lymphocytes showed only limited exchange between the parabionts (Fig. 2B). In line with a weak circulatory dynamic of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells, we also observed a limited exchange of splenic IL-17-producing $\gamma\delta$ T cells between parabiotic mice (Fig. 2C). To examine whether these results were stable over time, we performed longer-term parabiosis experiments (Fig. 2D). Eight weeks post-surgery, in all studied SLOs, Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes still showed limited exchange between the paired mice (Fig. 2E). Altogether, these findings suggest strongly that an important proportion of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes corresponds to long-term SLO-resident cells in the steady state.

Macrophages are responsible for the long-term trapping of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes within SLOs in the steady state

We then attempted to identify the cellular actors involved in the retention of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells within SLOs. We first studied the potential involvement of B cells in this process. B-cell-deficient or -proficient C57BL/6 mice were treated or not with integrin-neutralizing antibodies and analyzed 48 hours later (Fig. 3A). The retention rate (recovery) of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells in pLNs and mLNs was comparable in both mouse strains, 48 hours after blocking T-cell entry (Fig 3B). Thus, B lymphocytes do not seem to be involved in the trapping of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells within LNs.

We next assessed whether dendritic cells (DCs) played a role in the retention of this $\gamma\delta$ T-cell subset in LNs. For this purpose, C57BL/6 CD11c.DTR mice (designated CD11c.DOG), that allow, following Diphtheria Toxin (DT) injections, a continuous *in vivo* depletion of DCs,

245 were injected or not with anti- α L and anti- α 4 antibodies (Fig. 3C). We first assessed the
246 depletion efficiency of such a protocol (Fig. S2A-B). Depletion was quite efficient for
247 plasmacytoid DCs in all LNs and better for the other DC subsets in mLNs than in pLNs. The
248 recovery of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells in both pLNs and mLNs 48 hours after blocking T-cell
249 entry into LNs was not diminished by DC depletion (Fig. 3D) suggesting that these latter cells
250 are not crucially involved in the retention of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells within SLOs.

251 Finally, we examined whether macrophages could contribute to Ly-6C⁻ CD44^{hi} $\gamma\delta$ T-cell
252 retention within SLOs. To test this hypothesis, C57BL/6 mice were fed with mouse chow
253 containing an inhibitor of CSF1R (PLX3397, provided by Plexxikon Inc.) for 7 days (Fig.
254 3E). We first assessed the depletion efficiency of such a protocol (Fig. S1C). This protocol led
255 to the depletion of the majority of subcapsular (F4/80⁻ CD169⁺) and medullary (F4/80⁺
256 CD169⁺) sinus macrophages in both pLNs and mLNs whereas F4/80⁺ CD169⁻ macrophages
257 were only poorly affected especially in pLNs. We found that the absolute numbers of Ly-6C⁻
258 CD44^{hi} $\gamma\delta$ T cells in pLNs, mLNs and the spleen dropped dramatically in mice fed 7 days
259 with PLX3397 chow (Fig. 3F). Although the absolute numbers of the other $\gamma\delta$ T-cell subsets
260 were also affected, the observed decreases were far less pronounced (Fig. 3F).

261 We hypothesized that macrophages would be required for Ly-6C⁻ CD44^{hi} $\gamma\delta$ T-cell retention
262 within SLOs. In such a model, macrophage depletion would lead to the egress of Ly-6C⁻
263 CD44^{hi} $\gamma\delta$ T cells from SLOs and that would account for the significant drop in absolute
264 numbers observed in mice treated 7 days with PLX3397. The membrane receptor S1PR1, by
265 allowing T cells to respond to the sphingolipid S1P present within efferent lymph, is the
266 central mediator of lymphocyte egress from SLOs (31, 32). First, we studied S1PR1
267 expression by $\gamma\delta$ T-cell subsets recovered from pLNs, mLNs and the spleen (Fig. S3). We
268 found that Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells from peripheral and mesenteric LNs exhibited higher

S1PR1 surface levels than the other $\gamma\delta$ T-cell subsets. Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells should be thus highly sensitive to S1P gradients. Then, C57BL/6 mice were fed with PLX3397 chow and daily injected or not for 7 days with FTY720 (Fig. 3G). Interestingly, FTY720 injections fully abrogated the strong decrease in Ly-6C⁻ CD44^{hi} $\gamma\delta$ T-cell absolute numbers provoked by the PLX3397 treatment in both pLNs and mLNs (Fig. 3H). These results suggest that CSF1R inhibitor administration is not toxic to $\gamma\delta$ T cells but rather allow them to egress from LNs through a S1PR1 dependent mechanism. Of note, FTY720 administration did not abolish the effect of the PLX3397 treatment in the spleen but rather accentuated the observed decrease of the absolute number of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T-cells (Fig. 3G). That could be explained by the duration of the S1PR1 agonist treatment. More precisely, it has already been described that whereas a single injection of FTY720 induces T-cell sequestration in the spleen (33 , 34), a prolonged FTY720 treatment rather leads, in this SLO, to a decrease in the absolute numbers of T lymphocytes (34, 35, 36). Taken together, these last results suggest that macrophages play a crucial role in the long-term trapping of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes within SLOs.

Membrane blebs reveal the close intimacy between Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes and macrophages in SLOs

It has recently been shown that an important fraction of IL-17-committed T lymphocytes from pLNs could acquire membrane blebs from subcapsular sinus macrophages, reflecting the intimacy of the crosstalk between these 2 cell types (37). We thus examined the proportion of Ly-6C⁻ CD44^{hi}, Ly-6C⁺ CD44^{hi} and CD44^{lo} $\gamma\delta$ T lymphocytes expressing macrophage markers such as CD11b, F4/80 and CD169 (Fig. 4A-B). Expression of these 3 molecules was solely detected on the cell surface of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells. Interestingly, the surface expression of CD11b, F4/80 and CD169 by Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes seemed to vary depending

on the location of these cells (pLNs vs mLNs vs the spleen; Fig. 4B) suggesting the ability of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes to initiate intimate crosstalk with various macrophage subsets such as subcapsular sinus macrophages (CD11b⁺ F4/80⁻ CD169⁺), medullary sinus macrophages (CD11b⁺ F4/80⁺ CD169⁺) or spleen red pulp macrophages (CD11b⁻ F4/80⁺ CD169⁻) (38 , 39, 40). More precisely, the observed proportions of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells acquiring CD11b, F4/80 and CD169 expression suggest that these cells interact preferentially with subcapsular and medullary sinus macrophages within pLNs (30% of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells from pLNs expressed CD11b and CD169 whereas only 15% expressed F4/80), with medullary sinus macrophages in mLNs [equal proportions (15%) of cells from mLNs expressing CD11b, F4/80 and CD169] and with red pulp macrophages in the spleen (Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells only expressed F4/80) (Fig. 4B and Fig. S4).

Using the PLX3397⁺ feeding strategy, we then studied the consequences of macrophage depletion on the expression of macrophage markers by Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes (Fig. 4C). Following PLX3397 treatment, there was a gradual decrease with time in the proportion of SLO Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells expressing CD11b, F4/80 or CD169 (Fig. 4D). These latter results confirm that the expression of CD11b, F4/80 and CD169 by this $\gamma\delta$ T-cell subset results from the transfer of membrane blebs from macrophages to $\gamma\delta$ T cells.

Altogether, our results suggest that Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes initiate strong adhesive interactions with macrophages that may lead to their long-term retention within SLOs.

ROR γ t expression allows to determine the localization of resident $\gamma\delta$ T cells within pLNs

We have described above a clear enrichment in IL-17-producing cells among $\gamma\delta$ T cells in LNs, 48 hours after blocking T-cell entry into these SLOs (Fig. 1E-F). We thus hypothesized that the expression of the transcription factor ROR γ t may allow a precise identification of resident $\gamma\delta$ T cells in SLOs. We first evaluated the proportion of Ly-6C⁻ CD44^{hi}, Ly-6C⁺

317 CD44^{hi} and CD44^{lo} $\gamma\delta$ T lymphocytes expressing ROR γ t in SLOs by using ROR γ t-GFP
318 transgenic mice (Fig. 5A-B). In agreement with the fact that only Ly-6C⁻ CD44^{hi} $\gamma\delta$ T
319 lymphocytes are able to produce IL-17 (15), we found that ROR γ t expression was restricted to
320 these cells. Surprisingly, the proportion of ROR γ t⁺ cells within Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells
321 differed considerably depending on the organ studied. Indeed, whereas the vast majority
322 (~80%) of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes recovered from pLNs expressed ROR γ t, this
323 proportion was quite lower in mLNs and the spleen (Fig. 5B). We then examined the
324 expression of ROR γ t by LN-resident $\gamma\delta$ T lymphocytes 48 hours after blocking T-cell entry
325 (Fig. 5C-E). In pLNs, after treatment, the proportion of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells expressing
326 ROR γ t was significantly higher than in pLNs from control mice (Fig. 5D). Accordingly, 48
327 hours after blocking T-cell entry, the recovery of ROR γ t⁺ Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells was far
328 better than the recovery observed for their ROR γ t⁻ cell counterparts (Fig. 5E). This was not
329 the case for mLNs as in this SLO, ROR γ t⁻ Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells were more efficiently
330 retained after treatment than ROR γ t⁺ Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells (Fig. 5D-E).

331 Altogether, these results suggest that the expression of ROR γ t can be considered as a reliable
332 marker to identify resident $\gamma\delta$ T cells within pLNs (but not in mLNs). Thus, in order to study
333 the location of resident $\gamma\delta$ T cells in pLNs, we imaged pLN slices recovered from ROR γ t-GFP
334 mice by immunofluorescence microscopy (Fig. 5F-H). In line with the capacity of Ly-6C⁻
335 CD44^{hi} $\gamma\delta$ T lymphocytes to acquire membrane blebs from subcapsular and medullary sinus
336 macrophages, we clearly observed that ROR γ t⁺ $\gamma\delta$ T cells (the majority of which are resident
337 cells within pLNs) were localized close to CD169⁺ sinus macrophages (Fig. 5F-G).
338 Quantification of the distance between ROR γ t⁻ or ROR γ t⁺ $\gamma\delta$ T cells and CD169⁺ cells
339 confirmed that ROR γ t⁺ $\gamma\delta$ T cells were very significantly far closer to CD169⁺ cells than their
340 ROR γ t⁻ cell counterparts (Fig. 5H). Such a close vicinity may help explaining the capacity of

341 Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells to acquire macrophage-related surface markers and such interactions
342 may lead to the long-term retention of these cells within SLOs.

343 **Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes from SLOs respond rapidly *in vivo* to LPS challenge**

344 As described above, our results suggest that Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes initiate strong
345 adhesive interactions with macrophages that may lead to their long-term retention within
346 SLOs. In addition, we observed that resident ROR γ t⁺ $\gamma\delta$ T cells were localized close to
347 CD169⁺ sinus macrophages within pLNs. We thus hypothesized that SLO resident $\gamma\delta$ T cells
348 could represent sentinels surveying the pathogen-exposed surface of SLOs.

349 To address this issue, ROR γ t-GFP transgenic mice were injected i.v. with LPS. IL-17 and
350 IFN γ productions by Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells recovered from pLNs, mLNs and the spleen
351 were then analyzed 3 hours later (Fig. 5I). We observed that, in all SLOs, around 20% of Ly-
352 6C⁻ CD44^{hi} $\gamma\delta$ T cells were already producing IL-17 in response to LPS challenge at this early
353 time-point (Fig. 5J). In addition, we noticed that IL-17 production was restricted to ROR γ t
354 expressing cells (Fig. 5K). Of note, in the same setting, we detected a small proportion of
355 IFN γ -producing Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells in the spleen and this IFN γ production was
356 restricted to ROR γ t⁺ cells (Fig. 5J-K). The capacity of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells to rapidly
357 produce IL-17 upon LPS challenge strongly suggest that these SLO resident $\gamma\delta$ T cells could
358 play a crucial surveying role to protect these organs from infections.

DISCUSSION

To achieve their surveying task for foreign antigens and survival signals, peripheral T cells have been described as having a nomadic existence between blood, SLOs and lymph in the steady state (16 , 17). Transit times within SLOs should be (a) not too long as T cells must continuously scan antigen-presenting cells (APCs) from different LNs in order to find where the next pathogen will cross the epithelial barrier, but also (b) not too rapid to avoid missing the rare APCs presenting their specific antigen in SLOs in the early phases of an infection. However, in parallel to that model, recent findings highlight the existence of T cells residing for long periods of time within non-lymphoid tissues (41). More precisely, subsets of CD8⁺ and CD4⁺ αβ T cells but also subsets of NK cells, NKT cells and γδ T cells have been shown to reside within non-lymphoid tissues such as the skin, gut, lungs or adipose tissue providing a rapid and efficient protection against reinfection by tissue-tropic pathogens but also promoting the repair of damaged tissues (42 , 43 , 44 , 45 , 46 , 47).

In the present paper, using injection of integrin-neutralizing antibodies and parabiosis strategies, we highlight the existence of γδ T cells residing in the steady state in the SLOs of specific pathogen-free mice. In line with our results, Zhang et al. have recently shown that innate-like T cells defined as expressing high surface levels of CD127 and CCR6, among which about one fourth corresponds to γδ T cells, accumulated near the subcapsular sinus in pLNs (32). Here, we confirm and extend these observations. First, while Zhang et al. examined the amount of cell exchange 2 weeks after surgery in parabiotic mice or 2 days after T-cell photoconversion in photoconvertible fluorescence protein Kaede transgenic mice, our parabiosis experiments clearly showed that full chimerism between the 2 parabionts was still not reached in pLNs for Ly-6C⁻ CD44^{hi} γδ T cells at least up to 2 months after surgery (Fig. 2). Thus, we here demonstrate the long-term residency of these cells within pLNs. Second, we showed that the long-term retention of Ly-6C⁻ CD44^{hi} γδ T cells was not a peculiar feature of

pLNs as it also applies to mLNs and the spleen. Third, while we confirmed that in pLNs, innate-like Ly-6C⁻ CD44^{hi} γδ T lymphocytes were located close to subcapsular sinus macrophages and were able to acquire, from this vicinity, the apparent expression of CD11b and CD169, our results strongly suggest that other subsets of macrophages are involved in the retention of these cells within SLOs (see below). Finally, we have also studied the behavior of the other γδ T-cell subsets (i.e. Ly-6C⁺ CD44^{hi} and Ly-6C^{-/+} CD44^{low} γδ T cells) in SLOs and showed that whatever their naïve-like or memory-like phenotype, they corresponded to circulating cells.

In addition to the CD11b and CD169 markers, about half of CD11b-expressing innate-like γδ T cells in pLNs and all of them in mLNs were also acquiring F4/80 expression (Fig. 4B). Like for CD11b and CD169, F4/80 surface expression by γδ T cells decreased gradually following *in vivo* macrophage depletion suggesting that innate-like γδ T cells are not only interacting with subcapsular (CD11b⁺ F4/80⁻ CD169⁺) sinus macrophages but also with medullary sinus (CD11b⁺ F4/80⁺ CD169⁺) macrophages. Finally, our parabiosis experiments show that a fraction of Ly-6C⁻ CD44^{hi} γδ T cells are also residing for long periods of time in the spleen. In this SLO, only F4/80 was detected at the cell surface of Ly-6C⁻ CD44^{hi} γδ T cells. This latter result suggests that resident γδ T lymphocytes are interacting with splenic red pulp (CD11b⁻ F4/80⁺ CD169⁻) macrophages. Such a hypothesis would be in agreement with previous studies that have observed a preferential localization of human and mice γδ T lymphocytes within the red pulp of the spleen (48 , 49). Interactions between tissue resident γδ T cells and F4/80⁺ macrophages have been reported in the lungs in mice (50). Indeed, it has been shown that pulmonary resident γδ T cells had an intrinsic preference to interact with F4/80⁺ macrophages (51). These continuous interactions would be important for their protective role as sentinels of airways and lung tissues.

409 Altogether, our data suggest that the precise “nature” of resident $\gamma\delta$ T cells but also of the
410 macrophages involved in their retention varies according to the secondary lymphoid organs
411 analyzed (pLNs vs mLNs vs the spleen). Indeed, whereas in pLNs, the residence of Ly-6C⁻
412 CD44^{hi} $\gamma\delta$ T cells was mostly restricted to ROR γ t⁺ cells, ROR γ t⁻ cells were more efficiently
413 trapped in mLNs than their ROR γ t⁺ cell counterparts. Furthermore, whereas both subcapsular
414 and medullary sinus macrophages would be involved in the retention of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T
415 cells in pLNs, the role of subcapsular sinus macrophages would be less crucial in mLNs and
416 the spleen. Indeed, medullary sinus and red pulp macrophages appear as the main actors
417 implied in the retention of $\gamma\delta$ T cells in mLNs and the spleen respectively. Sinus and red pulp
418 macrophages have a propensity to capture particulate material arriving in the lymph and the
419 blood (52-54). Thus, we hypothesize that resident Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells could play a
420 critical surveillance role in protecting SLOs from infections. In our previous study, we have
421 showed that Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells were possessing innate-like properties such as the
422 ability to respond directly to TLR ligands *in vitro* (15). Here, we have found that Ly-6C⁻
423 CD44^{hi} $\gamma\delta$ T cells from SLOs were rapidly able to produce IL-17 *in vivo* in response to LPS
424 challenge. In this setting, LPS may activate them directly or indirectly through the induction
425 of IL-23 and IL-1beta production by neighboring macrophages as it has been described that
426 ROR γ t⁺ $\gamma\delta$ T cells express IL-23R and produce IL-17 in response to IL-1beta and IL-23,
427 without T-cell receptor engagement (6). We are thus proposing that resident $\gamma\delta$ T cells and
428 macrophages could have complementary roles and may represent a major barrier blocking the
429 systemic spread of pathogens within SLOs by providing rapid innate responses.

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434

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592

593 **FIGURE LEGENDS**

594 **Figure 1: An important proportion of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes is trapped in** 595 **LNs in the steady state**

596 C57BL/6 mice have been injected i.p. with integrin-neutralizing anti-LFA-1 (α L) and anti-
597 VLA-4 (α 4) antibodies. Two days after treatment, pLNs, mLNs and spleen (Spl.) were
598 recovered separately. (A) Diagram illustrating the experimental model. (B) CD44/Ly-6C dot-
599 plots for gated TCR $\gamma\delta^+$ cells recovered from pLNs of representative control and treated mice.
600 Numbers on FACS dot-plots indicate the percentage of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells among the
601 $\gamma\delta$ T lymphocyte compartment (C) Pie charts illustrating the proportions of $\gamma\delta$ T-cell subsets
602 recovered from pLNs, mLNs and spleen (Spl.) of control (Ctrl.) and treated mice. (D)
603 Percentage of $\gamma\delta$ T cell-subset recovery within pLNs, mLNs and spleen (Spl.) of anti- α L and
604 anti- α 4 treated mice. The percentage of recovery was calculated by dividing the absolute
605 numbers in treated mice by the mean absolute number obtained in untreated animals. (E)
606 Percentage of V γ 1.1⁺ and V γ 2⁺ cells among Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells recovered from pLNs
607 and mLNs of control (Ctrl.) and anti- α L and anti- α 4 treated mice. (F) IL-17/IFN γ dot-plots
608 for gated TCR $\gamma\delta^+$ cells recovered from pLNs of representative control and treated mice. (G)
609 Proportion of IL-17- and IFN γ -producing TCR $\gamma\delta^+$ cells recovered from peripheral and
610 mesenteric LNs of control and treated mice. (H-I) C57BL/6 mice have been injected i.p. every
611 2 days during 6 days with 200 μ g of both integrin-neutralizing Abs and analyzed 24 hours
612 later. (H) Diagram illustrating the experimental model. (I) Percentage of $\gamma\delta$ T-cell subset
613 recovery within pLNs, mLNs and spleen (Spl.) of 6 days treated mice.
614 (C, D, E, G, I) Results are shown as means $\pm$ s.e.m. for at least two mice per group per
615 experiment, from at least three independent experiments and each point indicates an
616 individual mouse. Significance of differences between two series of results was assessed

using a two-tailed paired (D, H) or unpaired (C, F) Student's t-test. Values of $P < 0.05$ were considered as statistically significant (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$).

Figure 2: Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes are largely long-term SLO-resident cells in the steady state

Parabiotic mice were established between CD45.1 and CD45.2 C57BL/6 mice. Four weeks after surgery, parabiotic pairs were analyzed. (A) Diagram illustrating the experimental model. (B) Proportions of host cells, CD45.1⁺ for the CD45.1 parabiont and CD45.2⁺ for the CD45.2 parabiont, among the indicated $\gamma\delta$ T-cell subsets recovered from pLNs, mLNs and spleen (Spl.) from parabiotic pairs. (C) IL-17 and IFN γ dot-plots for gated TCR $\gamma\delta$ ⁺ cells recovered from spleen of representative parabiotic mice and percentage of host cells among these IL-17- and IFN γ -producing $\gamma\delta$ T cells. (D-E) Parabiotic mice were established between CD45.1 and CD45.2 C57BL/6 mice and analyzed 8 weeks after surgery. (D) Diagram illustrating the experimental model. (E) Proportions of host cells among the indicated $\gamma\delta$ T-cell subsets recovered from pLNs, mLNs and spleen (Spl.) from parabiotic pairs. Results are shown as means $\pm$ s.e.m. for at least three mice per group per experiment, from at least three independent experiments.

Figure 3: Macrophages are responsible for the long-term trapping of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes within SLOs in the steady state

(A-B) C57BL/6 and C57BL/6 μ MT mice have been treated or not with integrin-neutralizing antibodies and analyzed 48 hours later. (A) Diagram illustrating the experimental model. (B) Percentage of $\gamma\delta$ T cell-subset recovery (calculated as in figure 1D) within pLNs and mLNs of anti- α L and anti- α 4 treated C57BL/6 and C57BL/6 μ MT mice. (C-D) C57BL/6 CD11c.DTR

mice (designated CD11c.DOG) have been injected or not with integrin-neutralizing antibodies and Diphtheria Toxin (DT). (C) Diagram illustrating the experimental model. (D) Percentage of $\gamma\delta$ T cell-subset recovery within pLNs and mLNs of anti- α L and anti- α 4 treated control (Ctrl.) and DT injected mice. (E-F) C57BL/6 mice were fed with mouse chow containing or not an inhibitor of CSF1R (PLX3397). Seven days after PLX3397 feeding, pLNs, mLNs and spleen were recovered separately. (E) Diagram illustrating the experimental model. (F) Percentage of $\gamma\delta$ T-cell subset recovery within pLNs, mLNs and spleen (Spl.) of PLX3397 fed mice. (G-H) C57BL/6 mice were fed with mouse chow containing or not PLX3397 and daily injected or not with FTY720. Seven days after PLX3397 and FTY treatments, pLNs, mLNs and spleen were recovered separately. (G) Diagram illustrating the experimental model. (H) Absolute numbers of Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells recovered from pLNs, mLNs and spleen of control (Ctrl.), PLX3397 and PLX3397+FTY720 treated mice. (B, D, F, H) Results are shown as means $\pm$ s.e.m. for at least three mice per group per experiment, from at least two independent experiments.

Figure 4: Membrane blebs reveal the close intimacy between Ly-6C⁻ CD44^{hi} $\gamma\delta$ T lymphocytes and macrophage subsets in SLOs

(A) CD11b, F4/80 and CD169 fluorescence histograms of $\gamma\delta$ T-cell subsets recovered from pLNs are shown for a representative C57BL/6 mouse. Numbers in each histogram represent the percentage of CD11b⁺, F4/80⁺ or CD169⁺ cells among $\gamma\delta$ T-cell subsets. (B) The proportions of CD11b⁺, F4/80⁺ or CD169⁺ cells among $\gamma\delta$ T-cell subsets recovered from pLNs, mLNs and spleen (Spl.) of C57BL/6 mice were calculated. (C-D) C57BL/6 mice were fed with mouse chow containing or not an inhibitor of CSF1R (PLX3397). Two, four and seven days after PLX3397 feeding, pLNs, mLNs and spleen were recovered separately. (C) Diagram illustrating the experimental model. (D) The proportions of CD11b⁺, F4/80⁺ or

CD169⁺ cells among Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells recovered from pLNs, mLNs and spleen (Spl.) of C57BL/6 mice were calculated. Results are shown as means $\pm$ s.e.m. for at least three mice per group per experiment, from at least three independent experiments.

Figure 5: ROR γ t expression allows to determine the location of resident $\gamma\delta$ T cells within pLNs

(A) ROR γ t/TCR $\gamma\delta$ dot-plots for gated $\gamma\delta$ T-cell subsets recovered from pLNs of a representative C57BL/6 ROR γ t-GFP mouse. (B) The proportions of ROR γ t⁺ cells among $\gamma\delta$ T-cell subsets recovered from pLNs, mLNs and spleen (Spl.) of C57BL/6 ROR γ t-GFP mice were calculated. (C-E) C57BL/6 ROR γ t-GFP mice have been injected i.p. with integrin-neutralizing anti-LFA-1 (α L) and anti-VLA-4 (α 4) antibodies. Two days after treatment, pLNs and mLNs were recovered separately. (C) Diagram illustrating the experimental model. (D) The proportions of ROR γ t⁺ cells among Ly-6C⁻ CD44^{hi} $\gamma\delta$ T cells recovered from pLNs and mLNs of treated or untreated mice were calculated. (E) Percentage of ROR γ t⁺ and ROR γ t⁻ Ly-6C⁻ CD44^{hi} $\gamma\delta$ T-cell recovery (calculated as in figure 1) within pLNs and mLNs of anti- α L and anti- α 4 treated mice. Results are shown as means $\pm$ s.e.m. for at least three mice per group per experiment, from at least three independent experiments.

(F-H) Location of ROR γ t⁻ and ROR γ t⁺ $\gamma\delta$ T cells within pLNs from C57BL/6 ROR γ t-GFP mouse has been examined. (F) Representative immunofluorescence TCR $\gamma\delta$, CD169 and ROR γ t-GFP staining of fixed pLNs slices (maxillary LN is shown here). ROR γ t⁺ $\gamma\delta$ T cells appear in yellow. Squares indicate zoomed areas presented on the figure. (G) Location modeling of ROR γ t⁻ and ROR γ t⁺ $\gamma\delta$ T lymphocytes within pLNs (maxillary LN is shown here). Coordinates of all ROR γ t⁻ and ROR γ t⁺ $\gamma\delta$ T cells were calculated from immunofluorescence staining shown in (F) and were then re-applied on CD169 stained LN

slice. (H) Distance between $\text{ROR}\gamma\text{t}^-$ and $\text{ROR}\gamma\text{t}^+$ $\gamma\delta$ T lymphocytes and CD169^+ cells were
calculated. (I, J, K) C57BL/6 $\text{ROR}\gamma\text{t}$ -GFP mice were injected i.v. with LPS. Three hours later,
cells recovered from pLNs, mLNs and spleen were incubated during 2 hours with Brefeldin A
for determination of intracellular cytokine production. (I) Diagram illustrating the
experimental model. (J) Percentage of IL-17- and $\text{IFN}\gamma$ -producing cells among $\text{Ly-6C}^- \text{CD44}^{\text{hi}}$
 $\gamma\delta$ T cells recovered from pLNs, mLNs and spleen of control (Ctrl.) and LPS treated mice.
(K) Percentage of IL-17- and $\text{IFN}\gamma$ -producing cells among $\text{ROR}\gamma\text{t}^-$ and $\text{ROR}\gamma\text{t}^+$ $\text{Ly-6C}^- \text{CD44}^{\text{hi}}$
 $\gamma\delta$ T cells recovered from pLNs, mLNs and spleen of LPS treated mice. Results are shown as
 $\text{means} \pm \text{s.e.m.}$ for at least three different LNs slices from three different mice.

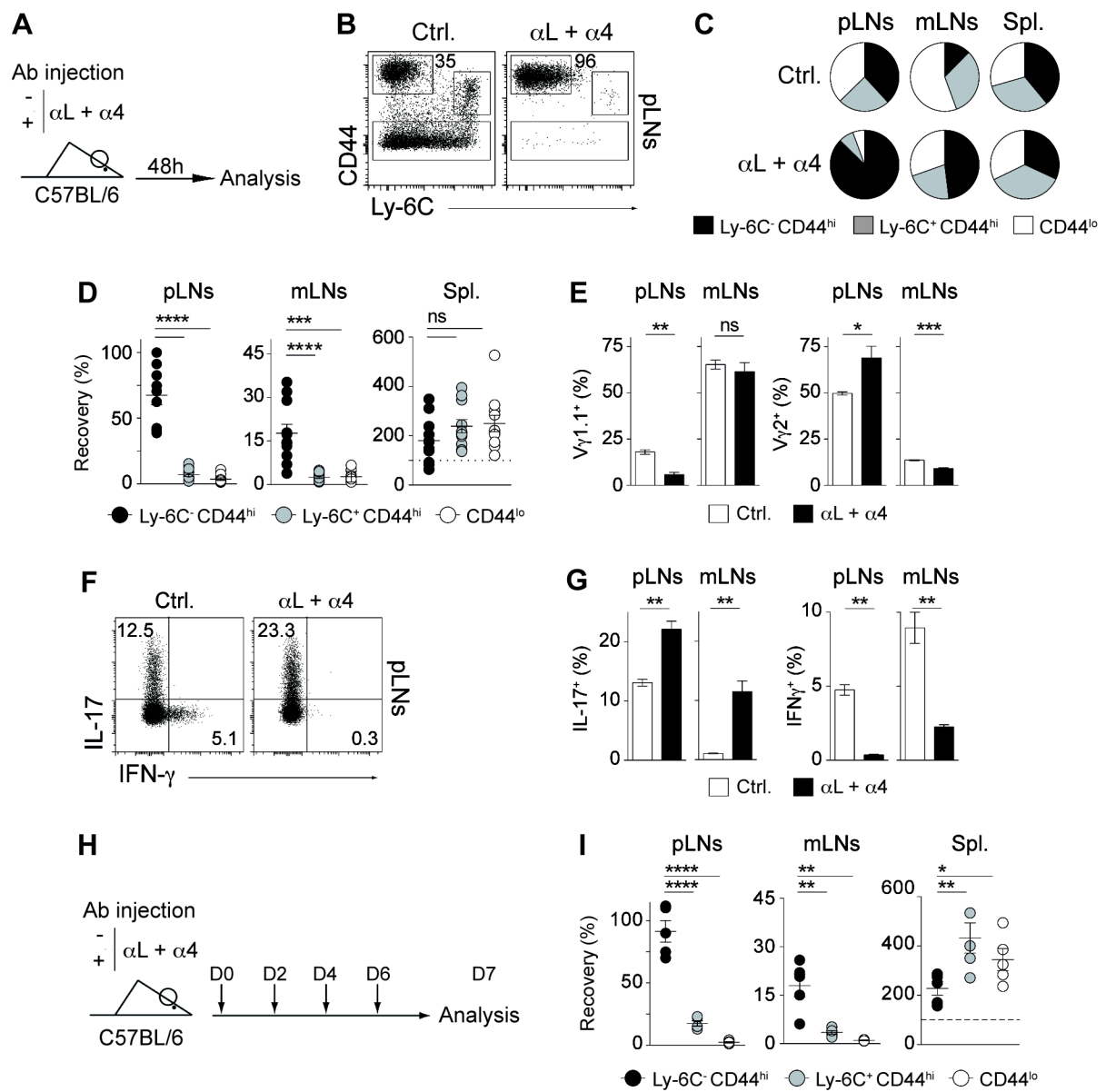


Figure 1

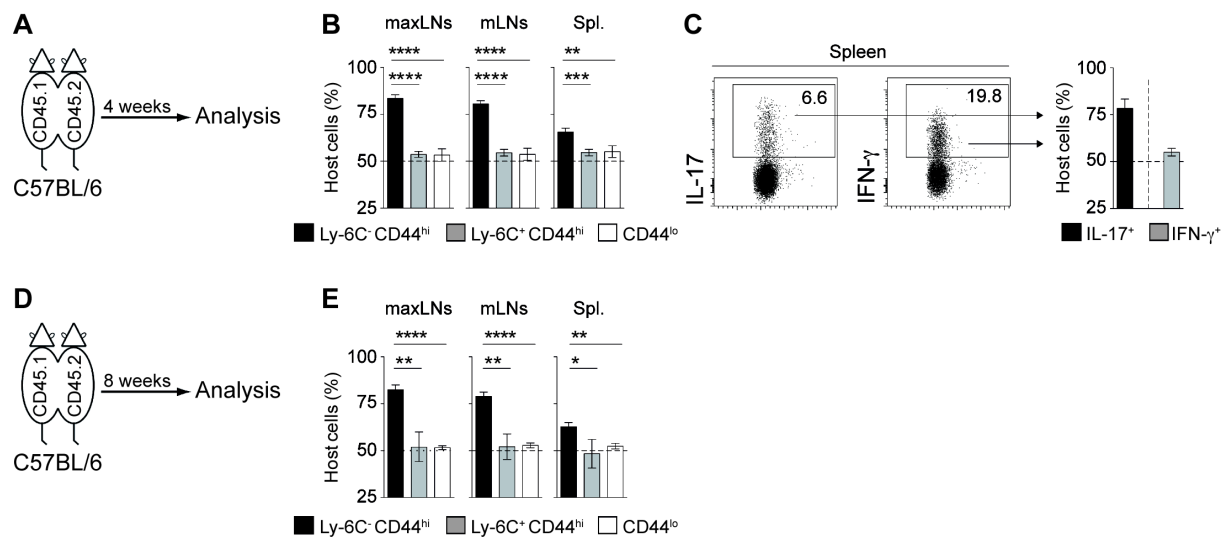


Figure 2

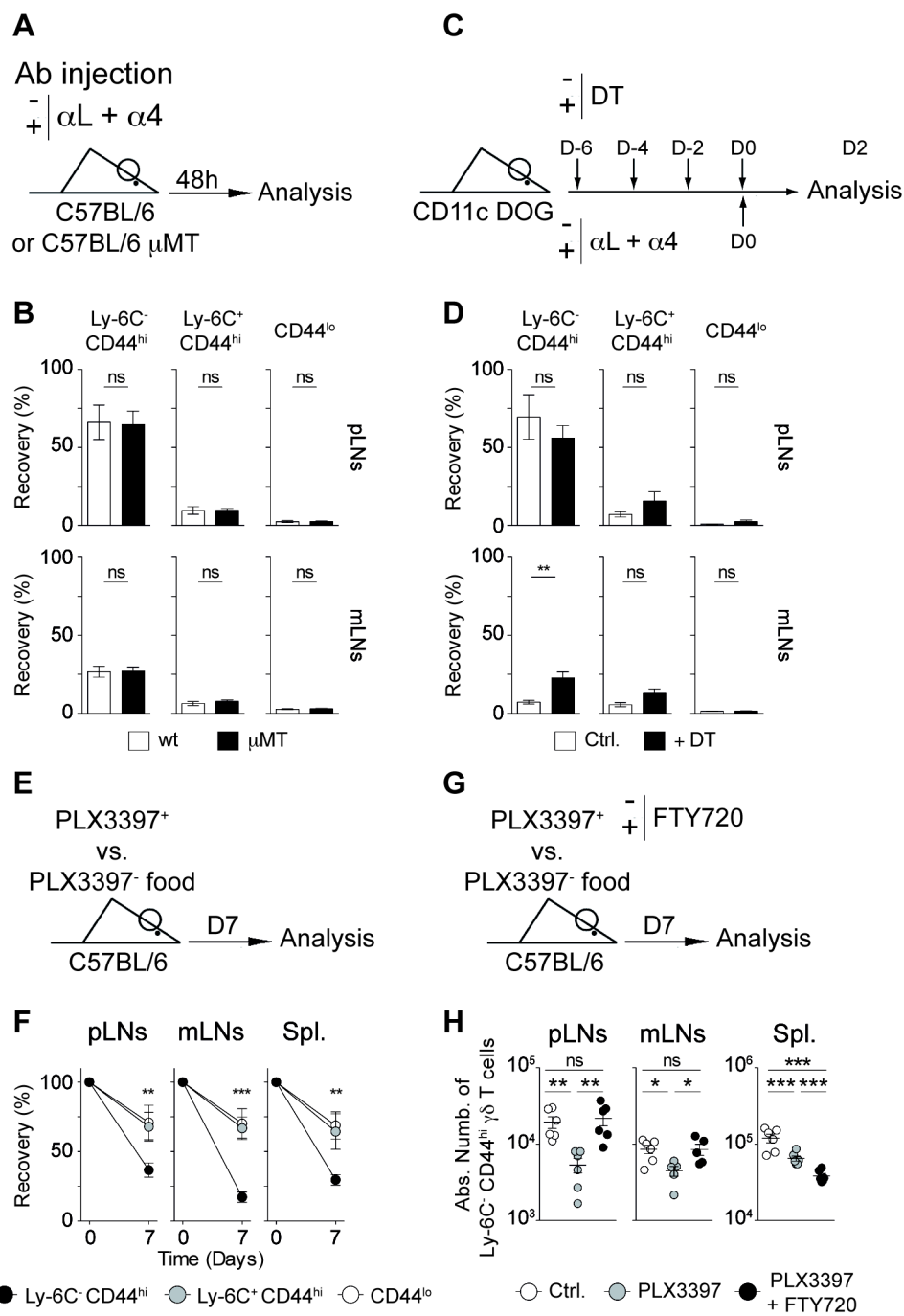


Figure 3

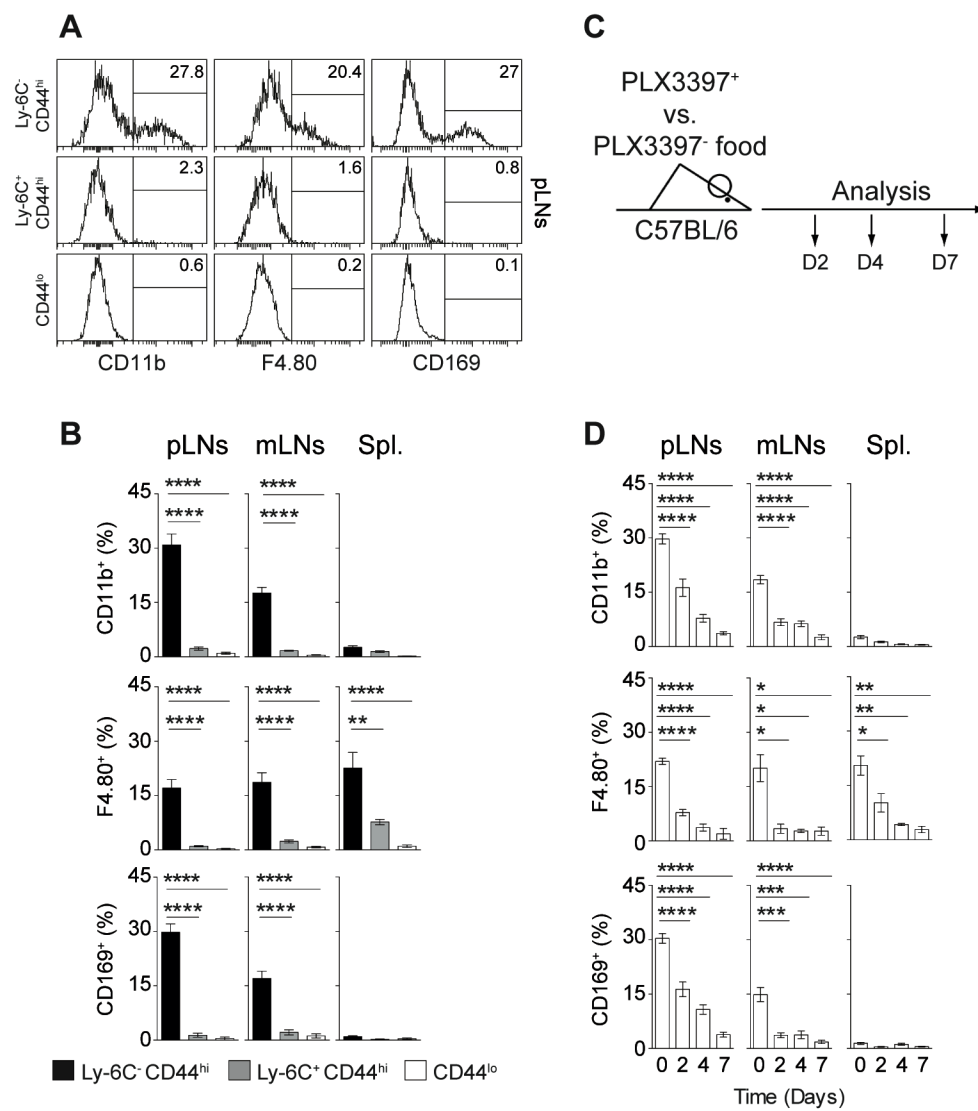


Figure 4

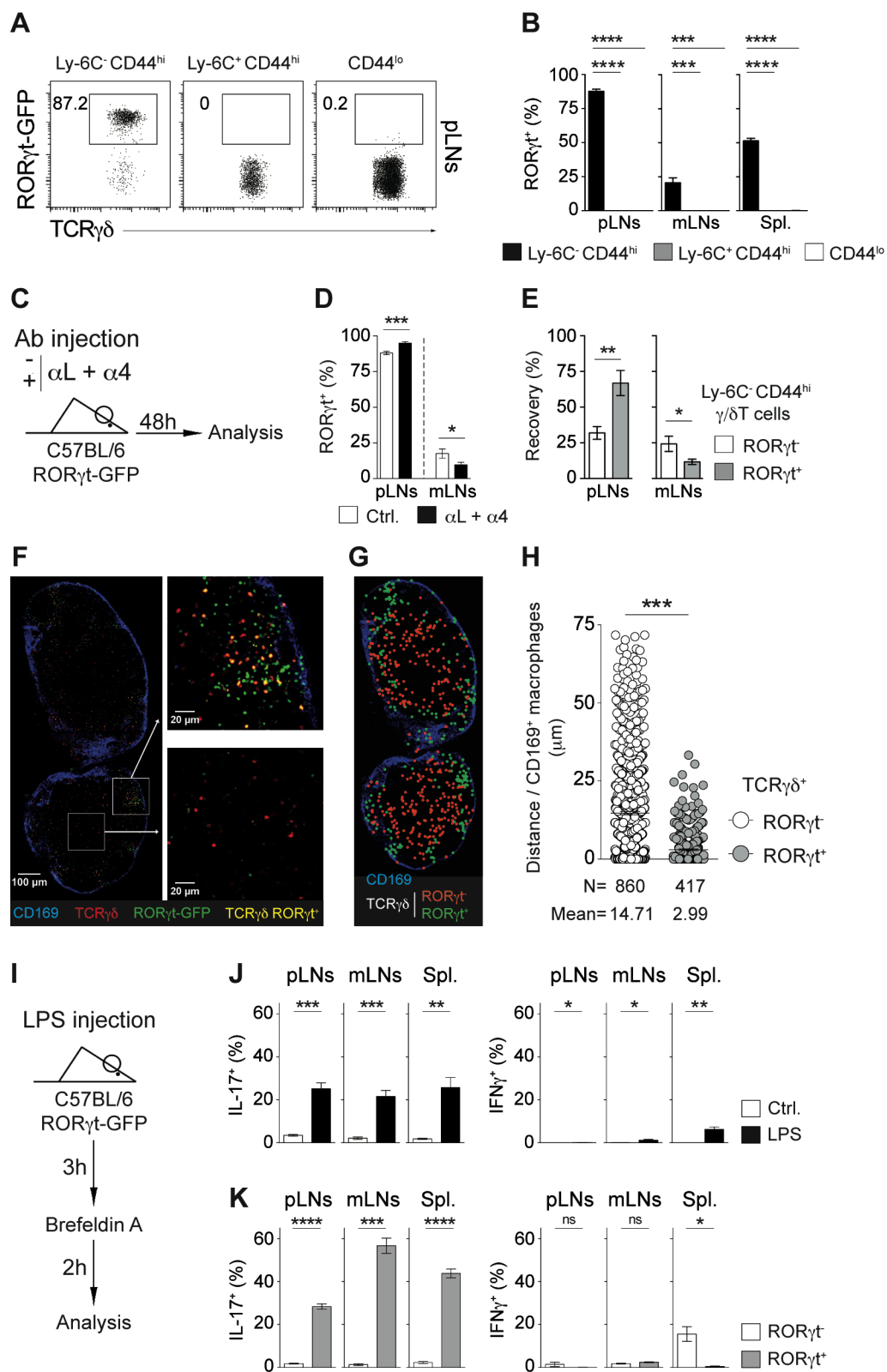


Figure 5