ImmunoHorizons

RESEARCH ARTICLE

Adaptive Immunity

No Major Impact of Two Homologous Proteins Ly6C1 and Ly6C2 on Immune Homeostasis

Junko Morimoto,* Minoru Matsumoto,*,†,‡ Ryuichiro Miyazawa,* Takeshi Oya,† Koichi Tsuneyama,‡ and Mitsuru Matsumoto*

*Division of Molecular Immunology, Institute for Enzyme Research, Tokushima University, Tokushima, Japan; †Department of Molecular Pathology, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan; and ‡Department of Pathology and Laboratory Medicine, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan

ABSTRACT

Ly6C comprises two homologous components of Ly6C1 and Ly6C2, and the expression of either of the Ly6C molecules defines unique functional subsets of monocytes. Ly6C is also expressed by other immune cell types, including Aire-expressing medullary thymic epithelial cells. Because the role of Ly6C expression in determining the functional subsets remains unclear, we generated mice deficient for both Ly6C1 and Ly6C2 with CRISPR-Cas9–mediated deletion. Mice deficient for Ly6C1/Ly6C2 showed no major alterations in the subsets and function of monocyte and other immune cells, including the cells involved in the dextran sulfate sodium salt–induced colitis model. By generating the mice deficient for Ly6C1 alone, we have also investigated the expression pattern of Ly6C1 and Ly6C2 in immune cells. Except for medullary thymic epithelial cells and CD4 single-positive T cells, immune cells predominantly expressed Ly6C2. Thus, despite the importance as a marker with a unique differential expression pattern, the Ly6C molecules have no major impact on determining the functional subsets and maintaining immune homeostasis. *ImmunoHorizons*, 2022, 6: 202–210.

INTRODUCTION

Monocytes and macrophages comprise an ontologically diverse family of mononuclear phagocytes (1–3). In mice, monocyte subsets can be discriminated by the surface expression of Ly6C (4–6), a member of a multigene family of GPI-anchored cell surface glycoproteins (7, 8). Specifically, Ly6C^{high} (i.e., Ly6C^{+}) monocytes are considered the progenitors of inflammatory macrophages, whereas Ly6C^{low} (i.e., Ly6C^{-}) monocytes reside in and maintain homeostasis in the vascular endothelium (9, 10) and have been shown to control tumor metastasis in the lung (11). Interestingly, Ly6C^{high} and Ly6C^{low} monocytes exhibit different developmental requirements for the transcription factor Nr4a1 (Nur77). Nr4a1 plays an important role in Ly6C^{low} monocyte development, but deletion of Nr4a1 does not affect Ly6C^{high} monocytes (12). Consistent with this finding, deletion of the Nr4a1 superenhancer ablated the development of Ly6C^{low} monocytes while preserving Ly6C^{high} monocytes (13). C/EBPβ was also found to be required for Ly6C^{low} but not Ly6C^{high} monocyte development (14). Thus, Ly6C expression plays a unique role in

Received for publication December 15, 2021. Accepted for publication February 25, 2022.

Address correspondence and reprint requests to: Prof. Mitsuru Matsumoto, Division of Molecular Immunology, Institute for Enzyme Research, Tokushima University, 3-18-15 Kuramoto, Tokushima 770-8503, Japan. E-mail address: mitsuru@tokushima-u.ac.jp

ORCIDs: 0000-0002-1955-6821 (Minoru Matsumoto); 0000-0001-5892-7480 (R.M.); 0000-0002-0670-9868 (K.T.); 0000-0003-3844-8110 (Mitsuru Matsumoto).

This work was supported in part by Japan Society for the Promotion of Science KAKENHI Grants 16H06496, 16K21731, 18K19564, and 19H03699 (to Mitsuru Matsumoto), and 19K07626 (to J.M.).

J.M. and Mitsuru Matsumoto designed the experiments. J.M., Minoru Matsumoto, R.M., and Mitsuru Matsumoto conducted the experiments. J.M., Minoru Matsumoto, T.O., and K.T. evaluated the pathological changes in mice. J.M. and Mitsuru Matsumoto wrote the paper.

Abbreviations used in this article: BM, bone marrow; cMOP, common monocyte progenitor; DC, dendritic cell; DSS, dextran sulfate sodium salt; DT, diphtheria toxin; EpCAM, epithelial cell adhesion molecule 1; ES cell, embryonic stem cell; gRNA, guide RNA; KO, knockout; Lin, lineage; Ly6C1/6C2-deficient mice, mice deficient for Ly6C1/Ly6C2; Ly6C1-KO, Ly6C1 knockout; mTEC, medullary thymic epithelial cell; SP, single-positive; UEA-1, *Ulex europaeus* agglutinin 1; WT, wild-type.

The online version of this article contains supplemental material.

https://doi.org/10.4049/immunohorizons.2100114

ImmunoHorizons is published by The American Association of Immunologists, Inc.

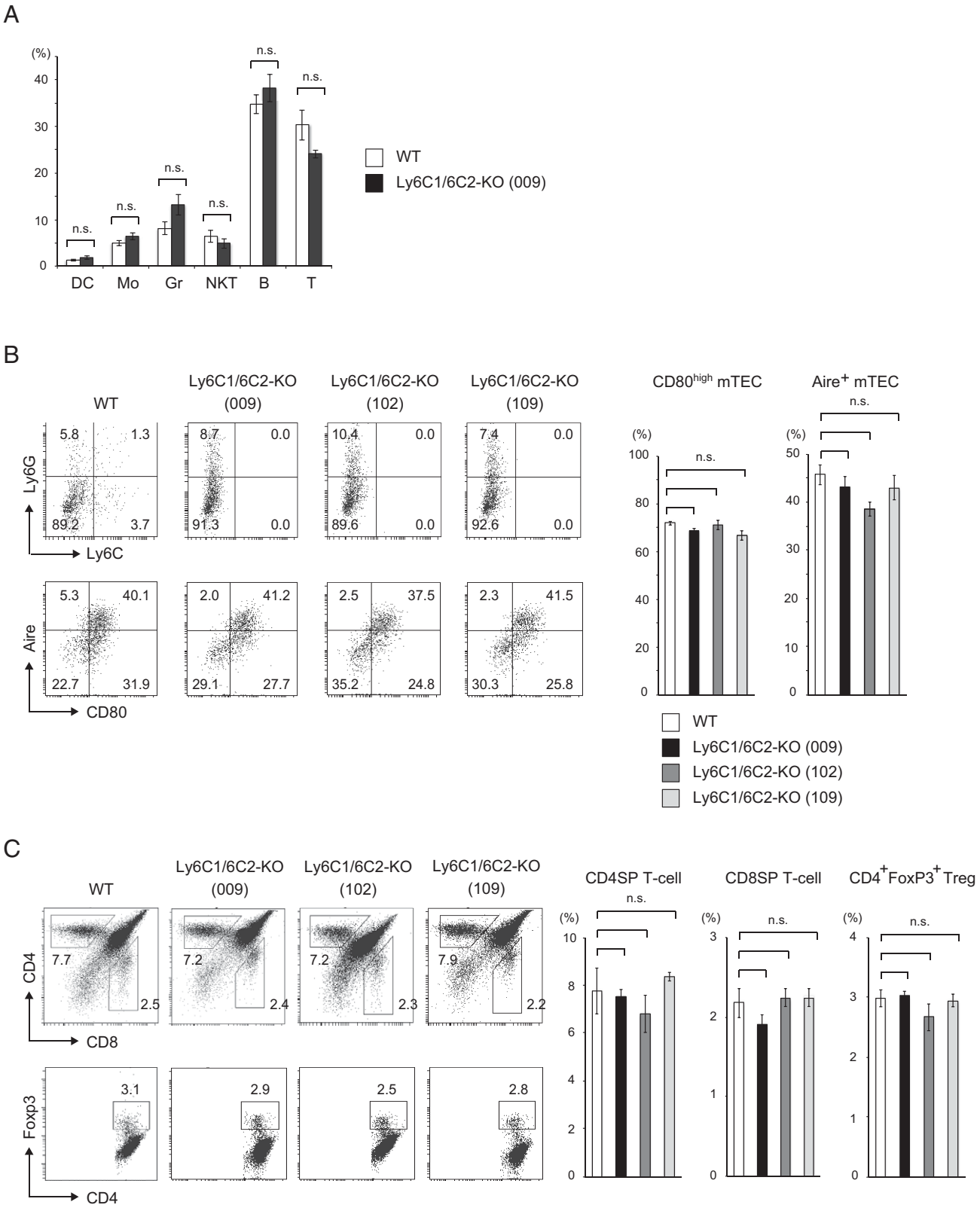

FIGURE 1. Phenotypic analysis of Ly6C1/6C2-deficient mice

(**A**) The abrogation of Ly6C1/Ly6C2 did not affect the composition of peripheral blood, including monocytes. Mo, monocyte; Gr, granulocyte; NKT, NKT cell. Ly6C1/6C2-deficient mice (n = 4) and littermate controls (n = 5) were investigated. The results shown were obtained from line 009. (**B**) The abrogation of Ly6C1/Ly6C2 did not affect the composition of mature CD80^{high} mTECs and Aire-expressing mTECs ***(Continued)***

https://doi.org/10.4049/immunohorizons.2100114

defining monocyte subsets. In addition to monocytes/macrophages, Ly6C is expressed from other immune cells such as $CD8^+$ T cells (15–17) and NK cells (18, 19). Stromal cells including intestinal epithelial cells (20) and medullary thymic epithelial cells (mTECs) containing $Aire^+$ mTECs also express Ly6C (21). However, the role of the Ly6C molecules in determining the functional subsets and their possible corresponding functions remains unknown.

The difficulty in elucidating the function of Ly6C by generating mice deficient for Ly6C has been attributed to the presence of two highly homologous proteins, Ly6C1 and Ly6C2 (22): the corresponding genes of these proteins are located in close proximity in the genome (22), and mAbs currently available cannot distinguish between Ly6C1 and Ly6C2. Accordingly, information on the exact distribution of each Ly6C protein in immune cells is lacking. Because deletion of *Ly6c1* and *Ly6c2* individually by homologous recombination in embryonic stem cells (ES cells) and crossing the two knockout strains to generate total Ly6C null mice is not a feasible approach, we used CRISPR-Cas9–mediated deletion in fertilized oocytes to investigate the role of Ly6C proteins for immune homeostasis. Furthermore, by combining with the mice deficient for Ly6C1 alone, which have been generated by conventional gene knockout technology, we have provided information on the differential expression of Ly6C1 and Ly6C2 from various immune cells, hoping that information from our genetic approach will further extend the utility of Ly6C as a functional marker for broader types of immune cells.

MATERIALS AND METHODS

Mice

Mice deficient for both *Ly6c1* and *Ly6c2* were generated via CRISPR-Cas9–mediated deletion in fertilized oocytes. Briefly, two common target sequences located in exon 3 of *Ly6c1* and *Ly6c2* were selected to disrupt both genes: 5′-CTGCAGTGCTACGAGTGCTA-3′ for guide RNA (gRNA)gg1 and 5′-GAATCCATCAGAGGCGCGGC-3′ for gRNAcc2. BLAT (BLAST-like alignment tool) was used to ensure that the candidate gRNA sequences had a low predicted incidence of off-target mutations. The two gRNAs and hsCas9 mRNA (System Biosciences, catalog no. CAS500A-1) sequences were cloned into the T7 gRNA cloning and production vector (System Biosciences, catalog no. CAS510A-1), transcribed in vitro, and injected into the cytoplasm of fertilized C57BL/6 oocytes (CLEA Japan) at a gRNA1/gRNA2/hsCas9 ratio of 1:1:2. To eliminate phenotypes derived from off-target mutations, analyses were performed on mice that were at least two generations removed from the founder.

Ly6C1-deficient mice were generated by homologous recombination in ES cells established from C57BL/6 mice. A knock-in cassette containing flox-3 × poly(A)-flox/diphtheria toxin (DT)/frt-Neo^r-frt was inserted into exon 2 of the *Ly6c1* locus. After targeted cells were injected into morula-stage embryos, the resulting chimeric male mice were mated with C57BL/6 females (CLEA Japan) to establish germline transmission. Neo^r was removed by crossing the mice with a transgenic line expressing the general deleter flippase. All mice were maintained under pathogen-free conditions and handled in accordance with the Guidelines for Animal Experimentation of Tokushima University School of Medicine.

Pathology

Formalin-fixed tissue sections were subjected to H&E staining, and two pathologists independently evaluated the histology without being informed of the detailed condition of the individual mouse.

Cell preparation and flow cytometric analysis

Thymic epithelial cells (TECs) were prepared via enzymatic digestion of thymi, and subjected to flow cytometric analysis using a FACSCalibur (BD Biosciences) and a FACSAria II (BD Biosciences) as described previously (21, 23, 24). In brief, TECs were defined as $CD45^-$epithelial cell adhesion molecule 1 $(EpCAM)^+$ cells, and mTECs were defined as $CD45^-EpCAM^+$ *Ulex europaeus agglutinin* 1 $(UEA\text{-}1)^+$ cells. Bone marrow (BM) cells were harvested from bilateral femurs, and RBCs were lysed before flow cytometric analysis. Cells in the peritoneal cavity were harvested by washing the peritoneal cavity with cold PBS containing 0.5 mM EDTA twice. mAbs against CD45 (clone 30-F11), EpCAM (clone G8.8), Ly51 (clone 6C3), CD80 (clone 16-10A1), I-A/I-E (clone M5/114.15.2), Ly6C (clone HK1.4), Ly6G (clone 1A8), CD11b (clone M1/70), CD11c (clone N418), CD3 (clone 145-2C11), CD19 (clone 6D5), CD49b (clone HMa2), CD4 (clone L3T4), CD8 (clone 53-6.7), Foxp3 (clone FJK-16S), CD115 (clone AFS98), CD117 (clone 2B8), CD135 (clone A2F10), F4/80 (clone BM8), CD62L (clone MEL-14), CD44 (clone IM7), CD64 (clone X54-5/7.1), NK1.1 (clone PK136), and CD49f (clone GoH3) were purchased from BioLegend and eBioscience. Anti-Ly6C mAbs and clones ER-MP 20 and AL-21 were from Abcam and BD Biosciences, respectively. A rat anti-mouse Aire mAb (clone MM-525) from

($Aire^+$ mTECs) that develop among $CD80^{high}$ mTECs (left). A summary of the percentages of $CD80^{high}$ mTECs and $Aire^+$ mTECs in three independent knockout lines ($n = 5$ for each line) are shown on the right. Representative profiles of flow cytometry are shown on the left. Plots were gated for $CD45^-EpCAM^+UEA\text{-}1^+$ cells. (**C**) The abrogation of Ly6C1/Ly6C2 did not affect the development of thymocytes (left upper), including regulatory T cells (Tregs; left lower). A summary of the results from three independent knockout lines is shown on the right ($n = 5$ for each line). Representative profiles of flow cytometry are shown on the left. Plots were gated for $CD45^+$ cells (upper) and CD4SP thymocytes (lower). Bands indicate mean ± SEM. The significance was determined using an unpaired two-tailed Student *t* test. n.s., not significant.

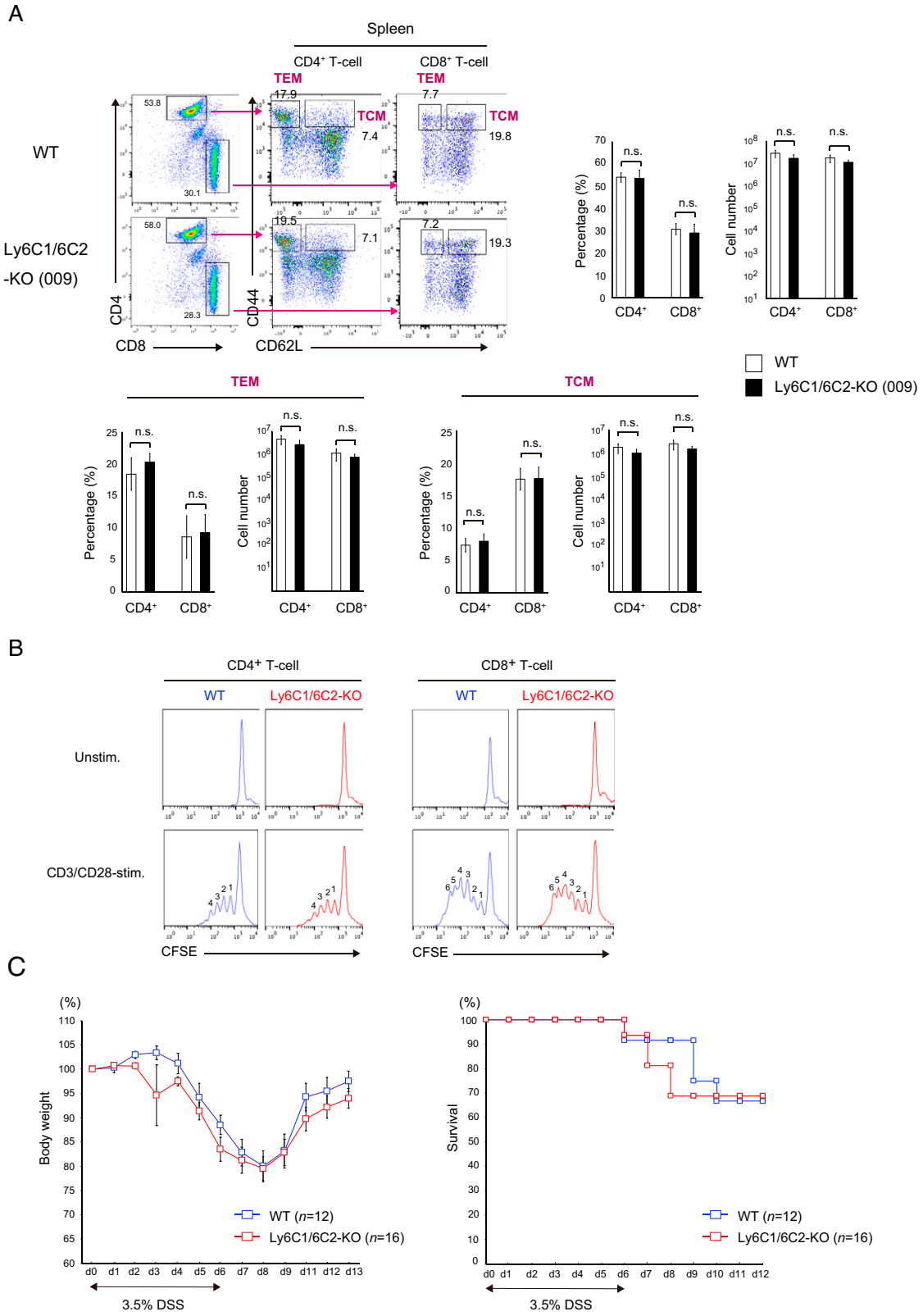

FIGURE 2. Unaltered immunological properties of Ly6C1/6C2-deficient mice

(**A**) Phenotypic analysis of $CD4^+$ SP and $CD8^+$ SP T cells from the spleen in WT ($n = 5$) and Ly6C1/6C2-deficient mice ($n = 4$) (upper right). Percentages and absolute cell numbers of effector memory T cells (TEMs) and central memory T cells (TCMs) as identified $CD44^{high}CD62L^{low}$ and $CD44^{high}CD62L^{high}$, respectively, were analyzed (lower). Representative profiles of flow cytometry are shown on the upper left. ***(Continued)***

https://doi.org/10.4049/immunohorizons.2100114

BD Biosciences was also used. UEA-1 was obtained from Vector Laboratories.

Ly6C$^+$ monocytes in BM were identified as lineage (Lin)$^-$ (CD3$^-$CD19$^-$CD49b$^-$Ly6G$^-$)CD115$^+$CD135$^-$CD117$^-$Ly6C$^+$ cells. BM monocytes were identified as Lin$^-$CD115$^+$ cells. Common monocyte progenitors (cMOPs) were identified as Lin$^-$CD115$^+$CD135$^-$CD117$^+$Ly6C$^+$ cells. In the spleen, dendritic cells (DCs), macrophages, and monocytes were identified as MHC class II$^+$CD11c^{high}CD11b$^-$, F4/80$^+$CD11b$^+$, and Lin$^-$CD11b$^+$CD115$^+$, respectively. In the peripheral blood, DCs, monocytes, granulocytes, NKT cells, B cells, and T cells were identified as CD11c^{high}, Lin$^-$CD115$^+$CD117$^-$, Ly6G$^+$, CD3$^+$NK1.1$^+$, CD19$^+$, and CD3$^+$ cells, respectively. Migratory monocytes/macrophages and resident macrophages in the peritoneal cavity were identified as CD19$^-$CD11b$^+$Ly6G$^-$CD64$^-$CD49f$^-$ cells and CD19$^-$CD11b$^+$Ly6G$^-$CD64$^+$CD49f$^+$ cells, respectively.

T cell proliferation assay

Splenocytes from wild-type mice (WT) and mice deficient for Ly6C1/Ly6C2 (Ly6C1/6C2-deficient mice) were labeled with CFSE and stimulated with Dynabeads mouse T-activator CD3/CD28 (Thermo Fisher Scientific). CFSE dilution in CD4$^+$ T cells and CD8$^+$ T cells was assessed 72 h later.

Induction of colitis

For colitis induction, WT and Ly6C1/6C2-deficient mice were given 3.5% dextran sulfate sodium salt (DSS) (MP Biomedicals) dissolved in drinking water for 6 d, followed by the provision of ordinary water. Mice were weighed daily from day 0 to day 13.

Statistical analysis

All results are expressed as mean ± SEM or mean ± SD. The Student two-tailed unpaired *t* test was used for statistical analysis. Differences were considered significant at $p < 0.05$.

RESULTS

Dispensable role of Ly6C proteins in immune homeostasis

Because *Ly6c1* and *Ly6c2* share an extremely high level of homology, mice deficient for both *Ly6c1* and *Ly6c2* were generated using two gRNAs for common target sequences located in exon 3 of *Ly6c1* and *Ly6c2*: the gRNAs were designed for destroying all of the isoforms of *Ly6c1* (National Center for Biotechnology Information gene ID: 17067) and *Ly6c2* (National Center for Biotechnology Information gene ID: 100041546). We generated three independent lines (lines 009, 102, and 109), each of which successfully transmitted the mutations in the germline (J.M. and Mitsuru Matsumoto, unpublished observation). All lines lacked Ly6C expression (detected by Ly6C-specific mAb, which is reactive with both Ly6C1 and Ly6C2; clone HK1.4) from granulocytes as well as lymphocytes in peripheral blood, whereas Ly6G expression (detected by Ly6G-specific mAb; clone 1A8) from granulocytes was retained (Supplemental Fig. 1). Two other mAbs specific for Ly6C, clones ER-MP 20 and AL-21, showed the same results (J.M. and Mitsuru Matsumoto, unpublished observation), indicating the successful disruption of both *Ly6c1* and *Ly6c2*.

We first examined the composition of various immune cells in Ly6C1/6C2-deficient mice. Abrogation of Ly6C1/Ly6C2 did not affect the compositions of peripheral blood, including monocytes (Fig. 1A). The production of prototypic Ly6C-expressing cells, such as CD11c^{high} DCs and F4/80$^+$CD11b^{int} macrophages in the spleen (Supplemental Fig. 2A), CD115$^+$CD11b$^+$ monocytes in the spleen (Supplemental Fig. 2B), and percentages of Lin$^-$CD115$^+$ or Lin$^-$CD115$^+$CD117$^-$ monocytes in the BM (Supplemental Fig. 2C) were also unaffected by the loss of Ly6C1/Ly6C2. Cells in the peritoneal lavage including migratory monocytes/macrophages (CD19$^-$CD11b$^+$Ly6G$^-$CD49f$^-$CD64$^-$) and resident macrophages (CD19$^-$CD11b$^+$Ly6G$^-$CD49f$^+$CD64$^+$) (25) were also unaffected (Supplemental Fig. 3A).

Beyond the monocytes and macrophages, other immune cells also express Ly6C. We have previously reported that Gr-1 mAb (clone RB6-8C5), which recognizes Ly6G as an authentic Ag but cross-reacts with Ly6C, targets a unique subset of mTECs. mTECs are classified into immature mTECs (CD80^{low} mTECs) and mature mTECs (CD80^{high} mTECs) based on the expression levels of CD80 (23, 24). By using the mAbs specific for Ly6C (clone HK1.4) and Ly6G (clone 1A8), we have demonstrated that Ly6C$^+$/Ly6G$^-$, Ly6C$^-$/Ly6G$^+$, and Ly6C$^+$/Ly6G$^+$ subsets of mature CD80^{high} mTECs exist with various proportions depending on the genetic background of mice (i.e., 5–15% in total) (21). These Ly6C- and/or Ly6G-expressing mTECs localized preferentially at the cortico-medullary junction and expressed high levels of tissue-restricted self-antigens and thymocyte-attracting chemokines. Remarkably, these Gr-1–reactive mTECs were absent in mice deficient for Aire, a gene responsible for the hereditary type of autoimmune disease (OMIM 240300) (26). The results suggested that this novel Gr-1–reactive mTEC subset is strongly associated with Aire-mediated tolerogenic function (21). However, the role of the Ly6C molecules in the tolerogenic function of Gr-1–reactive mTECs remained unknown. The abrogation of Ly6C1/Ly6C2 did not affect the composition of mTECs, including mature CD80^{high} mTECs,

Cells were gated for CD3$^+$ cells. Bands indicate mean ± SD. The significance was determined using an unpaired two-tailed Student *t* test. n.s., not significant. (**B**) Proliferation of splenic CD4$^+$ and CD8$^+$ T cells upon stimulation with anti-CD3/anti-CD28 mAbs (lower). Unstimulated CD4$^+$ and CD8$^+$ T cells served as controls (upper). CFSE-labeled T cells were analyzed 72 h after stimulation. The numbers of cell divisions are included in the lower panels. One representative result from a total of three repeats is shown. (**C**) Body weight (left) and survival (right) of DSS-treated mice were monitored. Ly6C1/6C2-deficient mice ($n = 16$) and littermate controls ($n = 12$) were examined.

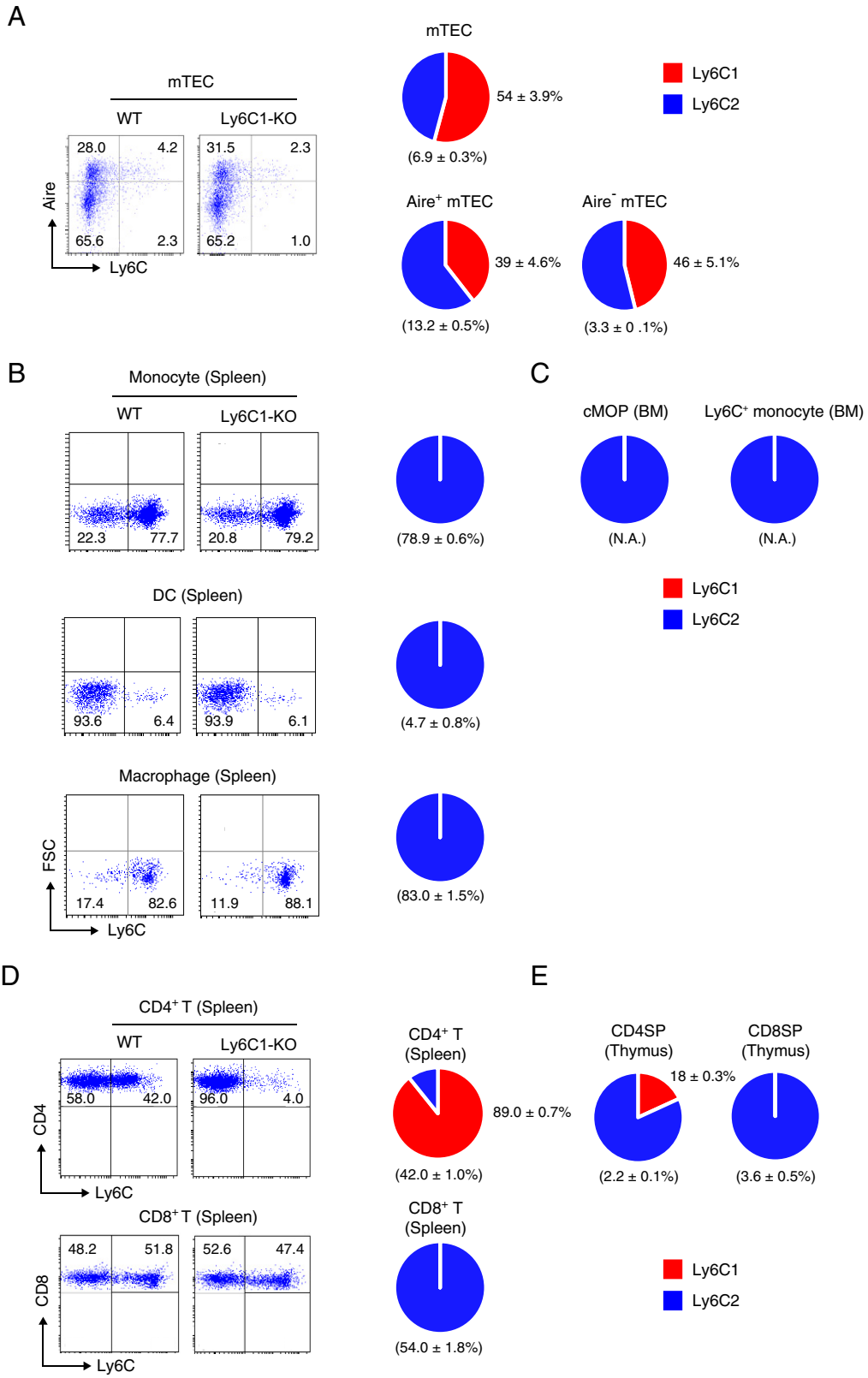

FIGURE 3. Differential expression of Ly6C1 and Ly6C2 from Ly6C$^+$ immune cells

(**A**) Ly6C$^+$ mTECs were labeled with an anti-Ly6C mAb (clone HK1.4) that reacts with both Ly6C1 and Ly6C2. Aire$^+$ mTECs were simultaneously detected using an anti-Aire mAb (left). The relative expression of Ly6C1 and Ly6C2 among total Ly6C$^+$ mTECs (right upper), as well as Aire$^+$ and Aire$^-$ mTECs (right lower), was calculated by the detection of Ly6C$^+$ cells from Ly6C1-deficient mice. The percentages of total ***(Continued)***

https://doi.org/10.4049/immunohorizons.2100114

in any of the Ly6C1/6C2-deficient mouse lines (Fig. 1B). Aire is expressed from a portion of mature CD80^{high} mTECs (23, 24), and the percentages of Aire$^+$ mTECs were not also altered by the lack of Ly6C1/Ly6C2 (Fig. 1B). Similarly, the fractions of developing thymocytes (CD4/CD8 double-positive, CD4 single-positive [CD4SP], and CD8SP T cells) were not altered by the abrogation of Ly6C1/Ly6C2 (Fig. 1C, upper panels). Production of CD4$^+$Foxp3$^+$ regulatory T cells also was not changed (Fig. 1C, lower panels). Consistent with these findings, Ly6C1/6C2-deficient mice did not exhibit any obvious inflammatory lesions in major organs and production of autoantibodies (J.M., Minoru Matsumoto, T.O, K.T., and Mitsuru Matsumoto, unpublished observation). Thus, although Ly6C- and/or Ly6G-expressing (Gr-1–reactive) mTECs are considered a tolerogenic population, the Ly6C molecules do not appear to play a direct role in establishing central tolerance. Rather, Ly6C/Ly6G expression, as a kind of marker, may denote an mTEC population that is important for preventing autoimmunity (21).

In the periphery, Ly6C has been suggested to regulate endothelial adhesion and homing of CD8$^+$ T cells (15). However, Ly6C1/6C2-deficient mice showed no alterations in the ratios and the absolute numbers of CD4$^+$ and CD8$^+$ T cells from both spleen and inguinal lymph nodes (Fig. 2A, Supplemental Fig. 3B, upper panels). Ly6C has been also suggested to support the preferential homing of central memory CD8$^+$ T cells into lymph nodes (16). In contrast to the results from these Ab-based studies, Ly6C1/6C2-deficient mice showed no alterations of the composition of CD44$^+$CD62L^{high} central memory T cells as well as the CD44$^+$CD62L^{low} effector memory T cells for both CD4$^+$ and CD8$^+$ T cells in the spleen (Fig. 2A), inguinal lymph nodes (Supplemental Fig. 3B), and mesenteric lymph nodes (J.M. and Mitsuru Matsumoto, unpublished observation). Furthermore, although a previous study suggested an inhibitory role of Ly6C in the proliferation of anti-CD3–stimulated CD8$^+$ T cells and IL-2 production from CD4$^+$ T cells (17), both CD4$^+$ and CD8$^+$ T cells from Ly6C1/6C2-deficient mice showed unaltered proliferation upon anti-CD3/CD28 stimulation (Fig. 2B).

In addition to the lymphocyte function, Ly6C expression from intestinal epithelial cells has been implicated in the immunopathogenesis of inflammatory bowel disease (20). However, the loss of Ly6C1/Ly6C2 showed no major impact on the DSS-induced inflammatory colitis in terms of body weight loss (Fig. 2C, left) and survival rate (Fig. 2C, right). Thus, despite the importance as a functional marker for the unique subset of immune cells, the Ly6C molecules, so far studied, have no major impact on determining the functional subsets and maintenance of their authentic function. Further studies are required, however, to draw the whole picture of the role of Ly6C molecules in immune homeostasis because our current studies are still the beginning of this task.

Differential expression of Ly6C1 and Ly6C2 from immune cells

Ly6c1 and *Ly6c2* share high sequence homology, with a nucleotide identity of up to 97% (802/822), and currently available mAbs cannot distinguish the corresponding two proteins. Accordingly, there is no information about which types of Ly6C molecules are expressed from Ly6C$^+$ cells. In addition to Ly6C1/6C2-deficient mice, we generated Ly6C1-specific knockout mice by homologous recombination in ES cells to determine which immune cells express Ly6C1 and/or Ly6C2 (Supplemental Fig. 4). Although we initially intended to deplete Ly6C1-expressing cells upon Cre-mediated DT expression with this strain, DT-mediated cell depletion was not successful for unknown reasons. Instead, a knock-in cassette inserted into the *Ly6c1* locus disrupted Ly6C1 expression in homozygous animals (J.M. and Mitsuru Matsumoto, unpublished observation). A flow cytometric analysis of WT mice using a mAb that recognizes both Ly6C1 and Ly6C2 (clone HK1.4) revealed the presence of 6.9 ± 0.3% of Ly6C$^+$ mTECs on average among total mTECs (Fig. 3A, left). However, the percentages of Ly6C$^+$ mTECs were reduced in Ly6C1-deficient mice: e.g., 6.5% (4.2 + 2.3%) in WT versus 3.3% (2.3 + 1.0%) in Ly6C1 knockout (Ly6C1-KO) (Fig. 3A, left). Given that deletion of Ly6C1/Ly6C2 did not affect the composition of mTECs (Fig. 1B), this reduction was thought to correspond to the percentages of cells expressing Ly6C1. Accordingly, the remaining HK1.4-reactive cells were considered to express Ly6C2. Following this idea, we estimated that 54 ± 3.9% of Ly6C$^+$ mTECs on average had been expressing Ly6C1 and the rest of Ly6C$^+$ mTECs expressed Ly6C2 (Fig. 3A, right upper).

Because Ly6C is mainly expressed from mature CD80^{high} mTECs, and Ly6C$^+$ mTECs are absent in Aire-deficient mice (21), we further evaluated the differential expression of Ly6C1 and Ly6C2 after dividing the mature CD80^{high} mTECs into Aire$^+$ and Aire$^-$ mTECs (23, 24). The percentages of Ly6C$^+$ mTECs from WT were higher in Aire$^+$ mTECs (13.2 ± 0.5%) than Aire$^-$ mTECs (3.3 ± 0.1%) (Fig. 3A, right lower). Ly6C1-deficient mice showed reduced percentages of HK1.4-reactive cells: for example, 4.2% (WT) versus 2.3% (Ly6C1-KO) for Aire$^+$ mTECs; 2.3% (WT) versus 1.0% (Ly6C1-KO) for

Ly6C$^+$ cells are shown in parentheses. Percentages of Ly6C1-expressing cells were indicated on the right of each pie chart. Numbers in the parentheses below the pie chart indicate the percentages of total Ly6C-expressing cells among the corresponding cells analyzed in WT. Representative profiles of flow cytometry are shown on the left. Cells were gated for CD45$^-$EpCAM$^+$UEA-1$^+$ cells. (**B** and **C**) Monocytes, DCs, and macrophages in the spleen (B) and cMOPs and monocytes in the BM (C) were analyzed as described in (A). All of the Ly6C-expressing cells expressed Ly6C2 but not Ly6C1. Numbers in the parentheses below the pie chart indicate the percentages of total Ly6C-expressing cells among the corresponding cells analyzed. N.A., not assessed. (**D** and **E**) CD4$^+$ and CD8$^+$ T cells in the spleen (D) and CD4SP and CD8SP T cells in the thymus (E) were analyzed as described in (A). WT (n = 4) and Ly6C1-KO (n = 4) mice were analyzed in (A)–(E).

Aire$^-$ mTECs (Fig. 3A, left). From this reduction in HK1.4-reactive cells, we estimated that Aire$^+$ mTECs and Aire$^-$ mTECs expressed Ly6C1 at 39 ± 4.6% and 46 ± 5.1% among the Ly6C$^+$ cells, respectively, and the rest of Ly6C$^+$ mTECs expressed Ly6C2 (Fig. 3A, right lower). Although we have not taken into account the cells expressing both Ly6C1 and Ly6C2 in this calculation, it was clear that mTECs expressed Ly6C1 at significant percentages.

We further examined the differential expression of Ly6C1 and Ly6C2 from other immune cells. In contrast to mTECs, other Ly6C$^+$ cell populations such as monocytes, DCs, and macrophages in the spleen (Fig. 3B), as well as cMOPs and monocytes in the BM (Fig. 3C), were found to mostly comprise Ly6C2-expressing cells because the percentages of Ly6C$^+$ cells were not changed by the deletion of *Ly6c1* in these cell types. The exceptions for the discrete Ly6C1 expression among Ly6C1$^+$ cells were found for CD4$^+$ T cells in the spleen (89.0 ± 0.7%) (Fig. 3D) and CD4SP T cells in the thymus (18.0 ± 0.3%) (Fig. 3E). In these cases, the percentages of Ly6C-expressing cells in the spleen were 42.0 ± 1.0% and 54.0 ± 1.8% for CD4$^+$ and CD8$^+$ T cells, respectively (Fig. 3D). Ly6C-expressing CD4SP and CD8SP thymocytes were 2.2 ± 0.1% and 3.6 ± 0.5%, respectively (Fig. 3E). Thus, mTECs and CD4$^+$ T cells expressed Ly6C1 at significant percentages. In contrast, other prototypic Ly6C-expressing cell types such as monocytes and macrophages primarily expressed Ly6C2, although we cannot exclude the possibility that the cells expressing both Ly6C1 and Ly6C2 might have been missed by the current approach.

DISCUSSION

Although expression of Ly6C molecules defines unique functional subsets of monocyte and mTECs, mice deficient for Ly6C1/Ly6C2 showed no major alterations in the subsets and function of these cell types. For example, mice deficient for Ly6C1/Ly6C2 showed no alteration of the monocyte/macrophage subsets in the peripheral blood, spleen, BM, and peritoneum, as well as the cells involved in the colitis model. Similarly, although Ly6C$^+$ mTECs showed higher expression of molecules relevant to the thymic tolerance induction such as MHC class II, costimulatory receptors, adhesion molecules, and chemokines (21), mice deficient for Ly6C1/Ly6C2 showed no signs of autoimmunity. Lack of Ly6C1/Ly6C2 also showed no major impact on the development and function of other immune cells such as thymocytes and peripheral T cells. The results so far favor a model that Ly6C molecules are the markers rather than the functional molecules for the subsets. The immediate question of why the expression of Ly6C defines a unique subset for the immune cells remains to be solved by future studies.

Interestingly, note that Ly6C$^+$ monocytes and Ly6C$^+$ mTECs have some common features. Lineage tracing experiments suggested that Ly6C$^-$ monocytes do not represent a distinct lineage, but instead arise from the conversion of Ly6C$^+$ cells (27). Similarly, Ly6C$^+$ and Ly6C$^-$ mTECs are not distinct lineages, but instead represent an interchangeable cellular state (21): we observed bidirectional conversion between Ly6C$^+$ and Ly6C$^-$ mTECs within a thymic microenvironment mimicked by reaggregated thymic organ culture. Currently, the factors controlling the conversion between the Ly6C$^+$ and Ly6C$^-$ populations remain unknown in both monocytes and mTECs. However, we assume that those factors are unique to each cell type because our RNA sequencing analysis indicated that *Nr4a1*, which encodes a transcription factor essential for the development of Ly6C^{low} monocytes (10, 12, 13), was rather upregulated in Ly6C$^+$ mTECs compared with Ly6C$^-$ mTECs (21). This finding clearly indicates that different intrinsic factors regulate the expression of Ly6C and/or the production of Ly6C-expressing populations in monocytes and mTECs.

In contrast to the monocytes (4–6) and mTECs (21), the exact functional difference between Ly6C$^+$ and Ly6C$^-$ cells in other cell types remains largely unknown. The reasons for the differential expression of Ly6C1 and Ly6C2 depending on the cell types also need to be addressed. Although we have observed no major phenotypes in Ly6C null mice so far, further studies are required to fully reveal the significance of Ly6C expression from particular cell subsets in inflammation, autoimmunity, and tumor immunity.

DISCLOSURES

The authors have no financial conflicts of interest.

REFERENCES

1. Ginhoux, F., and S. Jung. 2014. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat. Rev. Immunol.* 14: 392–404.
2. Perdiguero, E. G., and F. Geissmann. 2016. The development and maintenance of resident macrophages. *Nat. Immunol.* 17: 2–8.
3. Gordon, S., and P. R. Taylor. 2005. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* 5: 953–964.
4. Sunderkötter, C., T. Nikolic, M. J. Dillon, N. Van Rooijen, M. Stehling, D. A. Drevets, and P. J. Leenen. 2004. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J. Immunol.* 172: 4410–4417.
5. Auffray, C., M. H. Sieweke, and F. Geissmann. 2009. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu. Rev. Immunol.* 27: 669–692.
6. Geissmann, F., M. G. Manz, S. Jung, M. H. Sieweke, M. Merad, and K. Ley. 2010. Development of monocytes, macrophages, and dendritic cells. *Science* 327: 656–661.
7. LeClair, K. P., M. Rabin, M. N. Nesbitt, D. Pravtcheva, F. H. Ruddle, R. G. Palfree, and A. Bothwell. 1987. Murine Ly-6 multigene family is located on chromosome 15. *Proc. Natl. Acad. Sci. USA* 84: 1638–1642.
8. Spindler, K. R., A. R. Welton, E. S. Lim, S. Duvvuru, I. W. Althaus, J. E. Imperiale, A. I. Daoud, and E. J. Chesler. 2010. The major locus for mouse adenovirus susceptibility maps to genes of the hematopoietic cell surface-expressed LY6 family. *J. Immunol.* 184: 3055–3062.
9. Shi, C., and E. G. Pamer. 2011. Monocyte recruitment during infection and inflammation. *Nat. Rev. Immunol.* 11: 762–774.

10. Carlin, L. M., E. G. Stamatiades, C. Auffray, R. N. Hanna, L. Glover, G. Vizcay-Barrena, C. C. Hedrick, H. T. Cook, S. Diebold, and F. Geissmann. 2013. *Nr4a1*-dependent Ly6C^{low} monocytes monitor endothelial cells and orchestrate their disposal. *Cell* 153: 362–375.
11. Hanna, R. N., C. Cekic, D. Sag, R. Tacke, G. D. Thomas, H. Nowyhed, E. Herrley, N. Rasquinha, S. McArdle, R. Wu, et al. 2015. Patrolling monocytes control tumor metastasis to the lung. *Science* 350: 985–990.
12. Hanna, R. N., L. M. Carlin, H. G. Hubbeling, D. Nackiewicz, A. M. Green, J. A. Punt, F. Geissmann, and C. C. Hedrick. 2011. The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C$^-$ monocytes. *Nat. Immunol.* 12: 778–785.
13. Thomas, G. D., R. N. Hanna, N. T. Vasudevan, A. A. Hamers, C. E. Romanoski, S. McArdle, K. D. Ross, A. Blatchley, D. Yoakum, B. A. Hamilton, et al. 2016. Deleting an *Nr4a1* super-enhancer subdomain ablates Ly6C^{low} monocytes while preserving macrophage gene function. *Immunity* 45: 975–987.
14. Mildner, A., J. Schönheit, A. Giladi, E. David, D. Lara-Astiaso, E. Lorenzo-Vivas, F. Paul, L. Chappell-Maor, J. Priller, A. Leutz, et al. 2017. Genomic characterization of murine monocytes reveals C/EBPβ transcription factor dependence of Ly6C$^-$ cells. *Immunity* 46: 849–862.e7.
15. Jaakkola, I., M. Merinen, S. Jalkanen, and A. Hänninen. 2003. Ly6C induces clustering of LFA-1 (CD11a/CD18) and is involved in subtype-specific adhesion of CD8 T cells. *J. Immunol.* 170: 1283–1290.
16. Hänninen, A., M. Maksimow, C. Alam, D. J. Morgan, and S. Jalkanen. 2011. Ly6C supports preferential homing of central memory CD8$^+$ T cells into lymph nodes. *Eur. J. Immunol.* 41: 634–644.
17. Yamanouchi, S., K. Kuwahara, A. Sakata, T. Ezaki, S. Matsuoka, J. Miyazaki, S. Hirose, T. Tamura, H. Nariuchi, and N. Sakaguchi. 1998. A T cell activation antigen, Ly6C, induced on CD4$^+$ Th1 cells mediates an inhibitory signal for secretion of IL-2 and proliferation in peripheral immune responses. *Eur. J. Immunol.* 28: 696–707.
18. Egan, C. E., W. Sukhumavasi, A. L. Bierly, and E. Y. Denkers. 2008. Understanding the multiple functions of Gr-1$^+$ cell subpopulations during microbial infection. *Immunol. Res.* 40: 35–48.
19. Omi, A., Y. Enomoto, T. Kiniwa, N. Miyata, and A. Miyajima. 2014. Mature resting Ly6C^{high} natural killer cells can be reactivated by IL-15. *Eur. J. Immunol.* 44: 2638–2647.
20. Flanagan, K., Z. Modrusan, J. Cornelius, A. Chavali, I. Kasman, L. Komuves, L. Mo, and L. Diehl. 2008. Intestinal epithelial cell up-regulation of LY6 molecules during colitis results in enhanced chemokine secretion. *J. Immunol.* 180: 3874–3881.
21. Morimoto, J., Y. Nishikawa, T. Kakimoto, K. Furutani, N. Kihara, M. Matsumoto, K. Tsuneyama, Y. Kozono, H. Kozono, K. Hozumi, et al. 2018. Aire controls in *Trans* the production of medullary thymic epithelial cells expressing Ly-6C/Ly-6G. *J. Immunol.* 201: 3244–3257.
22. Lee, P. Y., J. X. Wang, E. Parisini, C. C. Dascher, and P. A. Nigrovic. 2013. Ly6 family proteins in neutrophil biology. *J. Leukoc. Biol.* 94: 585–594.
23. Morimoto, J., M. Matsumoto, R. Miyazawa, H. Yoshida, K. Tsuneyama, and M. Matsumoto. 2022. Aire suppresses CTLA-4 expression from the thymic stroma to control autoimmunity. *Cell Rep.* 38: 110384.
24. Nishijima, H., M. Matsumoto, J. Morimoto, K. Hosomichi, N. Akiyama, T. Akiyama, T. Oya, K. Tsuneyama, H. Yoshida, and M. Matsumoto. 2022. Aire controls heterogeneity of medullary thymic epithelial cells for the expression of self-antigens. *J. Immunol.* 208: 303–320.
25. Galbas, T., M. Raymond, A. Sabourin, M. C. Bourgeois-Daigneault, F. Guimont-Desrochers, T. J. Yun, J. F. Cailhier, S. Ishido, S. Lesage, C. Cheong, and J. Thibodeau. 2017. MARCH1 E3 ubiquitin ligase dampens the innate inflammatory response by modulating monocyte functions in mice. *J. Immunol.* 198: 852–861.
26. Matsumoto, M. 2011. Contrasting models for the roles of Aire in the differentiation program of epithelial cells in the thymic medulla. *Eur. J. Immunol.* 41: 12–17.
27. Yona, S., K. W. Kim, Y. Wolf, A. Mildner, D. Varol, M. Breker, D. Strauss-Ayali, S. Viukov, M. Guilliams, A. Misharin, et al. 2013. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. [Published erratum appears in 2013 *Immunity* 38: 1073–1079.] *Immunity* 38: 79–91.