



DCIR3 and DCIR4 are widely expressed among tissue-resident macrophages with the exception of microglia and alveolar macrophages

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ABSTRACT

Dendritic cell inhibitory receptor 3 (DCIR3, Clec4a3) and dendritic cell inhibitory receptor 4 (DCIR4, Clec4a1) are C-type lectin receptors that belong to mouse dendritic cell immunoreceptor (DCIR) family. We recently showed that DCIR3 and DCIR4 are co-expressed on inflammatory and patrolling monocytes. In this study, we investigated the expression of DCIR3 and DCIR4 on tissue-resident macrophages. We found that spleen red pulp macrophages, liver Kupffer cells, large and small peritoneal macrophages and small intestinal macrophages expressed both DCIR3 and DCIR4. By contrast, lung alveolar macrophages expressed DCIR3 but not DCIR4 and brain microglia expressed neither DCIR3 nor DCIR4. Considerable part of tissue-resident macrophages are derived from embryonic precursors. We, therefore, examined the expression of DCIR3 and DCIR4 on the embryonic precursors. Yolk-sac macrophages from embryonic day (E) 8.5 embryos expressed both DCIR3 and DCIR4, while DCIR3 and DCIR4 were expressed on subpopulations of fetal liver monocytes from E14.5 embryos. Our results, together with previous data, indicate that the expression of DCIR3 and DCIR4 is widely shared by mononuclear phagocytes, including monocytes and macrophages, and that the expression of DCIR3 and DCIR4 on the embryonic precursors are not always retained by their progenies, suggesting that expression of DCIR3 and DCIR4 on tissue-resident macrophages might be regulated by environment of the tissues where the embryonic precursors differentiate into macrophages.

1. Introduction

Dendritic cell inhibitory receptor 3 (DCIR3¹, Clec4a3) and dendritic cell inhibitory receptor 4 (DCIR4, Clec4a1) are C-type lectin receptors that belong to mouse dendritic cell immunoreceptor (DCIR) family [1]. Mouse DCIR family consists of four inhibitory receptors, DCIR1 ~ 4, and two activating receptors, DCAR1 and DCAR2 [1]. We recently found that DCIR3 and DCIR4 are co-expressed on Ly-6C⁺ inflammatory monocytes and Ly-6C⁻ patrolling monocytes [2,3]. Monocytes have a capacity to differentiate into dendritic cells (DCs) and macrophages *in vitro* and *in vivo*. *In vitro* culture of mouse Ly-6C⁺ monocytes in the presence of GM-CSF and IL-4 induces differentiation into DCs and macrophages, while that in the presence of M-CSF induces differentiation into macrophages [4]. When Ly-6C⁺ DCIR4⁺ monocytes differentiate into DCs, DCIR4 expression is diminished, whereas when they differentiate into macrophages, DCIR4 expression is retained [2]. These observations raise the possibility that DCIR3 and DCIR4 might be co-expressed on

macrophages *in vivo*.

The majority of tissues in the body contain tissue-resident macrophages, which exert tissue-specific functions [5]. Most tissue-resident macrophages develop from embryonic precursors in the fetal stage and considerable part of them are maintained by self-renewal in adults [6–8]. Recently, Liu et al. showed that lung alveolar macrophages (AMs), spleen red pulp macrophages, large peritoneal macrophages and small intestinal macrophages are replaced by the macrophages derived from adult bone marrow monocytes to some extent after birth, resulting in a mixture of embryo- and adult-derived macrophages in adult mice, while brain microglia and liver Kupffer cells maintain their population by self-renewal after differentiation from embryonic precursors and are hardly replaced by the macrophages derived from adult bone marrow monocytes [9].

In this study, we investigated the expression of DCIR3 and DCIR4 on various tissue-resident macrophages and their embryonic precursors. We demonstrate that DCIR3 and DCIR4 are co-expressed by majority of

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¹ Abbreviations: AM, alveolar macrophage; BM, bone marrow; BMDMs, bone marrow-derived macrophages; DCIR, dendritic cell immunoreceptor; DCIR3, dendritic cell inhibitory receptor 3; DCIR4, dendritic cell inhibitory receptor 4; FL-MOs, fetal liver monocytes; YS-MFs, yolk-sac macrophages.

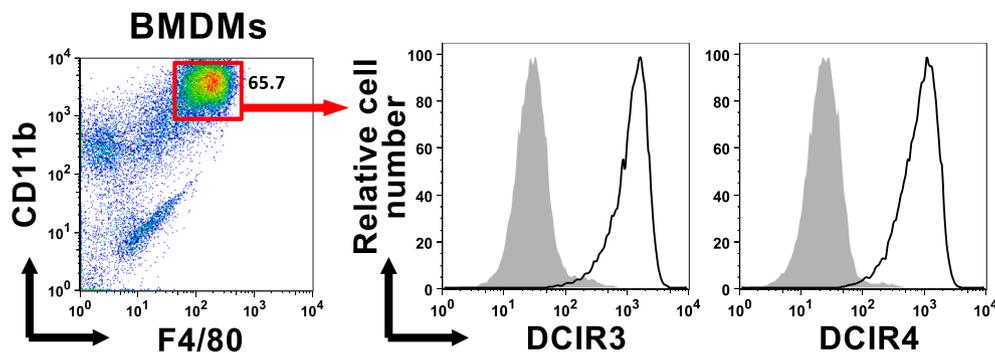


Fig. 1. Expression of DCIR3 and DCIR4 on bone marrow-derived macrophages (BMDM).

BMDMs were stained with the indicated mAbs and analyzed by flow cytometry. Expression of DCIR3 or DCIR4 (solid line histograms) on CD11b⁺F4/80⁺ BMDMs are shown. Shaded histograms are staining with isotype control mAbs. Data shown are one of three independent experiments with similar results.

tissue-resident macrophages except brain microglia and lung AMs. We also show that the expression of DCIR3 and DCIR4 on the embryonic precursors is not always retained by their progenies. Our data suggest that the expression of DCIR3 and DCIR4 on tissue-resident macrophages is independent of DCIR3 and DCIR4 expression on their precursors, but might be influenced by the environments of the tissues where precursors differentiate into macrophages.

2. Materials and methods

2.1. Animals

C57BL/6J mice were obtained from Nippon SLC (Shizuoka, Japan) and housed under specific pathogen-free conditions. B6. SJL-*Ptprc*^a/BoyYuoRbrc mice obtained from Bioresource Research Center of RIKEN (Ibaraki, Japan) were bred and housed under specific pathogen-free conditions. All the animal experiments were conducted in accordance with the University of Tokyo's rules regarding animal experimentation and the University's animal-experimentation manual.

2.2. Cells

Mouse fibroblast cell line L929 was obtained from Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). L929 cells were maintained in RPMI1640 medium (Sigma-Aldrich, St Louis, MO) containing 10% heat-inactivated fetal calf serum (FCS, Sigma-Aldrich), 50 μ M 2-mercaptoethanol (Sigma-Aldrich), 100 U/mL of penicillin G (Sigma-Aldrich) and 100 μ g/mL of streptomycin sulfate (Sigma-Aldrich). The culture supernatants from L929 cells were prepared by culturing 80–90% confluent L929 cells in the medium described above for 2 or 3 days. The collected culture supernatants were filtered through a 0.22 μ m filter (Merck Millipore Ltd., Co. Cork, IRL) and used for preparation of bone marrow-derived macrophages (BMDMs).

2.3. Preparation of BMDMs

BMDMs were prepared as described by Zhang et al. [10]. In brief, bone marrow (BM) cells from C57BL/6J mice were cultured in the RPMI1640-based medium as described above in the presence of 20% culture supernatant from L929 fibroblasts. The medium was changed every 2 or 3 days and the adherent cells obtained after 7 days of culture were used for flow cytometry analysis.

2.4. Preparation of single cell suspensions of immune cells from various organs and mouse embryos

8–10 weeks-old female C57BL/6J mice were used. To obtain immune cells from small intestine, small intestines removed of Peyer's patches

were longitudinally opened, cut into 10 mm pieces and incubated in PBS containing 1.3 mM EDTA at 37 $^{\circ}$ C to remove epithelial cells, then digested with collagenase in the collagenase solution (RPMI1640 containing 100 U/mL of type IV collagenase (Sigma-Aldrich), 40 μ g/mL of DNaseI (Sigma-Aldrich), 10% heat-inactivated FCS, 50 μ M 2-mercaptoethanol, 100 U/mL of penicillin G and 100 μ g/mL of streptomycin sulfate) for 1 h at 37 $^{\circ}$ C. Single cell suspension from the small intestines were prepared by passing through cell strainers. The cells were layered on a 30/50% Percoll (GE Healthcare Japan Corporation, Tokyo, Japan) density-gradient and centrifuged for 20 min at 600 \times g. The cells enriched in the 30/50% interface were collected.

Lungs, brains and livers from mice were minced and digested with collagenase as described above. Single cell suspensions from lungs, brains and livers were prepared by passing through cell strainers, followed by treatment with red blood cell lysis solution. The red blood cells-depleted lung cells were used for flow cytometry analysis.

The brain cells were layered on a 30/37/70% Percoll density-gradient and centrifuged for 40 min at 300 \times g. The cells enriched in the 37/70% interface, containing microglia, were collected and analyzed.

The red blood cells-depleted liver cells were suspended in 33% Percoll and centrifuged for 30 min at 800 \times g. The precipitated cells containing Kupffer cells were collected and analyzed.

Peritoneal exudate cells were collected by injecting PBS into the peritoneal cavity of mice and removing the fluid from the peritoneal cavity.

To obtain mouse embryos, C57BL/6J female mice (CD45.2) were crossed with B6. SJL-*Ptprc*^a/BoyYuoRbrc male mice (CD45.1). Yolk-sac was obtained from fetuses at 8.5 days after fertilization (E8.5) and fetal livers were obtained from fetuses at 14.5 days after fertilization (E14.5). Single cell suspensions from yolk-sac and fetal livers were prepared by digesting with collagenase as described above and passing through cell strainers followed by treatment with red blood cell lysis solution.

All the cells were suspended in PBS containing 1 mM EDTA, 0.1% BSA (Wako, Osaka, Japan) and 0.1% NaN₃.

2.5. Antibodies and flow cytometry

FITC-conjugated mAbs against mouse F4/80 (BM8), CD11c (N418) and Ly6C (HK1.4), and PE-conjugated mAbs against mouse F4/80 (BM8), CD11b (M1/70) and CD45.1 (A20), and APC-conjugated mAbs against mouse F4/80 (BM8) and CD64 (X54-5/7.1) were obtained from BioLegend (San Diego, CA). PE-conjugated mAb against Siglec-F (ES22-10D8) and MerTK (REA477) were from Milteny-Biotec (Bergisch Gladbach, Germany). mAbs against mouse Fc γ receptors (2.4G2) and CD11b (M1/70) were purified from supernatants of hybridomas obtained from ATCC (Manassas, VA) as described [2]. The purified anti-CD11b mAbs were then labeled with FITC (Sigma-Aldrich) or DyLight 650 NHS-ester

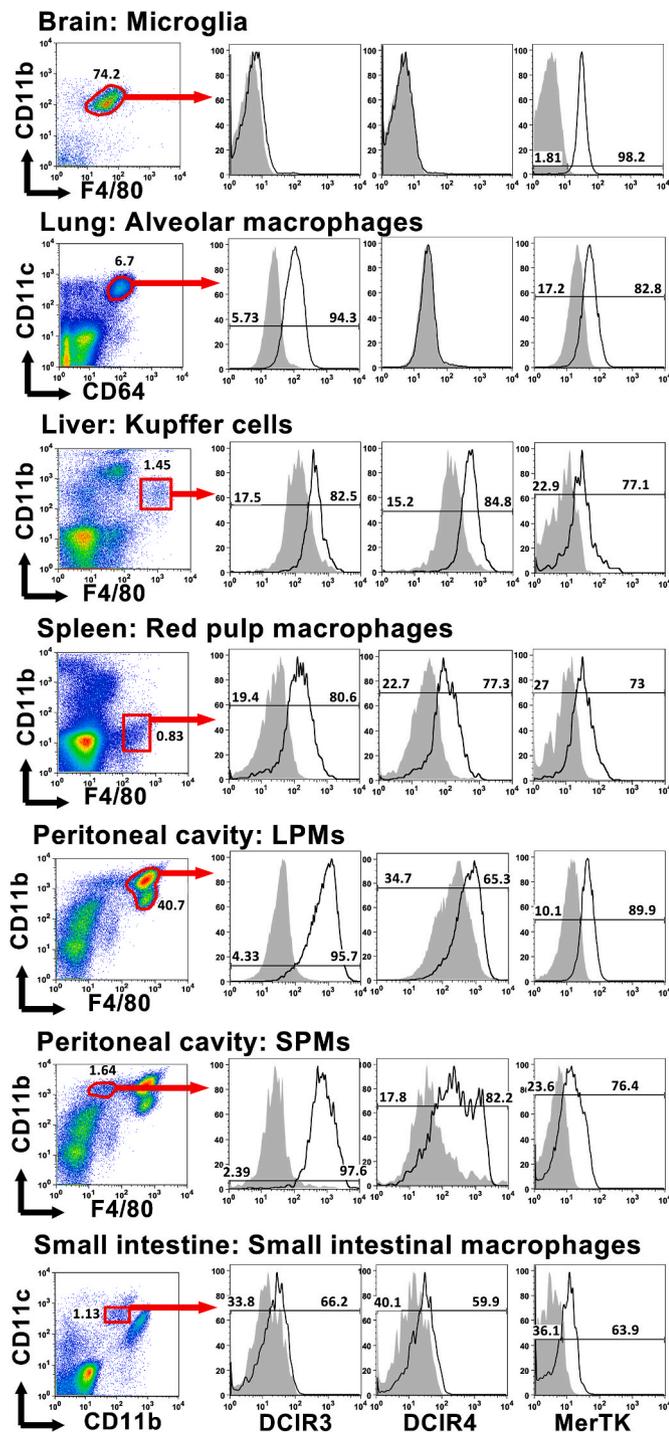


Fig. 2. Expression of DCIR3 and DCIR4 on various populations of tissue-resident macrophages. Immune cells prepared from various organs were stained with the indicated mAbs and analyzed by flow cytometry. Expression of DCIR3, DCIR4 or MerTK (solid line histograms) on each population of the tissue-resident macrophages are shown. Shaded histograms are staining with isotype control mAbs. Data shown are one of three independent experiments with similar results.

(Thermo-Fischer Scientific K-K., Tokyo, Japan). Biotinylated anti-DCIR3 (7E7F2) and anti-DCIR4 mAbs (MH7E7) were described previously [2, 3].

For staining of mouse immune cells, PBS containing 1 mM EDTA, 0.1% BSA and 0.1% NaN_3 was used. The single cell suspensions prepared from various organs were incubated with anti-mouse $\text{Fc}\gamma$ receptors mAb

(2.4G2) before the addition of mAbs. The binding of biotinylated anti-DCIR3 (7E7F2) and anti-DCIR4 (MH7E7) mAbs was detected with APC-conjugated streptavidin (BioLegend) or PE-conjugated streptavidin (BioLegend). Propidium iodide (Sigma-Aldrich) was added at the final concentration of 1 $\mu\text{g}/\text{mL}$ immediately before flow cytometry acquisition to exclude dead cells from analysis. The data were acquired with a FACSCalibur system (BD Biosciences, San Jose, CA) and were analyzed with FlowJo software (TreeStar, San Carlos, CA). Background staining was estimated using fluorochrome-conjugated or biotinylated isotype control mAbs: rat IgG2a (anti-human AICL, 3G72) or rat IgG1 control mAbs (anti-human KLRF1, H206), both of which were prepared in our laboratory [11], rat IgG1 (RTK2071), mouse IgG2a (eBM2a) or rat IgG2c (RTK4174) control mAbs obtained from BioLegend or REA control (REA293) obtained from Milteny-Biotec.

2.6. Microarray data analysis

To examine mRNA expression of DCIR3 and DCIR4 in embryonic precursors, BM monocytes and alveolar macrophages, a microarray data set normalized by quantile normalization (accession number: GSE66970) and a log2 converted microarray data set normalized by quantile normalization (accession number: GSE76999) were downloaded from the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/gds>). The data sets of GSE66970 were converted to log10 values. Heatmaps of the indicated mRNA expression were prepared by Multi Experiment Viewer (MeV) version 4.8.1 (TM4 Software Suite).

3. Results

3.1. Expression of DCIR3 and DCIR4 on bone marrow-derived macrophages (BMDMs)

We recently showed that macrophages induced from bone marrow (BM) Ly-6C^+ monocytes express DCIR4 [2]. To further extend this observation, we examined the expression of DCIR3 and DCIR4 on BMDMs. The BMDMs expressed both DCIR3 and DCIR4 (Fig. 1), suggesting the possibility that DCIR3 and DCIR4 might be expressed widely among macrophage populations, including tissue-resident macrophages.

3.2. Expression of DCIR3 and DCIR4 on various tissue-resident macrophages

To investigate the expression of DCIR3 and DCIR4 on tissue-resident macrophages, we prepared immune cells from various organs, including brain, lung, liver, spleen, peritoneal cavity and small intestine. According to the literatures, brain microglia are defined as $\text{CD11b}^+\text{F4/80}^{\text{Lo}}$ cells [12], lung alveolar macrophages (AMs) are defined as $\text{CD11c}^{\text{Hi}}\text{CD64}^+$ cells [13,14] and spleen red pulp macrophages, liver Kupffer cells are defined as $\text{F4/80}^+\text{CD11b}^+$ cells [15]. Macrophages in peritoneal cavity are divided into two subsets, large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs), which are defined as $\text{F4/80}^{\text{Hi}}\text{CD11b}^+$ and $\text{F4/80}^{\text{Lo}}\text{CD11b}^+$ cells, respectively [16]. Small intestinal lamina propria macrophages are defined as $\text{F4/80}^{\text{Hi}}\text{CD11b}^{\text{int}}$ cells [17]. We stained the immune cells obtained from the organs with mAbs against the indicated markers and anti-MerTK, anti-DCIR3 or anti-DCIR4 mAbs (Fig. 2). All the tissue-resident macrophages defined as above expressed MerTK, a marker of macrophages [14], indicating that they were *bona fide* macrophages. Red pulp macrophages, Kupffer cells, LPMs and SPMs expressed both DCIR3 and DCIR4. Small intestinal macrophages also expressed both DCIR3 and DCIR4, although their expression was low. By contrast, AMs expressed DCIR3 but not DCIR4 and microglia expressed neither DCIR3 nor DCIR4. These data indicate that DCIR3 and DCIR4 are widely expressed by tissue-resident macrophages, except brain microglia and lung AMs, in both of which expression of either one or both of DCIR3 and DCIR4 were absent.

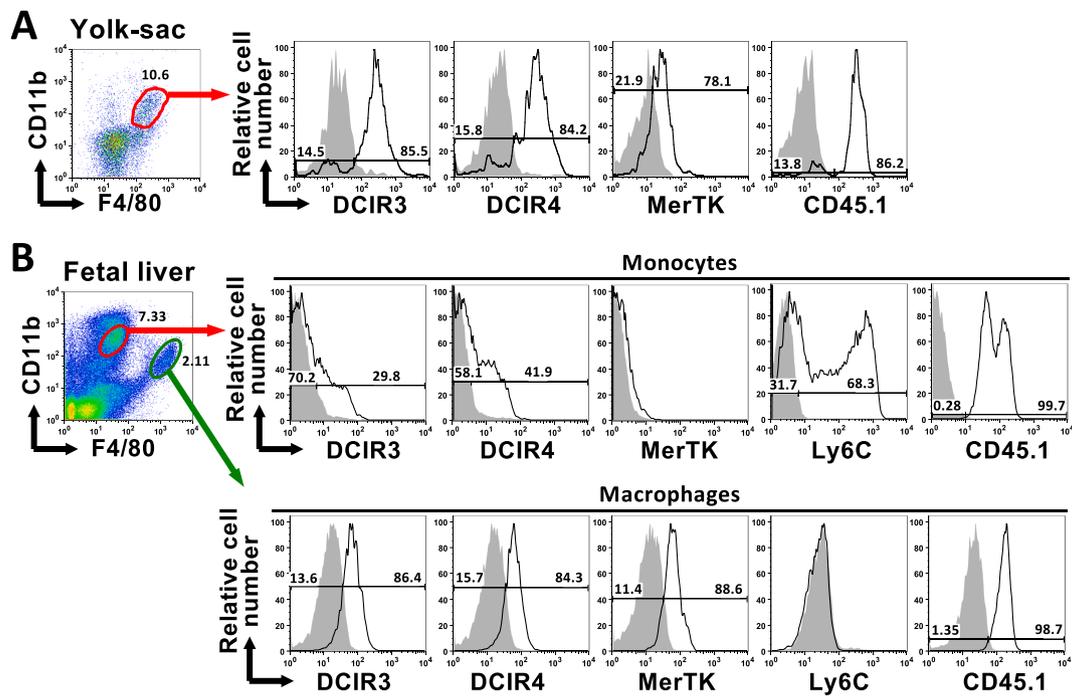


Fig. 3. Expression of DCIR3 and DCIR4 on embryonic precursors of tissue-resident macrophages. C57BL6/J female mice (CD45.2) were crossed with B6. SJL-*Ptprca*^d/BoyYuoRbrc male mice (CD45.1) to obtain fetuses. Single cell suspensions prepared from yolk-sac from E8.5 embryos (A) or fetal livers from E14.5 embryos (B) were stained with the indicated mAbs and analyzed by flow cytometry. Expressions of DCIR3, DCIR4, MerTK or CD45.1 on CD11b⁺F4/80⁺ YS-MFs, CD11b^{Hi}F4/80^{Lo} FL-MOs and CD11b^{Lo}F4/80^{Hi} fetal liver macrophages are shown. Expressions of Ly-6C on FL-MOs and fetal liver macrophages are also shown. Shaded histograms are staining with isotype control mAbs. Data shown are one of three independent experiments with similar results.

3.3. Expression of DCIR3 and DCIR4 on embryonic precursors of tissue-resident macrophages

Since microglia lacked the expression of DCIR3 and DCIR4 and AMs lacked that of DCIR4 (Fig. 2), we investigated the expression of DCIR3 and DCIR4 on their precursors.

In mice, microglia are derived from E7.5 ~ E9.5 yolk-sac macrophages (YS-MFs) [8,18,19]. To investigate the expression of DCIR3 and DCIR4 on YS-MFs, yolk-sac cells from E8.5 embryos were analyzed by flow cytometry (Fig. 3A). Despite the result that microglia expressed neither DCIR3 nor DCIR4 (Fig. 2), their precursor, CD11b⁺F4/80⁺ YS-MFs, expressed both DCIR3 and DCIR4.

The fetal liver begins to serve as the major hematopoietic organ around E11.5 and generates all hematopoietic cells [8,20]. CD11b^{Hi}F4/80^{Lo} cells generally called fetal liver monocytes (FL-MOs) and CD11b^{Lo}F4/80^{Hi}Ly6C⁺ cells defined as early tissue macrophages appear in the fetal liver after E12.5 [20]. FL-MOs are the major embryonic precursors of most tissue-resident macrophages except microglia [20]. To investigate the expression of DCIR3 and DCIR4 on FL-MOs, fetal liver cells from E14.5 embryos were analyzed by flow cytometry (Fig. 3B). Consistent with the previous report, the heterogeneous expression of Ly6C and the lack of the MerTK expression on CD11b^{Hi}F4/80^{Lo} cells and the MerTK expression on CD11b^{Lo}F4/80^{Hi} cells enabled us to distinguish FL-MOs from fetal liver macrophages. The CD45.1 expression on CD11b^{Hi}F4/80^{Lo} and CD11b^{Lo}F4/80^{Hi} cells indicate the fetal origin of these populations. The CD11b^{Lo}F4/80^{Hi} fetal liver macrophages expressed both DCIR3 and DCIR4, while CD11b^{Hi}F4/80^{Lo} FL-MOs were the mixture of DCIR3⁺ and DCIR3⁻ populations and, similarly, the mixture of DCIR4⁺ and DCIR4⁻ populations.

These results indicate that DCIR3 and DCIR4 are expressed on YS-MFs, which are the precursors of microglia, and on subpopulations of FL-MOs, which are the precursors of most tissue-resident macrophages.

3.4. DCIR3 and DCIR4 mRNA were expressed in FL-MOs and AMs derived from YS-MFs, FL-MOs and adult BM monocytes

Given that DCIR3 and DCIR4 were expressed on subpopulations of FL-MOs, we examined the expression of DCIR3 and DCIR4 at mRNA level in FL-MOs. G. Hoeffel et al. performed the microarray analysis of E14.5 Ly6C⁺ FL-MOs and adult Ly6C⁺ BM monocytes (BM-MOs) [20]. We obtained these data from the Gene Expression Omnibus database and analyzed the mRNA expression of DCIR3 (*Clec4a3*) and DCIR4 (*Clec4a1*). While *Mertk*, a macrophage marker, was minimally expressed in either BM-MOs or FL-MOs, *Clec4a3* and *Clec4a1* were expressed in adult BM-MOs and also in FL-MOs at levels lower than those in BM-MOs (*Clec4a3*: $p < 0.005$, *Clec4a1*: $p < 0.001$, Welch's *t*-test) (Fig. 4A), consistent with the result that DCIR3 and DCIR4 proteins were expressed on subpopulations of FL-MOs (Fig. 3B).

L. van de Laar et al. showed that not only FL-MOs, which are the embryonic precursors for AMs in wild type mice, but also YS-MFs and adult BM-MOs have capacity to differentiate into AMs when they are introduced into the empty AM niche of neonatal *Csf2rb*^{-/-} mice [21]. To get insight into the regulation of the DCIR3/DCIR4 expression during the differentiation of the precursors into AMs, we examined the expression of the *Clec4a3* and *Clec4a1* genes in AMs developed from YS-MFs, FL-MOs and adult BM-MOs by analyzing a microarray data set provided by L. van de Laar et al. (Fig. 4B). Importantly, *Clec4a3* was expressed in the AMs developed from any of the three precursors, while *Clec4a1* was negligibly expressed in the AMs. The expression profile of *Clec4a3* and *Clec4a1* genes in the AMs differentiated from YS-MFs, FL-MOs and adult BM-MOs were quite similar to that of DCIR3 and DCIR4 proteins in AMs from wild type mice (Fig. 2). These results suggest that the expression of DCIR3 and DCIR4 on AMs is independent of their precursors and is regulated by the environments of the niche where the precursors differentiate into AMs.

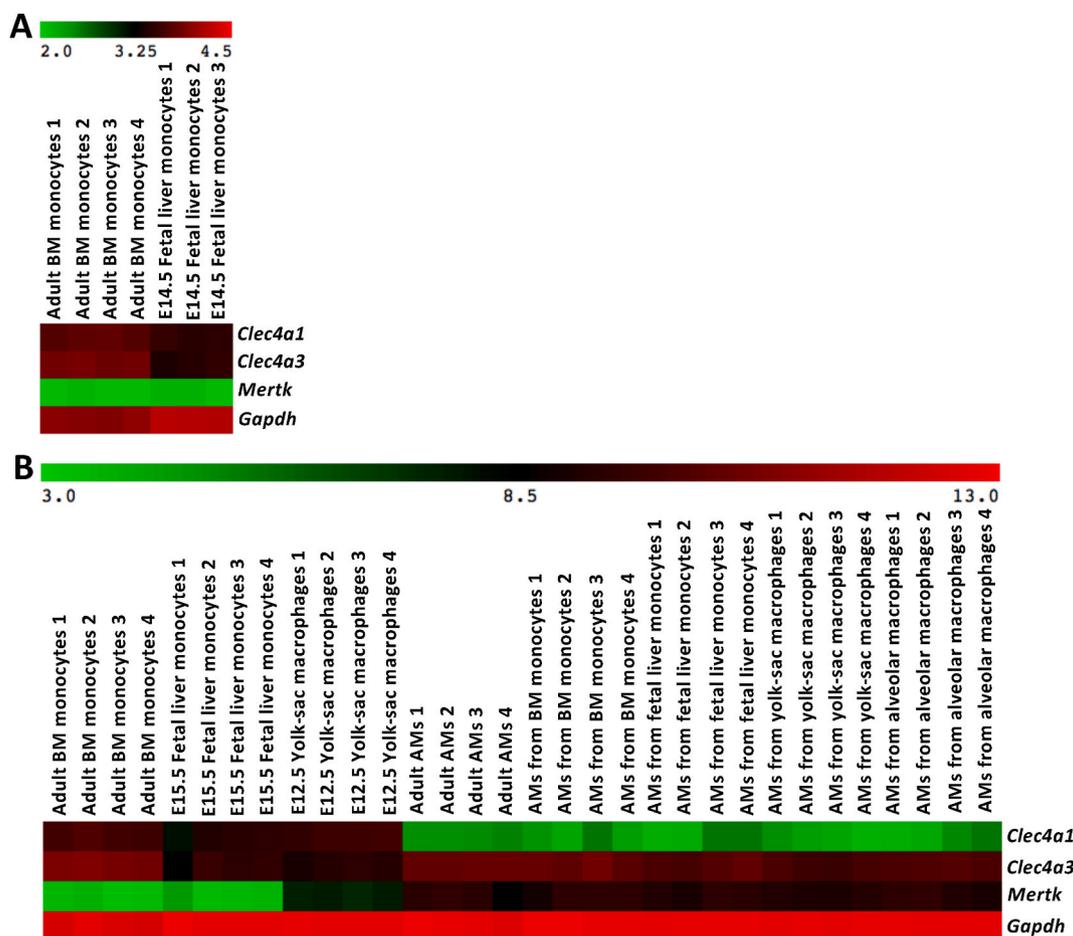


Fig. 4. mRNA expression of DCIR3 and DCIR4 in adult monocytes, FL-MOs and alveolar macrophages (AMs). A set of microarray data (accession number: GSE66970) of E14.5 Ly6C^{Hi} FL-MOs and adult Ly6C^{Hi} BM monocytes (BM-MOs), which were normalized by quantile normalization (A) and a set of log₂ converted microarray data (accession number: GSE76999) of E15.5 Ly6C^{Hi} FL-MOs, E12.5 YS-MFs, adult Ly6C^{Hi} BM-MOs, adult alveolar macrophages (AMs) and AMs differentiated from FL-MOs, YS-MFs, adult BM-MOs or adult lung AMs (B) were obtained from the GEO database and analyzed. The data of GSE66970 were converted to log₁₀ values.

4 Discussion

We recently showed that DCIR3 and DCIR4 are expressed on two major populations of monocytes, inflammatory and patrolling monocytes [2,3]. Our current study extends the observation in monocytes to that in macrophages to clearly demonstrate that DCIR3 and DCIR4 are widely expressed by macrophages including tissue-resident macrophages, except brain microglia and lung AMs (Fig. 2). Thus, our data together with the previous findings in monocytes indicate that DCIR3 and DCIR4 are widely distributed among phagocytic mononuclear cells. How DCIR3 and DCIR4 regulate the function of phagocytic mononuclear cells and what are the ligands recognized by DCIR3 and DCIR4 are important questions remain to be examined in future studies.

Our current study also showed that microglia lack the expression of DCIR3 and DCIR4, while AMs express DCIR3 but not DCIR4 (Fig. 2). The dichotomic expression of DCIR3 and DCIR4 in monocytes raised the possibility of coordinated regulation of DCIR3 and DCIR4 expressions [3], however, the current observation in AMs indicates that the expressions of DCIR3 and DCIR4 are independently regulated.

We also investigated the expression of DCIR3 and DCIR4 on fetal precursors of tissue-resident macrophages (Fig. 3). Microglia are derived from E7.5~E9.5 YS-MFs [8,18,19] and are hardly replaced by microglia derived from blood monocytes [9]. Interestingly, DCIR3 and DCIR4 were expressed on YS-MFs, but not on microglia (Figs. 2 and 3A). These data suggest that the expression of DCIR3 and DCIR4 is lost during differentiation of YS-MFs into microglia.

Z. Liu et al. showed that tissue-resident macrophages including AMs,

spleen red pulp macrophages, LPMs and small intestinal macrophages in adult mice are the mixture of macrophages derived from embryonic precursors and those derived from adult blood monocytes by the fate-mapping technique [9]. Notably, in 8–10 weeks-old mice, 10–20% of AMs were derived from adult blood monocytes [9]. This observation together with our current result that AMs from 8–10 weeks-old mice expressed DCIR3 but not DCIR4 (Fig. 2) suggests that, upon the differentiation of blood monocytes into AMs, the expression of DCIR3 is sustained, while the expression of DCIR4 is lost.

The data AMs derived from YS-MFs, FL-MOs and adult BM-MOs upon reconstitution in neonatal *Csf2rb*^{-/-} mice showed similar patterns regarding the expression of *Clec4a3* and *Clec4a1* genes (Fig. 4B) suggest that the expression of DCIR3 and DCIR4 might be influenced by the environment where precursors differentiate into macrophages irrespective of the types of the precursors.

The two populations of peritoneal macrophages, LPMs and SPMs, both expressed DCIR3 and DCIR4 (Fig. 2). LPMs are derived from embryonic precursors [6] and up to 40% of them are replaced with the macrophages derived from blood monocytes after birth [9], while SPMs are short-lived and the majority of them are originated from blood inflammatory monocytes [22,23].

Regarding the expression of DCIR3 and DCIR4, FL-MOs included positive and negative populations (Fig. 3B). This result precluded us to determine whether the precursors of the FL-MOs-derived tissue-resident macrophages express DCIR3 and DCIR4 or not. Fate mapping experiments by making *DCIR3-cre* or *DCIR4-cre* mice and crossing them with fluorescent reporters such as *Rosa26-LSL-YFP*, *Rosa26-LSL-RFP*, *Rosa26-*

LSL-GFP and *Rosa26-LSL-Tdtomato* lines to generate reporter mice would be useful to answer the question in the future, as used in the fate mapping studies of fetal monocytes/macrophages [24].

In contrast with the adult monocytes, which express both DCIR3 and DCIR4 [2,3], only subpopulations of FL-MOs expressed DCIR3 and DCIR4 (Fig. 3B), suggesting that FL-MOs might have gene expression profile distinct from adult monocytes. Indeed, mRNA expression levels of *Clec4a3* (DCIR3) and *Clec4a1* (DCIR4) were significantly lower in the FL-MOs than in the adult BM monocytes (Fig. 4). Moreover, Hoeffel et al. reported that FL-MOs selectively express genes related to cell cycle and differentiation, while adult monocytes selectively express genes related to immune responses and pathogen recognition [20].

Collectively, our results demonstrate that the majority of tissue-resident macrophages except microglia and AMs express DCIR3 and DCIR4. Our results also indicate that when the embryonic precursors differentiate into tissue-resident macrophages, they sometimes retain the expression of DCIR3 and DCIR4 and sometimes lose the expression of DCIR3 and/or DCIR4, depending on the organs where the embryonic precursors differentiate into tissue-resident macrophages. Our observations suggest that the expression of DCIR3 and DCIR4 on tissue-resident macrophages does not depend on the presence or absence of DCIR3 and DCIR4 expression on their precursors and that the expression of DCIR3 and DCIR4 might be influenced by environmental factors of the tissues where the precursors differentiate into macrophages.

Author contributions

Naoki Matsumoto conceived and designed the study. Ryo Okada acquired the data. Ryo Okada, Naoki Matsumoto and Kazuo Yamamoto analyzed and interpreted the data. Ryo Okada and Naoki Matsumoto drafted and revised the manuscript. Ryo Okada, Naoki Matsumoto and Kazuo Yamamoto approved the submission of the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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