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*J Immunol* 2013; 190:5296-5305; Prepublished online 15 April 2013;
doi: 10.4049/jimmunol.1201452
http://www.jimmunol.org/content/190/10/5296

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/04/15/jimmunol.1201452.DC1

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Intestinal Lin^-c-Kit^+NKp46^-CD4^- Population Strongly Produces IL-22 upon IL-1β Stimulation

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Small intestinal innate lymphoid cells (ILCs) regulate intestinal epithelial cell homeostasis and help to prevent pathogenic bacterial infections by producing IL-22. In a global gene-expression analysis comparing small intestinal ILCs (Lin^-c-Kit^-Sca-1^- cells) with non-ILCs (Lin^-c-Kit^-Sca-1^- cells), we found that Lin^-c-Kit^-Sca-1^- cells highly expressed the mRNAs for IL22, antimicrobial peptides, Csf2rb2 (Il3r), mast cell proteases, and Rorc. We then subdivided the Lin^-c-Kit^-Sca-1^- cells into three groups—Lin^-c-Kit^-NKp46^-CD4^+, Lin^-c-Kit^-NKp46^-CD4^- (CD4^- ILC-like cells), and Lin^-c-Kit^-NKp46^- (NKp46^- ILC22 cells)—and showed that the Lin^-c-Kit^-NKp46^-CD4^- cells produced the highest level of IL-22 protein after IL-1β, IL-23, or IL-1β and IL-23 stimulation. In addition, we showed that the majority of the Lin^-c-Kit^-NKp46^-CD4^- population was IL-7Rα^-CD34^-β^7int, cells, and IL-7Rα^-CD34^-β^7int, cells could be divided into three subsets (CD34^+β^7int, CD34^-β^7int, and CD34^-β^7hi cells). The IL-7Rα^-CD34^-β^7int cells strongly expressed the transcripts for Il17f and Il22 after costimulation with IL-1β and IL-23. The IL-7Rα^-CD34^-β^7int and IL-7Rα^-CD34^-β^7hi cells predominantly expressed the transcripts for mast cell proteases and differentiated almost entirely into mast cells after 1 wk in culture medium supplemented with a cytokine mixture, whereas the IL-7Rα^-CD34^-β^7hi cells highly expressed α-defensins and showed no differentiation. Taken together, these findings indicate that the IL-7Rα^-CD34^-β^7int and IL-7Rα^-CD34^-β^7hi populations are mast cell progenitors, and the IL-7Rα^-CD34^-β^7int (CD4^- ILC-like cells) and IL-7Rα^-CD34^-β^7hi populations within Lin^-c-Kit^-NKp46^-CD4^- cells may control intestinal homeostasis and provide intestinal protection by producing high levels of IL-22 and α-defensins, respectively. The Journal of Immunology, 2013, 190: 5296–5305.

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Received for publication May 30, 2012. Accepted for publication March 12, 2013.

This work was supported by Grant-in-Aid 23590570 (to M.H.J.) and KAKENHI Grant Number 24790474 (to Y.L.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. M.H.J. was supported by the World Class Universities project, National Research Foundation, Ministry of Education, Science and Technology, Korea (R31-10105).

The sequences presented in this article have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE40882.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; DC, dendritic cell; DEFA, α-defensin; EPO, erythropoietin; Fli-3L, Flt-3 ligand; ILC, innate lymphoid cell; LP, lamina propria; m; mouse; MC, mast cell; MCP, mast cell progenitor; MCPT, mast cell protease; Meg/E, megakaryocyte and/or erythroid; MNP, mononuclear phagocyte; SCF, stem cell factor; SDF-1, soluble CD40L; TPO, thrombopoietin.

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1 and -2 and low levels of heparin and histamine (5), and connective tissue-type MCs, which express high levels of MCPT4, -5, -6, -7 and CPA3, as well as high concentrations of heparin and histamine. MCPT1-deficient mice show delayed Trichinella spiralis expulsion, and CPA3-deficient mice have increased susceptibility to snake poison and show defective granules (6–9). MCPT4 regulates the degradation of extracellular matrix protein and intestinal epithelial permeability (10, 11).

The α-defensins (DEFAs) are an antimicrobial peptide family. In humans, DEFA1, DEFA3, and DEFA4 are expressed by neutrophils; in contrast, in mice all 26 DEFA genes (also called cryptdins) are predominantly expressed by Paneth cells, which reside in the mouse intestinal epithelial layer (12, 13). The small intestine is always in contact with commensal bacteria, so the release of antimicrobial peptides is likely to be important to maintain microbial balance. In human patients with ileal Crohn’s disease, DEFAS levels are reduced compared with normal control (14).

IL-22 is a key molecule in mucosal immune regulation, and it has a protective role against inflammatory bowel disease (15, 16). Treatment of primary keratinocytes with IL-22, along with IL-17A or IL-17F, synergistically induces the expression of β-defensin 2 and S100A9 and additively enhances that of S100A7 and S100A8 (17). IL-22 is also important for the proliferation and survival of intestinal epithelial cells (18). NKp46^- innate lymphoid cells (ILCs) of the ILC22 type are known to be the major source of IL-22 in the small intestinal lamina propria (LP) (19). Mice lacking ILC22 cells are highly susceptible to Citrobacter rodentium infection (20). A major inducer of IL-22 is IL-23, which is produced by intestinal macrophages and dendritic cells (DCs) activated by microbial products that cross the epithelial barrier (21).

In this study, we found that, within the Lin^-c-Kit^-NKp46^-CD4^- cells of the small intestinal LP, the IL-7Rα^-CD34^-β^7int population (CD4^- ILC-like cells) produces high levels of IL-17F.
and IL-22 upon stimulation with IL-1β and IL-23. In comparison, the IL-7Rα CD34⁺β7⁺ and IL-7Rα CD34⁺β7⁺ populations, which express high levels of MCPTs, can differentiate into MCs, and the IL-7Rα CD34⁺β7⁺ population strongly expresses DEFAs.

**Materials and Methods**

**Mice**

BALB/c female mice were purchased from SLC (Shizuoka, Japan). The mice were maintained under specific pathogen–free conditions and used in experiments at 8–10 wk of age. Animal experiments were approved by the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University).

**Abs and reagents**

Biotinylated anti-mouse lineage mixture was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-mouse c-Kit (CD117, clone 2B8), anti-mouse Ly-6A/E (Sca-1, clone D7), anti-mouse NKP46 (CD335, clone 29A1.4), anti-mouse CD4 (clone RM-45), anti-mouse IL-22 (CD122, clone 2A14), anti-mouse IL-17F (clone O79-289), anti-mouse IL-7Rα (CD127, clone AK734), anti-mouse CD34 (clone RAM34 or MECD14.7), anti-mouse β7 integrin (clone M293 or FI2B7), anti-mouse Mac-1 (CD11b, clone M1/70), anti-mouse CD11c (clone HL3), anti-mouse Ly-6G/Ly-6C (Gr-1; clone RB6-8C5), anti-mouse MHC class II (I-A/E) (clone M5/114.15.2), anti-mouse FcγRI (clone MAR-1), anti-mouse RORγt (clone B2D), isotype control Abs, and streptavidin were purchased from eBioscience (Franklin Lakes, NJ). Mouse recombinant proteins—IL-1β (R&D Systems, Minneapolis, MN), IL-2, IL-6, IL-7, IL-9, IL-10, IL-11, IL-23, Flt-3 ligand (Flt-3L), GM-CSF, erythropoietin (EPO), thrombopoietin (TPO), and SCF—were purchased from Wako Pure Chemical Industries (Osaka, Japan). The Bio-Plex Mouse Th17 8 Plex kit (Bio-Rad, Hercules, CA) was used for cytokine measurements, according to the manufacturer’s instructions.

**Cell culture and cell proliferation**

MCs were obtained by cultivating Lin⁺ c-Kit⁺NKP46⁺ CD4⁺ cells isolated from the small intestinal LP were generated in complete medium containing recombinant mouse IL-3 (10 ng/ml) and SCF (50 ng/ml) for 0–4 wk. For cultivating cells in complete medium containing a cytokine mixture, we used 10 ng/ml each of IL-1α, IL-3, IL-6, IL-7, IL-9, IL-10, IL-11, IL-23, Flt-3 ligand (Flt-3L), GM-CSF, erythropoietin (EPO), thrombopoietin (TPO), and SCF—were purchased from R&D Systems (Minneapolis, MN). BD GolgiStop was purchased from BD Biosciences.

**Cell sorting and FACS analysis**

Small intestinal LP cells were isolated as described previously, with minor modifications (22). In brief, mouse intestines were removed and placed in cold PBS. After removing the fat and Peyer’s patches, the intestine was cut along its entire length, washed with PBS three times, and cut into 1–2-cm pieces. The pieces of intestine were incubated with 50 ml FACS buffer [10% heat-inactivated FBS (SAFC Biosciences), 10 mM EDTA, 25 mM streptomycin, and 10 μg/ml polymyxin B (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 10 μg/ml gentamicin (Sigma-Aldrich), 50 μg/ml penicillin (Sigma-Aldrich), 2 mM L-glutamine (Life Technologies)] for 1–2 h at 25˚C. The LP cells were collected at the interphase of the Percoll gradient, overlaid on 5 ml 40% Percoll in a 15-ml conical tube. Percoll-gradient fractionation was measured with a spectrophotometer. cDNA was synthesized with an RT-Kit+Sca-1 (Sigma-Aldrich) and an RNeasy kit (QIAGEN, Valencia, CA). The RNA quality was measured with a spectrophotometer. cDNA was synthesized with an RT-PCR SensitiveScript Reverse Transcription kit (QIAGEN), according to the manufacturer’s protocol. Real-time PCR analysis was performed with the Thermal Cycler Dice Real-Time System TP800 instrument using SYBR Premix ExTag (both from TaKaRa Biotechnology, Otsu, Japan). PCR amplification consisted of a denaturation step at 95˚C for 30 s, 40 cycles of denaturation at 95˚C for 5 s, and annealing and extension at 60˚C for 30 s. The samples were analyzed in triplicate, and gene expression was normalized to the expression of Hprt1. All primers were purchased from Sigma Genosys (Tokyo, Japan), and the primer sequences are listed in Supplemental Table I.

**Cytokine assay**

The Bio-Plex Mouse Th17 8 Plex kit (Bio-Rad, Hercules, CA) was used for cytokine measurements, according to the manufacturer’s instructions.

**Statistical analysis**

Statistical analyses were performed using the Student t test. The p values < 0.05 were considered statistically significant. Results are presented as means ± SEM.

**Results**

Lin⁺c-Kit⁺Sca-1⁻ cells from the small intestinal LP strongly express the mRNAs for Il22, Csf2rb2, Defas, and Mcs

Although intestinal ILCs are known to produce IL-22 in response to pathogenic microbial infection, other functions of these cells and the lineal relationship between ILCs and lymphoid or myeloid cells are not well understood (23, 24). To examine the gene-expression profiles of ILCs within the small intestinal LP, we isolated Lin⁻ c-Kit⁻Sca-1⁻ cells (including NKP46⁺ ILC22 (Lin⁻ c-Kit⁺ Sca-1⁻ NKP46⁺ cells) and CD4⁺ LTI-like ILCs (Lin⁻ c-Kit⁺ Sca-1⁻ CD4⁺ cells)) as the ILC population and Lin⁻ c-Kit⁺ Sca-1⁻ cells as the non-ILC population from the small intestinal LP of 8–10-wk-old mice by FACS and then compared the gene-expression profiles between these two populations by microarray analysis. All raw and processed data files were deposited into the National Center for Biotechnology Information Gene Expression Omnibus dataset under accession number GSE40882 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?tag=GSE40882).

Lin⁻ c-Kit⁺Sca-1⁻ cells and Lin⁻ c-Kit⁺ Sca-1⁻ cells make up 2.08 ± 0.26% and 2.22 ± 1.12%, respectively, of the total lymphocytes in the small intestinal LP (Fig. 1A). May-Grünewald-Giemsa staining showed that the cells of both populations were small and round with a dark nucleus and scanty cytoplasm (Fig. 1B).
From the microarray results, we selected 251 genes that were upregulated and 219 genes that were downregulated in the Lin−c-Kit+Sca-1− cells compared with the Lin−c-Kit− Sca-1− cells. These genes were subjected to ontology analysis and annotated for biological processes. The Lin−c-Kit+Sca-1− cells strongly expressed the mRNAs for IL-22, chemokine receptors, antimicrobial peptides, and MCPTs (data not shown). These target genes were confirmed by real-time PCR, and most of the mRNA levels detected by PCR correlated with the microarray results. Many immune regulation–related genes, such as those encoding C4b, Muc2, Tnfsf11 (RANKL), Cxcr5, Cxcr7, Cerr6, and Csfr2b2 (Il3r), showed higher expression levels in the Lin−c-Kit+Sca-1− cells than in the Lin−c-Kit− Sca-1− cells, as did Il22 and Rorc, which were already known to be expressed at high levels in intestinal ILCs (23). In addition, the Lin−c-Kit+Sca-1− cells showed higher expression levels of the mRNAs for antimicrobial peptides [Defa4, -5, and -24; Defa-rs1, -rs2, and -rs7] and proteolysis-related genes (Mcp1, -2, -4, -5, -6, -8, -10, and Cpa3) than did the Lin−c-Kit− Sca-1− cells (Fig. 1C). These observations indicated that the Lin−c-Kit− Sca-1− cells might play important roles in the regulation of small intestine homeostasis and might contain MCPs.

Lin−c-Kit−NKp46−CD4− population in the small intestinal LP produces high levels of Defa, Mcpt2, IL-17F, and IL-22 under steady-state conditions

We divided the Lin−c-Kit− Sca-1− cells into three groups, according to their expression of NKp46 and CD4 (Lin−c-Kit− NKp46−CD4−, Lin−c-Kit−NKp46−CD4+), and NKp46+ ILC22 cells [Lin−c-Kit−NKp46+]) using a FACSAnia (Fig. 2A). Then, we compared the mRNA levels of Il22, Defas, and Mcpt2 among Lin−c-Kit−NKp46−CD4−, CD4+ LTi-like, and NKp46+ ILC22 cells under steady-state conditions, using real-time PCR. NKp46+ ILC22 cells are known to be a main source of IL-22 in the small intestinal LP (20). However, our results showed that the Il22 mRNA expression was higher in the Lin−c-Kit−NKp46−CD4− cells (21.8 ± 3.0-fold) and CD4+ LTi-like cells (38.0 ± 5.0-fold) than in the NKp46+ ILC22 cells (8.6 ± 1.0-fold), whereas the Kit and Rorc transcript levels were similar among these three groups (Fig. 2B). Intracellular staining showed that almost all of the Lin−c-Kit−NKp46− CD4− and CD4+ LTi-like cells expressed RORγt protein (Fig. 2D). In the Th17 Bio-Plex analysis, the levels of IL-17F and IL-22 protein were also higher in the Lin−c-Kit−NKp46−CD4+ cells (10.5 ± 1.6 pg/ml and 14.0 ± 1.9 pg/ml in 50,000 cells) and CD4+ LTi-like cells (38.4 ± 9.2 pg/ml and 43.8 ± 6.6 pg/ml) than in the NKp46+ ILC22 cells (2.4 ± 1.1 pg/ml and 2.7 ± 0.3 pg/ml) in the steady-state condition. However, the production of IL-21, IL-23p19, IL-31, IL-33, soluble CD40L (scD40L), and MIP-3x protein was either undetectable in all three groups or did not show a significant difference (Fig. 2C).

The Lin−c-Kit−NKp46−CD4− population also strongly expressed the mRNAs for Defa4, Defa5, Defa24, Defa-rs1, Defa-rs2, Defa-rs7, Mcpt2, Mcpt4, Cpa3, and Csfr2b2 (Fig. 2B). In contrast, the CD4+ LTi-like and NKp46+ ILC22 cells showed no expression of Mcpt2, Mcpt4, Cpa3, or Csfr2b2. The CD4+ LTi-like cells expressed low levels of Defas (Defa4, -5, and -24 and Defa-rs1, -rs2, and -rs7).

These observations collectively indicate that Lin−c-Kit− NKp46−CD4− and CD4+ LTi-like cells, rather than the NKp46+ ILC22 cells, produce higher levels of IL-17F and IL-22 protein. Moreover, the Lin−c-Kit−NKp46−CD4− population might contain MCPs that strongly express MCPTs (25).

Lin−c-Kit−NKp46−CD4− population in the small intestinal LP produces high levels of IL-17F and IL-22 protein after IL-1β or IL-23 treatment

The protective role of the IL-23/IL-22 axis in the inflamed intestine is well established (26), and the microbiota-induced IL-1β production is critical for the development of Th17 cells in the steady-state intestine (27, 28). To compare the production levels of IL-17F and IL-22 protein among the Lin−c-Kit−NKp46−CD4−, CD4+ LTi-like, and NKp46+ ILC22 cells under inflammatory conditions, we treated each population of sorted cells with rIL-1β protein or rIL-23 protein for 24 h. All three populations produced significantly higher levels of IL-22 upon stimulation with IL-1β or IL-23 compared with the unstimulated cells (Fig. 3A). The Lin−c-Kit−NKp46−CD4− population produced the highest levels of IL-22 protein when stimulated with IL-1β or IL-23 among the three groups. The levels of IL-17F protein were significantly increased by IL-1β or IL-23 treatment in Lin−c-Kit−NKp46−CD4− and CD4+ LTi-like cells, but the level was not significantly different between the two groups (Fig. 3A). The level of IL-21 protein
increasing their IL-17F and IL-22 production, and the Lin−c-Kit−
NKp46−CD4− cells still produced the highest levels of both of the
proteins (Fig. 3B) and their mRNAs (Supplemental Fig. 1). In ad-
dition, intracellular cytokine staining analysis showed that 79.4 ±
2.5% of the Lin−c-Kit−NKp46−CD4− cells produced IL-22 protein
under costimulation with IL-1β and IL-23, and, of these cells,
13.0 ± 3.0% coproduced IL-17F and IL-22 (Fig. 3C). Taken
together, these results indicate that the Lin−c-Kit−NKp46−CD4−
population is the major source of IL-17F and IL-22 in the small
intestinal LP under IL-1β- or IL-23-stimulated conditions.

Lin−c-Kit−NKp46−CD4− population can differentiate 
to MCs and proliferate in the presence of recombinant 
IL-3 and 

Among the three populations, only the Lin−c-Kit−NKp46−CD4−
population strongly expressed genes related to MCs, such as
Csfr2b1, c-Kit, and Mcpt5 (Fig. 2B). Csfr2b1 and c-Kit encode the
receptors for IL-3 and SCF, respectively, which are essential factors
for MC survival and proliferation. MCPTs are secreted from MCs
and play important roles in intestinal homeostasis regulation (29).
Previous studies showed that mouse intestinal MCPs are defined as
being Lin−, c-Kit+, Sca-1−, β7 integrin+, and FcεRI+, and splenic
progenitor cells are Lin−, c-Kit+, Sca-1+, β7 integrin+, and FcεRI+ 
(30, 31). However, we found that the Lin−c-Kit−NKp46−CD4−
population expressed no Sca-1 or FcεRI by FACS analysis (data
not shown).

Despite such discrepancies in surface marker expression, we
speculated that the Lin−c-Kit−NKp46−CD4− population might
contain early MCPs. Interestingly, only the Lin−c-Kit−NKp46−
CD4− population, among the three groups, began to differentiate
into MCs within 2 wk of culture in the presence of rIL-3 and rSCF.
May-Grünwald-Giemsa and Alcian blue staining showed that MC
granules increased in the cultured cells for 2 wk, and the high
granule numbers persisted for 3 wk in culture (Fig. 4A). In the
FACS analysis, these cells showed c-Kit and FcεRI coexpression
after 4 wk in culture (Fig. 4B). In addition, the Lin−c-Kit−
NKp46−CD4− cells, but not the NKp46+ILC22 cells, showed
increased growth rates at 1, 2, and 3 wk in the presence of rIL-3
and rSCF in vitro (Fig. 4C). These findings indicate that Lin−c-
Kit−NKp46−CD4− cells contain MCPs, which can differentiate
into and proliferate as MCs after stimulation with IL-3 and SCF.

Expression of Il22 and Defas decreases during the
differentiation of MCs

We next compared the mRNA expressions of Il22, Defas (Defa4,
-5, -24, -rs1, -rs2, and -rs7), Rorc, and Mcpt family members
(Mcpt1, -2, -4, -5, -8, and -10, and Cpa3) between the Lin−c-
Kit−NKp46−CD4− cells and mature MCs (samples at 2–4 wk) by real-
time PCR. The Lin−c-Kit−NKp46−CD4− cells strongly expressed
Il22, Defa4, Defa5, Defa24, Defa-rs1, Defa-rs2, and Defa-rs7.
However, none of these genes was detected after the cells differ-
entiated into MCs in the presence of IL-3 and SCF. Expression of
Mcpt family members (Mcpt1, -2, -4, -5, -8, and -10) also was
attenuated, whereas Mcpt5 and Cpa3 mRNAs were induced >100–
150-fold in the MCs from 2 wk after treatment with IL-3 and SCF
(Fig. 4D). It was reported that the expression of Mcpt5 is corre-
related with that of Cpa3, because Mcpt5-deficient mice also lacked
Cpa3 protein expression (32). CPA-deficient MCs do not fully
mature but show normal hexosaminidase release and histamine
content and release (9). In addition, we found that, compared with
control, the Lin−c'-Kit+'NKp46+CD4− cells expressed ~2–3-fold
higher levels of the mRNAs for Mcpt1 and Mcpt5 by IL-1β
stimulation and Mcpt1 by IL-23 stimulation (data not shown).
Our results show that the expressions of innate immunity–
related genes, such as Il22 and antimicrobial peptides (DEFAs), were not detected during MC maturation. In addition, Mcpt1, -2, -4, -8, and -10 mRNA were decreased in mature MCs, whereas Mcpt5 and Cpa3 mRNA increased in mature MCs and may play important roles in MC maturity.

Lin2c-Kit+NKp462CD42 population isolated from BM or LP shows different cell-differentiation patterns when cultured with the recombinant cytokine mixture

A previous study assessed the differentiation of Lin2c-Kit+Sca-12 or Lin2c-Kit+Sca-1+ cells isolated from BM into granulocytes/macrophages, megakaryocytes and/or erythroid (Meg/E) cells, or MCs when cultured with a cytokine mixture (33). To compare the characteristics of each Lin2c-Kit+NKp46+CD4− population from the BM differentiated into mononuclear phagocytes (MNPs), neutrophils (Mac-1+ Gr-1+ cells), or Meg/E cells (Mac-1− Gr-1−), but scarcely into MCs, which were very rare even at 2 wk. In contrast, the LP-derived Lin2c-Kit+NKp46+CD4− population could differentiate into MCs and MNPs, as assessed by both cytosin (Supplemental Fig. 2B) and FACS (Supplemental Fig. 2C) analyses. These data indicate that the LP-derived Lin2c-Kit+NKp46+CD4− population contains MCPs, which are already committed to become MCs, in contrast to the Lin2c-Kit+NKp46+CD4− population from the BM.

We performed single-cell cultivation analysis for the Lin2c-Kit+NKp46+CD4− population isolated from the BM or small intestinal LP in the presence of a cytokine mixture. The results showed a greater variety in cell size compared with that sorted from the LP (Supplemental Fig. 2B). After cultivation for 1–2 wk with a cytokine mixture containing of 10 ng/ml each of IL-1α, IL-3, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, Flt-3L, GM-CSF, EPO, TPO, and SCF, the Lin2c-Kit+NKp46+CD4− population from the BM differentiated into mononuclear phagocytes (MNPs), neutrophils (Mac-1+ Gr-1+ cells), or Meg/E cells (Mac-1− Gr-1−), but scarcely into MCs, which were very rare even at 2 wk. In contrast, the LP-derived Lin2c-Kit+NKp46+CD4− population could differentiate into MCs and MNPs, as assessed by both cytosin (Supplemental Fig. 2B) and FACS (Supplemental Fig. 2C) analyses. These data indicate that the LP-derived Lin2c-Kit+NKp46+CD4− population contains MCPs, which are already committed to become MCs, in contrast to the Lin2c-Kit+NKp46+CD4− population from the BM.

We performed single-cell cultivation analysis for the Lin2c-Kit+NKp46+CD4− population isolated from the BM or small intestinal LP in the presence of a cytokine mixture. The results showed...
that the Lin−c-Kit−NKp46−CD4+ population from the LP could almost differentiate into Kit+ cells in 2.1% of plating efficiency. In contrast, the by As determined by cytopsin analysis, the Lin−c-Kit−NKp46−CD4+ population represents MCs, whereas the Lin−c-Kit−NKp46−CD4+ cells represent MCPs, and the IL-7Rα+CD4+β7int population represents CD4− LTI-like cells.

We used FACS analysis to investigate what percentage of cells within the Lin−c-Kit−NKp46−CD4+ population expresses CD127 (IL-7Rα), CD34, and β7 integrin, because IL-22-producing ILCs express IL-7Rα (34), and mouse intestinal MCPs express CD34 and β7hi (30). We found that 95.0% of the Lin−c-Kit−NKp46−CD4+ population was IL-7Rα+CD34−β7int cells (subset IV), and the rest of the population consisted of IL-7Rα+CD34+β7int (subset I), IL-7Rα+CD34+β7int (subset II), and IL-7Rα+CD34+β7int (subset III) cells (Fig. 5A). May-Grünwald-Giemsa staining showed that the IL-7Rα+CD34+β7int (I) and IL-7Rα+CD34+β7int (II) cells had few or no granules (Fig. 5B). We cultured the four subsets in a cytokine mixture for 1 wk and found that the IL-7Rα+CD34+β7int (I) and IL-7Rα+CD34+β7int (III) cells differentiated almost entirely into MCs (c-Kit+FceRI+ cells), whereas a small percentage of the IL-7Rα+CD34+β7int cells (IV) differentiated into MNPcs (Mac-1+Gr-1− cells) (Fig. 5E, 5F). The IL-7Rα+CD34+β7int cells (II) showed low viability and did not differentiate into other cell types (data not shown).

In addition, the IL-7Rα+CD34+β7int (I) and IL-7Rα−CD34+β7int (III) populations strongly expressed MCPTs mRNA, whereas the IL-7Rα+CD34+β7int population (II) strongly expressed DEFAM mRNA, and the IL-7Rα+CD34−β7int population (IV) predominantly expressed II22 and Rorc mRNA (Fig. 5C). We next compared the mRNA expression of MCPT family members in the IL-7Rα+CD34+β7int (I) and IL-7Rα−CD34+β7int (III) cells, as well as mature MCs (samples at 1 wk). The IL-7Rα+CD34+β7int (III) cells expressed the highest levels of Mcpt1 and Mcpt2 mRNA among the four groups, whereas the mature MCs induced high levels of Mcpt5 and Cpa3 mRNA (Fig. 5G). Our results suggest that the IL-7Rα+CD34+β7int and IL-7Rα−CD34+β7int populations represent MCPs, and the IL-7Rα+CD34−β7int population represents CD4− LTI-like cells.

IL-7Rα+CD34+β7int population was the main source of IL-17F and IL-22 after stimulation with rIL-1β and rIL-23. We next compared the induction of II17f and II22 transcripts in the IL-7Rα+CD34+β7int (I), IL-7Rα−CD34+β7int (II), IL-7Rα−CD34+β7int (III), and IL-7Rα+CD34−β7int (IV) populations under costimulation with IL-1β and IL-23 for 16 h. We found that the costimulation induced II17f and II22 mRNA levels only in the IL-7Rα+CD34−β7int population (IV) (Fig. 5D). These data indicate that the main source of IL-17F and IL-22 production after stimulation with IL-1β and IL-23 is the IL-7Rα+CD34−β7int population (CD4− LTI-like cells).

In addition, we examined the effect of IL-3, which is a key factor for MC development, on IL-17F and IL-22 protein induction after stimulation with IL-1β or IL-23 in Lin−c-Kit−NKp46−CD4+ cells. We found that IL-3 slightly inhibited IL-17F and IL-22 induction by IL-1β and IL-23 (Supplemental Fig. 3).

Lin−c-Kit−NKp46−CD4+ population contains more immature MCPs than the Lin−CD45+CD34+FceRI+β7hi population. A previous study reported that intestinal MCPs, the Lin−CD45+CD34+FceRI+β7hi cells, contain a few scattered metachromatic granules and differentiate into pure MC colonies (31). We sorted the Lin−CD45+CD34+FceRI+β7hi population from small intestinal LP (Fig. 6A), cultured this population in the presence of a recombinant cytokine mixture for 0–2 wk, and analyzed the cell phenotype by...
cytospin and FACS. Our results agreed with the previous report: the sorted Lin<sup>−</sup>c-Kit<sup>−</sup>NKp46<sup>−</sup>CD4<sup>−</sup>CD45<sup>+</sup>CD34<sup>+</sup>FcεRI<sup>+</sup>bd7hi population (at 0 wk) already had a few granules in the cytoplasm (Fig. 6C); it showed higher side scatter compared with the IL-7Rα<sup>−</sup>CD34<sup>+</sup>bd7int(I) and IL-7Rα<sup>−</sup>CD34<sup>−</sup>bd7hi(III) populations (Fig. 6B), which do not express FcεRI (data not shown); and it could differentiate into pure MCs (Fig. 6C, 6D). These results suggest that the Lin<sup>−</sup>c-Kit<sup>−</sup>NKp46<sup>−</sup>CD4<sup>−</sup>CD45<sup>+</sup>CD34<sup>+</sup>FcεRI<sup>+</sup>bd7hi population consists of relatively more mature MCPs than do the IL-7Rα<sup>−</sup>CD34<sup>+</sup>bd7int and IL-7Rα<sup>−</sup>CD34<sup>−</sup>bd7hi populations (Fig. 7).

**Discussion**

In the current study, we observed that the Lin<sup>−</sup>c-Kit<sup>−</sup>NKp46<sup>−</sup>CD4<sup>−</sup>LTi-like population (CD4<sup>−</sup>LTi-like cells) produces significantly more IL-17F and IL-22 protein after costimulation with IL-1β and IL-23 than do the CD4<sup>+</sup> LTi-like or NKp46<sup>+</sup> ILC22 cells, which were previously reported to be a major source of IL-22 in the small intestinal LP (20, 35). We also confirmed that the IL-7Rα<sup>−</sup>CD34<sup>+</sup>bd7int population within Lin<sup>−</sup>c-Kit<sup>−</sup>NKp46<sup>−</sup>CD4<sup>−</sup> cells is the main source of DEFAs under steady-state conditions, whereas the IL-7Rα<sup>−</sup>CD34<sup>−</sup>bd7int and IL-7Rα<sup>−</sup>CD34<sup>−</sup>bd7hi populations strongly express MCPTs and can differentiate almost entirely into MCs in the presence of a cytokine mixture in vitro. IL-22, DEFAs, and MCPTs are known to regulate tissue homeostasis and innate immune responses (11, 36, 37). Our results suggest that IL-7Rα<sup>−</sup>CD34<sup>−</sup>bd7int (CD4<sup>−</sup>LTi-like cells), IL-7Rα<sup>−</sup>CD34<sup>−</sup>bd7int, IL-7Rα<sup>−</sup>CD34<sup>−</sup>bd7int, and IL-7Rα<sup>−</sup>CD34<sup>−</sup>bd7hi populations within the small intestinal LP play important roles in tissue homeostasis and mucosal innate immune responses.

Until now, the most-well known source of DEFAs was Paneth cells, which are specialized cells in the small intestinal epithelium...
FIGURE 6. The Lin-CD45^CD34^FceR1^β7hi population from the LP contains more mature MCPs than does the Lin-εKt^-NKp46^-CD4^- population. (A) The Lin-CD45^CD34^FceR1^β7hi population was sorted from the small intestinal LP with a FACSAria and cultured in the presence of 10 ng/ml each of IL-1α, IL-3, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, Flt-3L, GM-CSF, EPO, TPO, and SCF for 1–2 wk. (B) FACs analysis of the cytoplasmic granularity of the Lin-CD45^CD34^FceR1^β7. IL-7Rα^CD34^β7int (I), and IL-7Rα^CD34^β7hi (III) populations. (C) Differentiation of the Lin-CD45^CD34^FceR1^β7hi population into MCs after 1 wk in culture. The cells were stained with May-Grünwald-Giemsa solution (original magnification ×1000, scale bar, 10 μm). (D) FACs analysis of MCs differentiated from the Lin-CD45^CD34^FceR1^β7hi population after 2 wk in culture by gating on the markers c-Kit, FceRI, Gr-1, and Mac-1. Data are representative of two independent experiments with seven mice each.

(38). In this study, we found that IL-7Rα^-CD34^-β7int population also expresses high levels of DEFA mRNA under steady-state conditions. DEFAs have bactericidal activity against both Gram-negative and Gram-positive bacteria by disrupting the bacterial membrane integrity or interfering with the synthesis of bacterial cell walls (39). DEFAs secreted from Paneth cells may be localized to the small intestinal lumen (40), in contrast to DEFAs from the IL-7Rα^-CD34^-β7int population, which may be located specifically in the small intestinal LP. Our findings indicate that the IL-7Rα^-CD34^-β7int population within the small intestinal LP may have important antimicrobial activity.

NKp46^+ ILC22 cells were reported to be the main source of IL-22 in the small intestinal LP (20, 34). However, in this study, we compared the production of Th17 cytokines among CD4^-^LTi-like cells, NKp46^+ ILC22 cells, and CD4^-^LTi-like cells, and found that all three groups produced higher levels of IL-22 protein under stimulation with IL-1β compared with those treated with IL-23. They also showed synergistic effects on IL-17F and IL-22 production after co-stimulation with IL-1β and IL-23, with the CD4^-^LTi-like cells showing the highest level of IL-17F and IL-22 induction among the three groups. CD4^-^LTi-like cells also show synergistic effects on IL17a mRNA expression after co-stimulation with IL-1β and IL-23 but at a lower level compared with IL17f mRNA expression (Supplemental Fig. 1C). IL-17A is mainly produced in T cells, whereas IL-17F is produced in T cells, ILCs, and epithelial cells. IL-17F induces antimicrobial peptides and cytokines in epithelial cells (41). Although IL-1β and IL-23 are known to be key regulators of Th17 response induction, their functions seem to differ according to the tissue conditions (42). For example, IL-23 induces IL-22 under infectious conditions or in instances of tissue damage (19), whereas the IL-1β–dependent IL-22 increase occurs under homeostatic conditions (28, 43). Furthermore, microbiota-induced IL-1β plays a critical role in the development of steady-state Th17 cells in the intestine (27). We found that IL-31 (after stimulation with IL-23) and sCD40L (after stimulation with IL-1β) show induction in both CD4^-^LTi-like cells and NKp46^+ ILC22 cells, but the increases are not significant at low levels. Therefore, three populations (CD4^-^LTi-like cells, CD4^-^LTi-like cells, and NKp46^+ ILC22 cells) show different expression pattern of cytokines when stimulated with IL-1β or IL-23.

We also observed that none of the three cell groups showed any induction of IL-17F or IL-22 protein when stimulated with TLR2, TLR3, or TLR4 agonists. A previous study showed that CD3^-^CD127^- immune cells in the spleen and mucosa produce IL-17A, IL-17F, and IL-22 in a DC-dependent manner when treated with a TLR5 agonist (flagellin) (44). This means that TLR agonists cannot directly activate ILCs, whereas TLR5-mediated upregulation of IL-1β or IL-23 from DCs may induce Th17 cytokines from ILCs.

By subdividing the Lin^-c-Kit^-NKp46^-CD4^- population according to the expression of IL-7Rα, CD34, and β7 integrin, we found that IL-7Rα^-CD34^-β7int and IL-7Rα^-CD34^-β7hi cells can differentiate into pure MCs, whereas IL-7Rα^-CD34^-β7int cells show no differentiation into any cells in the presence of a cytokine mixture for 1 wk. Our data suggest that IL-7Rα^-CD34^-β7int and IL-7Rα^-CD34^-β7hi cells within the small intestinal LP are MCs, which are less mature (i.e., contain few or no granules and express no FceRI) than are the Lin^-CD45^-CD34^-FceR1^-β7hi cells reported to be intestinal MCs (30). MCs are also present in the common myeloid progenitor population (Sca-1^-^Lin^-c-Kit^-CD27^-Flk2^-) within the BM (33). We observed that the IL-7Rα^-CD34^-β7int and IL-7Rα^-CD34^-β7hi populations (both Sca-1^-) within the small intestinal LP are CD27^-Flk2^- and CD27^-Flk2^- cells, respectively (data not shown). Our data suggest that the MCs (Sca-1^-Lin^-c-Kit^-CD27^-Flk2^-) generated in the BM may lose the expression of Sca-1 after migration into the small intestinal LP. A study showed that IL-25–induced Lin^-c-Kit^- cells, termed MPP2 cells, in the mesenteric lymph nodes promote Th2 cytokine responses and differentiate into MCs, basophils, or macrophages. MPP2 cells express Sca-1 and intermediate levels of c-Kit (45). However, the IL-7Rα^-CD34^-β7int and IL-7Rα^-CD34^-β7hi populations that we identified exist in the
FIGURE 7. A schematic model of intestinal Lin e-Kit+NKp46 CD4⁺ CD4⁻ cell function. (A) Under steady-state conditions, the IL-7Rα⁺CD34⁺βint population (CD4⁻ LTi-like cells) produces IL-22, IL-7Rα⁺CD34⁺βint and IL-7Rα⁺CD34⁺βsh cells (MCPs) produce MCPTs, and IL-7Rα⁺CD34⁺βint cells produce DEFAs. (B) MCPs (IL-7Rα⁺CD34⁺βint and IL-7Rα⁺CD34⁺βsh cells) can differentiate into MCs in response to certain microenvironmental factors.

References


Acknowledgments

We thank Nori Yoshizumi (Immunology Frontier Research Center, Osaka University) for technical assistance and members of the DNA-chip Development Center for Infectious Diseases (Research Institute for Microbial Diseases, Osaka University) for technical advice.

Disclosures

The authors have no financial conflicts of interest.

small intestinal LP under steady-state conditions, express high levels of MCPTs and e-Kit, and differentiate only into MCs. In addition, we found that the IL-7Rα⁺CD34⁺βint population contains a small number of progenitors, which can differentiate into Mac-1⁺Gr-1⁻ cells, whereas the CD34⁺βint population from IL-7Rα⁻ deficient mice could also differentiate into Mac-1⁺Gr-1⁻ cells in our experiment (data not shown). These data suggest that the progenitor of Mac-1⁺Gr-1⁻ cells within the IL-7Rα⁺CD34⁺βint population is not of lymphoid origin. IL-7Rα⁺ progenitors are known to differentiate into lymphoid cells but not myeloid cells (46); however, a previous study reported that the IL-7Rα⁺e-Kit⁺Sca-1⁺ population from the fetal liver can give rise to macrophages at a fairly high frequency in vitro (47). Therefore, we need to identify more clearly the relationship between IL-7R expression and differentiation into Mac-1⁺Gr-1⁻ cells in a further study.

Finally, we found that the transcription levels of Mpt1 and Mpt2 are high in IL-7Rα⁺CD34⁺βint cells (MCPs), whereas Mpt5 and CPA3 transcript levels are high in mature MCs. MCP1 and Mpt2 are preferentially expressed in mucosal MCs, whereas MCP5 and CPA3 are mainly expressed in connective tissue MCs (48–50). MCP1 subtypes show functional differences (e.g., MCP1 can regulate the degradation of cell–cell junctions (51), MCP5 can control an ischemia-reperfusion injury of mouse skeletal muscle (52), and CPA3 can degrade sarafotoxin (snake poison)) (8). Our observations suggest that the main source of MCP1 and Mpt2 within the small intestinal LP may be MCPs rather than MCs; MCs are rarely detected in the steady-state condition, and MCPs mainly express Mpt1 and Mpt2 to maintain intestinal homeostasis under steady-state conditions, whereas mature MCs, which predominantly express Mpt5 and CPA3, may control inflammatory responses.

Taken together, our data show that the IL-7Rα⁺ population (CD4⁻ LTi-like cells) within Lin e-Kit+NKp46 CD4⁺ cells may contribute to tissue homeostasis and have a protective role against bacterial infection through their high expression of IL-17F and IL-22. The strong expression of DEFAs by the IL-7Rα⁺CD34⁺βint population in the steady-state condition may play an important role in maintaining tissue homeostasis with commensal bacteria. IL-7Rα⁺CD34⁺βint and IL-7Rα⁺CD34⁺βsh cells are MCPs, which can differentiate into MCs that may regulate inflammatory responses by expressing high levels of MCP5 and CPA3 (Fig. 7).