

Discussion

The demonstration of an association between ALDH and tumors was first shown in breast cancer (16) and subsequently in pancreatic (17), liver (18), colorectal (19), head and neck (19), thyroid (20) and lung (21) cancers. In lung cancer, ALDH activity has been shown to be selective for adenocarcinoma stem cells, depending on *Notch* signaling (21), in agreement with our observations of gastric CSCs. We demonstrated that in GC, *Notch* and *Shh* signaling may be important for both CSC maintenance and the generation of chemoresistance, providing the rationale for further study of therapy-resistant ALDH^{high} CSCs. ALDH is widely used as a marker to identify and isolate various types of normal stem cells and CSCs (22). In GC, several markers reportedly characterize CSCs: CD133 (23), CD44 (23-26), side-populations identified by FACS (27), CD44 and EpCAM (25), CD54 (26) and CD90 (28). Of these markers, CD44 and ALDH are involved in aerobic glycolysis during cancer metabolism. Although an association between ALDH and the clinicopathological features of GC has been reported (29), the relevance of ALDH to chemoresistance has yet to be fully investigated; another study detected no association between immunohistochemical staining for ALDH and prognosis in GC patients (23). In this study, we examined for the first time the involvement of ALDH in chemoresistance and identified a candidate underlying molecular mechanism for this resistance.

GC is the second major cause of cancer-related mortality worldwide and is prevalent across Asia. *Helicobacter pylori* (*H. pylori*) infection was identified in 1982 by Marshall and Warren in patients with chronic gastritis and gastric ulcers (30). *H. pylori*-associated GC has been investigated in order to elucidate the mechanisms underlying gastric tissue damage. In general, the two mechanisms by which *H. pylori* promotes cancer are as follows: i) enhanced production of free radicals proximal to the *H. pylori* infection site, increasing the host cell mutation rate; and ii) pregenetic factors that transform host cell phenotypes by altering adhesion proteins or inflammation-related cytokines/chemokines, such as tumor necrosis factor- α or interleukin-6. Thus, *H. pylori* infection causes enhanced migration or invasion of damaged epithelial cells, without additional tumor suppressor gene mutations (31). Those non-cell autonomous mechanisms are likely facilitated by the hypoxic microenvironment of tumors, since recent studies have implicated hypoxia in inflammatory reactions provoked by *H. pylori* infection (32). Indeed, hypoxia-inducible factor-1 α is mediated by the induction of a ROS-inducible protein (apurinic/apurimidinic endonuclease 1) and its enhanced interaction with the transcriptional coactivator, p300, leads to transformed phenotypes in *H. pylori*-infected gastric epithelia (33). Although *H. pylori* infection and related atrophic gastritis are closely associated with GC, hypoxia and its related metabolism play a critical role in tumor initiation and progression in the stomach and likely in other organs (34). Further studies are warranted to elucidate the association between *H. pylori* infection and ALDH-positive CSCs in hypoxic areas and to evaluate the eradication of *H. pylori* infection and GC treatment by surgery, chemotherapy and molecular targeting of therapy-resistant CSC functions.

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Increased Th17-Inducing Activity of CD14⁺ CD163^{low} Myeloid Cells in Intestinal Lamina Propria of Patients With Crohn's Disease

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BACKGROUND & AIMS: Abnormal activity of innate immune cells and T-helper (Th) 17 cells has been implicated in the pathogenesis of autoimmune and inflammatory diseases, including Crohn's disease (CD). Intestinal innate immune (myeloid) cells have been found to induce development of Th17 cells in mice, but it is not clear if this occurs in humans or in patients with CD. We investigated whether human intestinal lamina propria cells (LPCs) induce development of Th17 cells and whether these have a role in the pathogenesis of CD. **METHODS:** Normal intestinal mucosa samples were collected from patients with colorectal cancer and non-inflamed and inflamed regions of mucosa were collected from patients with CD. LPCs were isolated by enzymatic digestion and analyzed for expression of HLA-DR, lineage markers CD14 and CD163 using flow cytometry. **RESULTS:** Among HLA-DR^{high} Lin⁻ cells, we identified a subset of CD14⁺ CD163^{low} cells in intestinal LPCs; this subset expressed Toll-like receptor (*TLR*) 2, *TLR4*, and *TLR5* mRNAs and produced interleukin (IL)-6, IL-1 β , and tumor necrosis factor in response to lipopolysaccharide. In vitro co-culture with naïve T cells revealed that CD14⁺ CD163^{low} cells induced development of Th17 cells. CD14⁺ CD163^{low} cells from inflamed regions of mucosa of patients with CD expressed high levels of *IL-6*, *IL-23p19*, and tumor necrosis factor mRNAs, and strongly induced Th17 cells. CD14⁺ CD163^{low} cells from the noninflamed mucosa of patients with CD also had increased abilities to induce Th17 cells compared with those from normal intestinal mucosa. **CONCLUSIONS:** CD14⁺ CD163^{low} cells in intestinal LPCs from normal intestinal mucosa induce differentiation of naive T cells into Th17 cells; this activity is increased in mucosal samples from patients with CD. These findings show how intestinal myeloid cell types could contribute to pathogenesis of CD and possibly other Th17-associated diseases.

Keywords: Dendritic Cell; Macrophage; Helper T Cell; Inflammatory Bowel Disease.

Recent studies have suggested that innate immune cells, including macrophages and dendritic cells (DC) of the intestinal mucosa, play important roles in

maintaining gut homeostasis by protecting the host from foreign pathogens and by negatively regulating excess immune responses to commensal bacteria and dietary antigens.^{1,2} Inappropriate immune reactions to commensal bacteria can lead to development of inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis.^{3,4} The identification of interleukin (IL)-17-producing Th17 cells offers new insight into the induction and regulation of mucosal immunity, which is linked to IBD pathogenesis.^{5–7} Additionally, genome-wide association studies have shown significant associations of CD with Th17-related inflammatory pathways and innate microorganism sensors.^{8–13}

Several subsets of murine intestinal innate immune cells have been found to modulate intestinal homeostasis.¹⁴ In the CD11c⁺ cell subset, CD103⁺ and CX3CR1⁺ cells represent nonoverlapping populations with different precursors.^{15–17} CD103⁺ cells uptake luminal antigens in the lamina propria by a unique mechanism, move to the mesenteric lymph nodes (MLN), and activate T cells.^{18,19} They can induce expression of gut homing receptors (CCR9 and $\alpha 4\beta 7$ integrin) on T cells, activate CD8⁺ T cells, and induce differentiation of Foxp3-expressing regulatory T cells.^{20–22} CX3CR1⁺ cells can be divided into 2 subsets based on CX3CR1 expression level: CX3CR1^{int} and CX3CR1^{high} cells.^{15,23} CX3CR1^{int} cells are DCs that induce Th17 cells and migrate into the inflammatory regions,^{23–28} and CX3CR1^{high} cells possess anti-inflammatory properties and inhibit T-cell responses.^{26,29,30} These findings strongly support the notion that intestinal innate immune cells regulate gut homeostasis.

Compared with murine intestinal innate immune cells, human intestinal cells remain less well characterized. It

Abbreviations used in this paper: CD, Crohn's disease; CDi, inflamed region of CD; CDn, noninflamed region of CD; DC, dendritic cell; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; int, intermediate; LPC, lamina propria cell; MLN, mesenteric lymph node; mRNA, messenger RNA; NC, normal colon from colon cancer patients; slan, 6-sulfo LacNAC; TGF, transforming growth factor; Th, T-helper; TLR, Toll-like receptor; TNF, tumor necrosis factor.

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has been reported that resident intestinal macrophages fail to produce proinflammatory cytokines in response to Toll-like receptor (TLR) ligands, in spite of active phagocytic and bactericidal activities.^{31,32} A unique subset of macrophages that express macrophage (CD14, CD33, CD68) and DC (CD205, CD209) markers reportedly contributes to IBD pathogenesis through induction of IL-23–dependent interferon (IFN) gamma responses by T cells, as well as potent antigen-presenting activity.^{33,34} Similar to the mouse intestinal CD103⁺ cells, CD103⁺ DCs that can induce gut homing receptors have been identified in the human small intestine.²⁰ However, human intestinal lamina propria cells (LPCs) equivalent to mouse CX3CR1⁺ cells have not yet been characterized. Most recently, human LPCs have been classified into subsets based on the expressions of CD14, HLA-DR, CD209, CD163, and CD11c.²⁸ However, functional characterization of these human LPC subsets remains elusive.

In the present study, we show that HLA-DR^{high} Lin⁻ cells in human intestinal lamina propria comprise 4 subsets that are distinguished by differential expression patterns of CD14, CD11c, and CD163. We further show that HLA-DR^{high} Lin⁻ CD14⁺ CD163^{low} cells induce Th17 cell differentiation. CD patients exhibited exceedingly enhanced Th17 immunity induced by HLA-DR^{high} Lin⁻ CD14⁺ CD163^{low} cells, even in noninflamed intestine.

Methods

Tissue Samples

Normal intestinal mucosa was obtained from microscopically and macroscopically intact areas in patients with colorectal cancer. Intestinal mucosa was also obtained from surgically resected specimens from patients with CD who were diagnosed based on established clinical, bacteriological, radiologic, and endoscopic criteria. Histopathological analysis showed moderate to severe inflammation in all samples from patients with CD. This study was approved by the Ethical Committee of Osaka University School of Medicine and informed consent for specimen use was obtained from all patients.

Isolation of LPCs

To isolate LPCs, intestinal mucosa was washed in phosphate-buffered saline to remove feces. Then, intestinal mucosa was placed in RPMI 1640 containing 5 mM EDTA and incubated for 10 minutes in a 37°C shaking water bath. After washing in phosphate-buffered saline, the mucosa was cut into small pieces and incubated in RPMI 1640 containing 10% fetal bovine serum, 2 mg/mL collagenase D (Roche, Basel, Switzerland), 1 mg/mL dispase (Invitrogen, Carlsbad, CA), and 15 µg/mL DNase I (Roche) for 60 minutes in a 37°C shaking water bath. The digested tissues were then passed through a 40-µm cell strainer. The isolated cells were washed with RPMI 1640 containing 5 mM EDTA, resuspended in 5 mL 20% Percoll (GE Healthcare Japan, Tokyo, Japan), and then overlaid on 2.5 mL 40% Percoll in a 15-mL tube. Percoll gradient separation was performed by centrifugation at 500g for 30 min at 4°C. The LPCs

were collected from the interface of the Percoll gradient, then washed twice with phosphate-buffered saline containing 2% fetal bovine serum.

Flow Cytometry

The antibodies for flow cytometry are shown in the Supplementary Material. Flow cytometric analysis was performed using a FACSCantoII flow cytometer (BD Biosciences, San Jose, CA). LPCs were purified using a FACSARIAII system (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Morphological Analysis

Isolated LPC subsets were spread on glass slides and were air dried. The spread cells were fixed with methanol, stained by May-Grünwald-Giemsa method, and observed with a microscope (BZ-9000; KEYENCE, Itasca, IL).

Microarray Analysis

Total RNA derived from LPCs was reverse transcribed by using an Ovation Pico WTA System V2 (NuGen, San Carlos, CA) and labeled with cyanine-3 using a Genomic DNA Enzymatic Labeling Kit (Agilent Technologies, Santa Clara, CA). The labeled complementary DNA was hybridized to SurePrint G3 Human Gene Expression v2 8x60K Microarray (G4845A) (Agilent Technologies). The data have been deposited in GEO as GSE49066. See the Supplemental Material for more details.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA). RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and random primers (TOYOBO, Osaka, Japan) after treatment with RQ1DNase I (Promega). Quantitative real-time polymerase chain reaction was performed on a Light Cycler 2.0 (Roche) with the TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA). Amplification conditions were 95°C for 10 minutes, then 45 cycles of 95°C for 10 seconds, and 60°C for 30 seconds. All data were normalized to the expression of the glyceraldehyde 3-phosphate dehydrogenase gene. The primer sets and probes are shown in Supplementary Table 1.

Cytokine Assay

LPCs were cultured with 1 µg/mL FSL-1 (Invivogen), 1 µg/mL LPS (Sigma, St Louis, MO), or 1 µg/mL flagellin (Invivogen) for 72 hours at 37°C. The culture supernatants were collected and enzyme-linked immunosorbent assay was performed with the Human Th1/Th2 11plex FlowCytomix kit (eBioscience, San Diego, CA).

In Vitro T-Cell Differentiation

Allogeneic naive CD4⁺ T cells were co-cultured at 37°C with the indicated cells at a ratio of 1:5 in the presence of 10 U/mL IL-2 (Roche), 2 µg/mL anti-CD3 (BD Biosciences), and 5 µg/mL anti-CD28 (BD Biosciences). After 5 days, cells were analyzed by fluorescence-activated cell sorting. In cytokine blocking experiments, cells were cultured in the presence of 2.5 µg/mL anti-transforming growth factor (TGF)-β, 2.5 µg/mL

anti-IL-1 β , 2.5 μ g/mL anti-IL-6, or 0.5 μ g/mL anti-IL-23 (R&D Systems, Minneapolis, MN).

Statistical Analysis

Differences between the control and experimental groups were evaluated using the Student's *t* test. Statistical analyses were performed using JMP version 9.02 (SAS Institute, Cary, NC). *P* values < .01 were considered statistically significant.

Results

Phenotypic Characterization of Human Colonic LPC Subsets

We used a combination of surface markers to characterize LPC subsets in noninvaded colonic tissue samples from colorectal cancer patients. After LPC isolation from colonic tissues, single-cell suspensions were analyzed for expression of lineage markers (CD3, CD19, CD20, and CD56), HLA-DR, CD14, CD11c, and CD163 (Figure 1A). To characterize LPCs that were involved in

mediating T-cell differentiation and activation, we focused on HLA-DR^{high} cells. HLA-DR^{high} Lin⁻ cells comprised 3.7%–5.1% of total LPCs, and were divided into 3 subsets: CD14⁻ CD11c^{low}, CD14⁻ CD11c^{high}, and CD14⁺ CD11c⁺ cells. CD14⁻ CD11c^{low} and CD14⁻ CD11c^{high} cells each did not express CD163 (data not shown), and CD14⁺ CD11c⁺ cells were further subdivided into 2 populations: CD163^{low} CD14⁺ CD11c⁺ and CD163^{high} CD14⁺ CD11c⁺ cells; hereafter, these cells are designated CD14⁺ CD163^{low} and CD14⁺ CD163^{high} cells, respectively. In total, human colonic HLA-DR^{high} Lin⁻ cells comprised 4 subsets: CD14⁺ CD163^{low} (24.7% \pm 8.2% of all HLA-DR^{high} Lin⁻ cells), CD14⁺ CD163^{high} (14.7% \pm 4.7%), CD14⁻ CD11c^{high} (18.8% \pm 7.9%), and CD14⁻ CD11c^{low} (30.3% \pm 8.9%) cells (Figure 1B). CD14⁺ CD163^{low} and CD14⁺ CD163^{high} cells showed macrophage-like morphologies with numerous cytoplasmic vacuoles (Figure 1C).

We then analyzed whether these populations were present in other tissues (Figure 1D). In the small intestinal

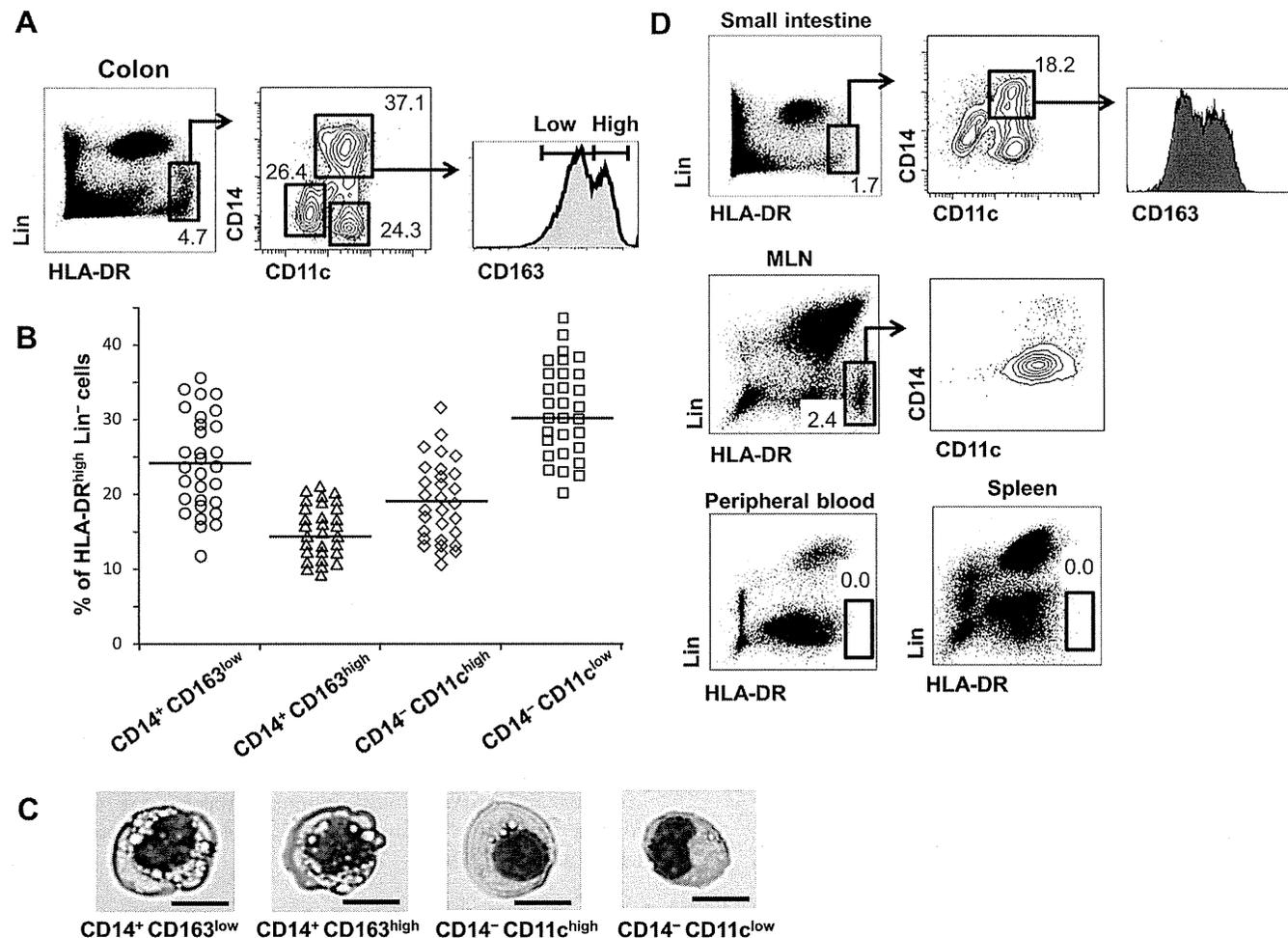


Figure 1. Characterization of LPC subsets. (A) Flow cytometry analysis of LPCs from normal intestine of colorectal cancer patients. Live HLA-DR^{high} Lin⁻ cells were gated and analyzed for expression of CD14 vs CD11c (Lin⁻; CD3, CD19, CD20, CD56). Three populations were defined (CD14⁻ CD11c⁻, CD14⁻ CD11c⁺, and CD14⁺ CD11c⁺), and the CD14⁺ CD11c⁺ population was further analyzed for CD163 expression. Representative results of 30 independent experiments are shown. (B) Percentages of LPC subsets among HLA-DR^{high} Lin⁻ cells of 30 patients; horizontal bars indicate mean. (C) Morphological analysis of LPC subsets by May-Grünwald-Giemsa staining. Scale bars = 10 μ m. (D) Characterization of cell subsets from human small intestine, MLN, peripheral blood, and spleen. Representative results of 10 independent experiments are shown. Numbers indicate the percentage of cells in indicated gates. Arrows show gating strategy.

lamina propria, we observed the same 4 populations, albeit with slightly different proportions: CD14⁺ CD163^{low} (11.7% ± 4.3% of all HLA-DR^{high} Lin⁻ cells), CD14⁺ CD163^{high} (9.9% ± 4.1%), CD14⁻ CD11c^{high} (35.1% ± 17.2%), and CD14⁻ CD11c^{low} (33.2% ± 11.1%) cells. In the MLN, we found only small numbers of CD14⁺ cells and increased numbers of CD14⁻ CD11c^{high} cells. HLA-DR^{high} Lin⁻ cells were not present in peripheral blood and spleen.

Next, we analyzed the surface expressions of several markers on these 4 subsets of human intestinal LPCs (Figure 2). CD14⁺ CD163^{low} and CD14⁺ CD163^{high} cells expressed several macrophage and DC markers, including molecules expressed on macrophages (CD16 and CD64),

on DCs (CD1c, CD141, CD209, and 6-sulfo LacNAc [slan]), on both macrophages and DCs (CD11b, CD172a, and CD206), and on mature DCs (CD80, CD83, and CD86). These 2 cell subpopulations showed almost the same expression patterns for all examined surface molecules, indicating that CD163 is a unique marker that can distinguish CD14⁺ cells subpopulations. CD14⁻ CD11c^{high} cells exhibited high expression of CD1c and CD172a as well as moderate expression of DC maturation markers, but were still heterogeneous because these cells included CD103⁺ and CD103⁻ populations. CD14⁻ CD11c^{low} cells were also heterogeneous, including CD141^{high} and CD141⁻ populations.

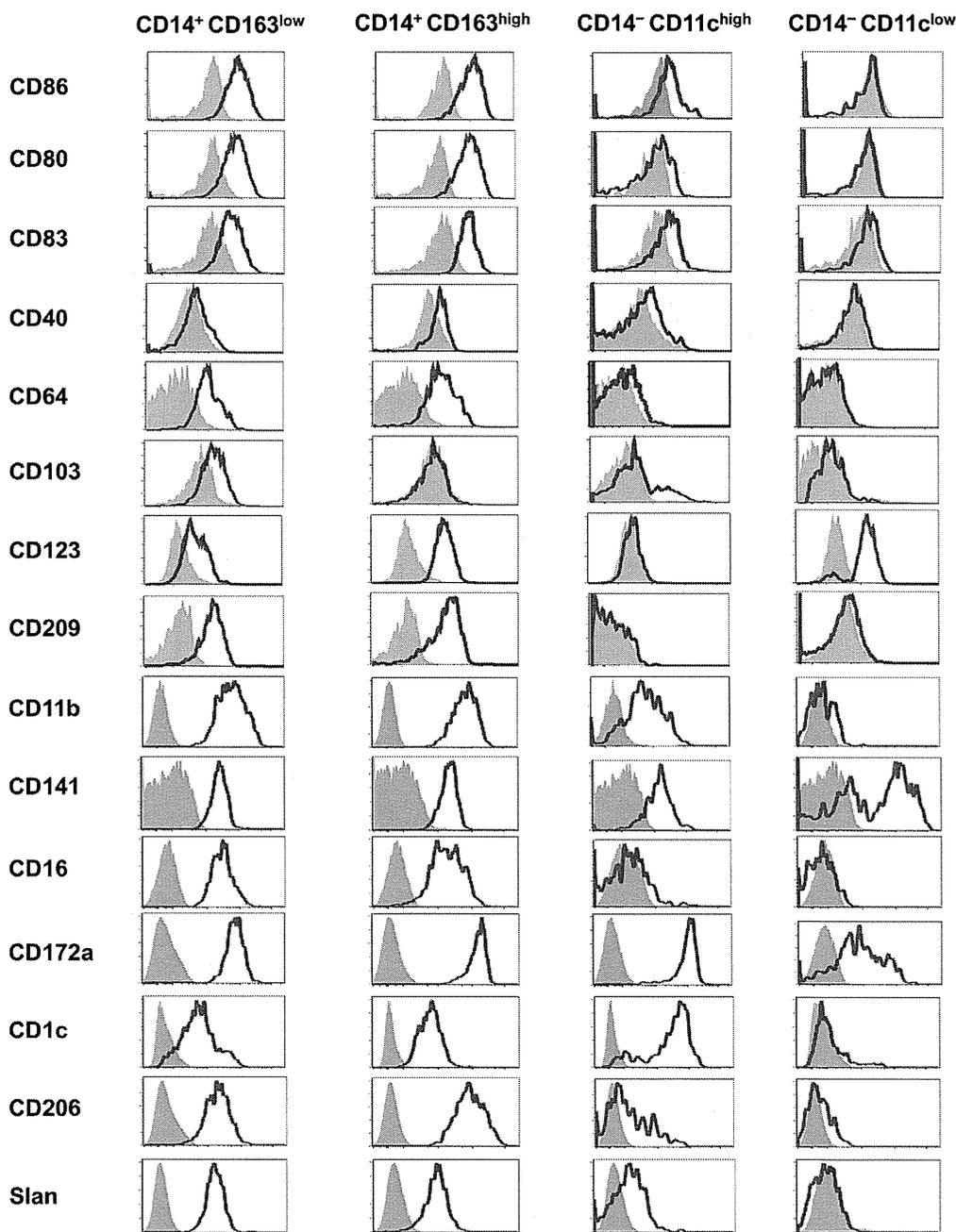


Figure 2. Phenotypic analysis of LPC subsets. Flow cytometry for surface expressions of CD86, CD80, CD83, CD40, CD64, CD103, CD123, CD209, CD11b, CD141, CD16, CD172a, CD1c, CD206, and slan in LPC subsets. The histogram shows the profiles of the indicated antibody staining, and the shaded histogram shows staining with isotype control. Representative results of 5–11 independent experiments are shown.

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Gene Expression Features of LPC Subsets

We performed microarray analysis to compare gene expression profiles among these subsets purified from normal colon of 10 colon cancer patients. To assess the reliability of the microarray analysis, we compared expression of mRNAs encoding glyceraldehyde 3-phosphate dehydrogenase, CD163, CD14, and CD11c across LPC subsets analyzed (Supplementary Figure 1). We found that mRNA expression of these genes correlated with the surface phenotype of each subset. Hierarchical clustering of about 15,000 protein-coding genes with >2-fold change in expression in at least 1 of the 6 pairwise comparisons showed that CD14⁺ CD163^{low} cells were more closely related to CD14⁺ CD163^{high} cells than to other subsets (Figure 3A). When we selected genes corresponding to the DC-related and monocyte/macrophage-related biological processes (antigen processing and

presentation [GO 0019882], phagocytosis [GO 0006909], monocyte differentiation [GO 0030224], or macrophage differentiation [GO 0030225]), the transcriptomic pathways of CD14⁺ CD163^{low} cells were found to be similar to those of CD14⁺ CD163^{high} cells. However, when focusing on inflammatory response to antigenic stimulus (GO 0002437), CD14⁺ CD163^{low} cells were close to CD14⁻ CD11c^{high} cells (Figure 3B and Supplementary Table 2). To gain more insight into the nature of LPC subsets, we analyzed expression of transcription factors and growth factor receptor, which are involved in function and development of DCs and macrophages (Supplementary Figure 2). Expression of mRNAs encoding *MAFB* and *CSFR1* (involved in macrophage differentiation) was high in CD14⁺ CD163^{low} and CD14⁺ CD163^{high} cells. CD14⁻ CD11c^{high} cells expressed the highest level of mRNAs encoding *IRF4* and *FLT3*, which are both involved in the

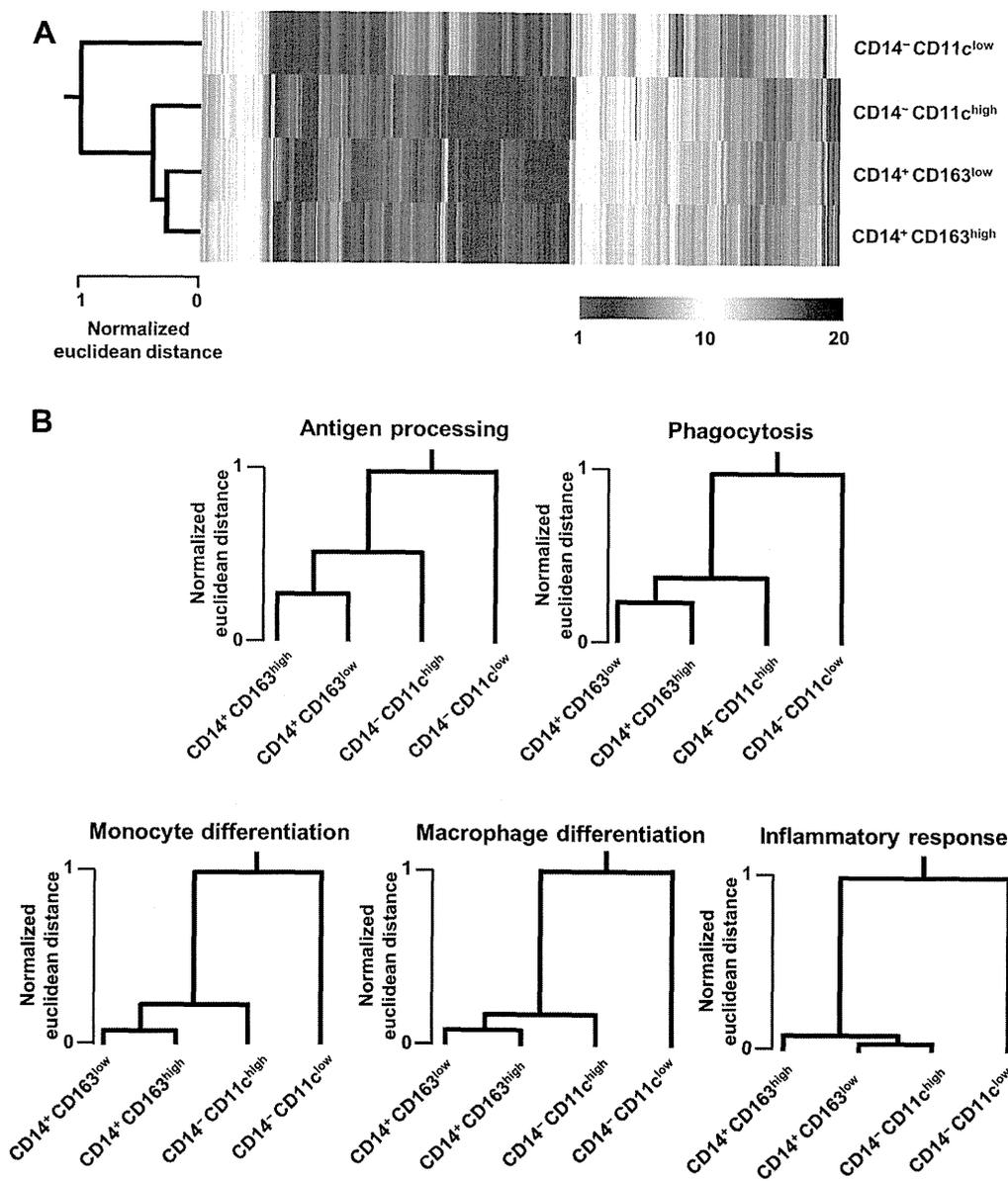


Figure 3. Gene expression profiles of LPC subsets. (A) Hierarchical clustering and heat map of LPC subsets was generated from about 15,000 protein-coding genes called detected in at least a subset, with >2-fold change in expression in at least 1 of the 6 possible pairwise comparisons. Euclidean distances are divided by the maximum to scale from 0 to 1. (B) Hierarchical clustering using sets of differentially expressed genes corresponding to the Gene Ontology biological functions of antigen processing and presentation (203 genes), phagocytosis (66 genes), monocyte differentiation (16 genes), macrophage differentiation (13 genes), or inflammatory response to antigenic stimulus (15 genes). Clustered heat maps were produced by using Euclidean distance. Euclidean distances are divided by the maximum to scale from 0 to 1.

DC differentiation process. CD14⁺ CD163^{low} cells showed higher levels of *IRF4* and *FLT3* expression than CD14⁺ CD163^{high} cells. Taken together, these results suggest that CD14⁺ CD163^{low} cells are closely related to CD14⁺ CD163^{high} cells and that the developmental pathway of CD14⁺ CD163^{low} cells have characteristics of both DC and macrophage development.

We next determined the *TLR* mRNA expression of these 4 subsets by quantitative real-time reverse transcription polymerase chain reaction (Figure 4A). CD14⁺ CD163^{low} cells expressed *TLR2* and *TLR4*, CD14⁺ CD163^{low}, and CD14⁺ CD163^{high} cells expressed *TLR5*, and CD14⁻

CD11c^{high} cells expressed *TLR9*. CD14⁻ CD11c^{low} cells did not express any *TLR*. We also examined the gene expressions of several inflammatory and anti-inflammatory mediators (Figure 4B). CD14⁺ CD163^{low} cells exhibited the highest expressions of *IL-6* and *IL-23p19*. CD14⁺ CD163^{high} cells uniquely expressed several anti-inflammatory markers, including *IL-10*, *HMOX1*, and *TGFB*. CD14⁻ CD11c^{high} cells expressed the highest level of *IFNG* among the 4 subsets. *IL-12p35* expression was observed only in CD14⁻ CD11c^{low} cells. Overall, the 4 subsets of intestinal LPCs showed differential expression patterns of inflammatory genes, and the CD14⁺

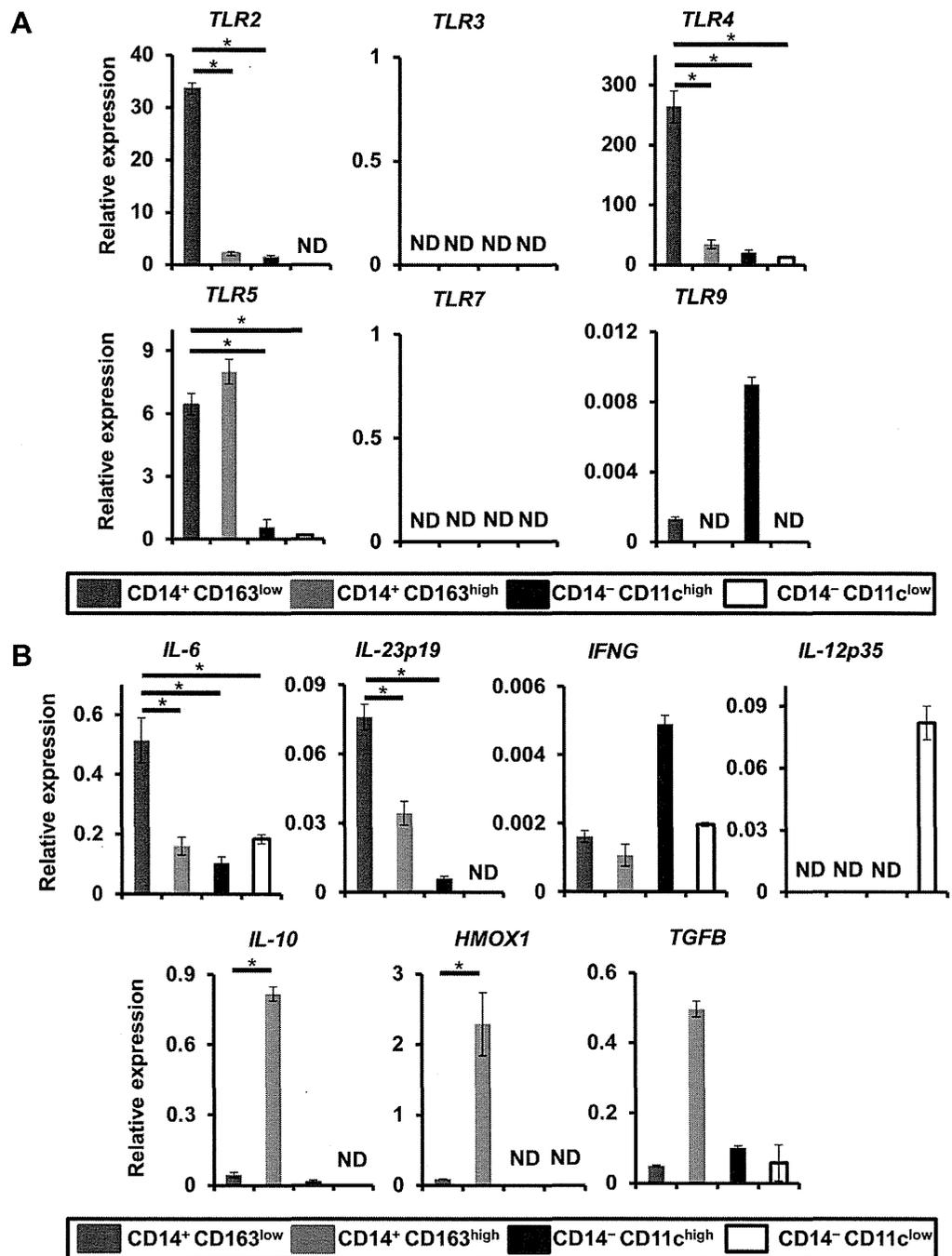


Figure 4. Various mRNA expression of LPC subsets. (A) Expression of *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, and *TLR9* in LPC subsets. (B) Expression of *IL-6*, *IL-23p19*, *IFNG*, *IL-12p35*, *IL-10*, *HMOX1*, and *TGFB* in LPC subsets. Results are mean ± SD from 5 independent experiments. ND, not detected. **P* < .01.

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CD163^{high} cells were unique in their expression of anti-inflammatory genes.

Cytokine Production of LPC Subsets in Response to TLR Stimulation

To analyze the functions of these 4 subsets, we used enzyme-linked immunosorbent assay to assess their cytokine productions in response to TLR stimulation (Figure 5). Based on the TLR mRNA expression patterns, we used the TLR2 ligand FSL-1, the TLR4 ligand LPS, and the TLR5 ligand flagellin. High production of inflammatory cytokines, such as IL-6, IL-1 β , and TNF- α , was observed in the CD14⁺ CD163^{low} cells, even without stimulation. CD14⁺ CD163^{low} cells produced increased amounts of IL-6, IL-1 β , and TNF- α in response to LPS, but not in response to FSL-1 or flagellin. CD14⁺ CD163^{high} cells showed constitutive high production of IL-10, and CD14⁻ CD11c^{low} cells produced IL-12p70 in response to LPS and flagellin. These findings revealed

differential patterns of cytokine production in the 4 LPC subsets, with the CD14⁺ CD163^{low} cells exhibiting LPS-induced inflammatory responses.

Induction of Th17 Cells by CD14⁺ CD163^{low} Cells

We next used the allogeneic mixed lymphocyte reaction technique to examine whether the 4 subsets induced Th1 or Th17 cell differentiation. After 5 days of co-culture, the IFN gamma and IL-17 productions of CD4⁺ T cells were analyzed by intracellular cytokine staining (Figure 6A). We used monocyte-derived DCs as a control, which induced IFN-gamma-producing CD4⁺ T cells (Th1 cells: 15.22% \pm 7.19%), but not IL-17-producing CD4⁺ T cells (Th17 cells: 0%). Naïve CD4⁺ T cells co-cultured with CD14⁺ CD163^{low} cells produced high amounts of IL-17 (0.52% \pm 0.17%) compared with those co-cultured with other subsets (Figure 6B), indicating that CD14⁺ CD163^{low} cells possess a high capacity

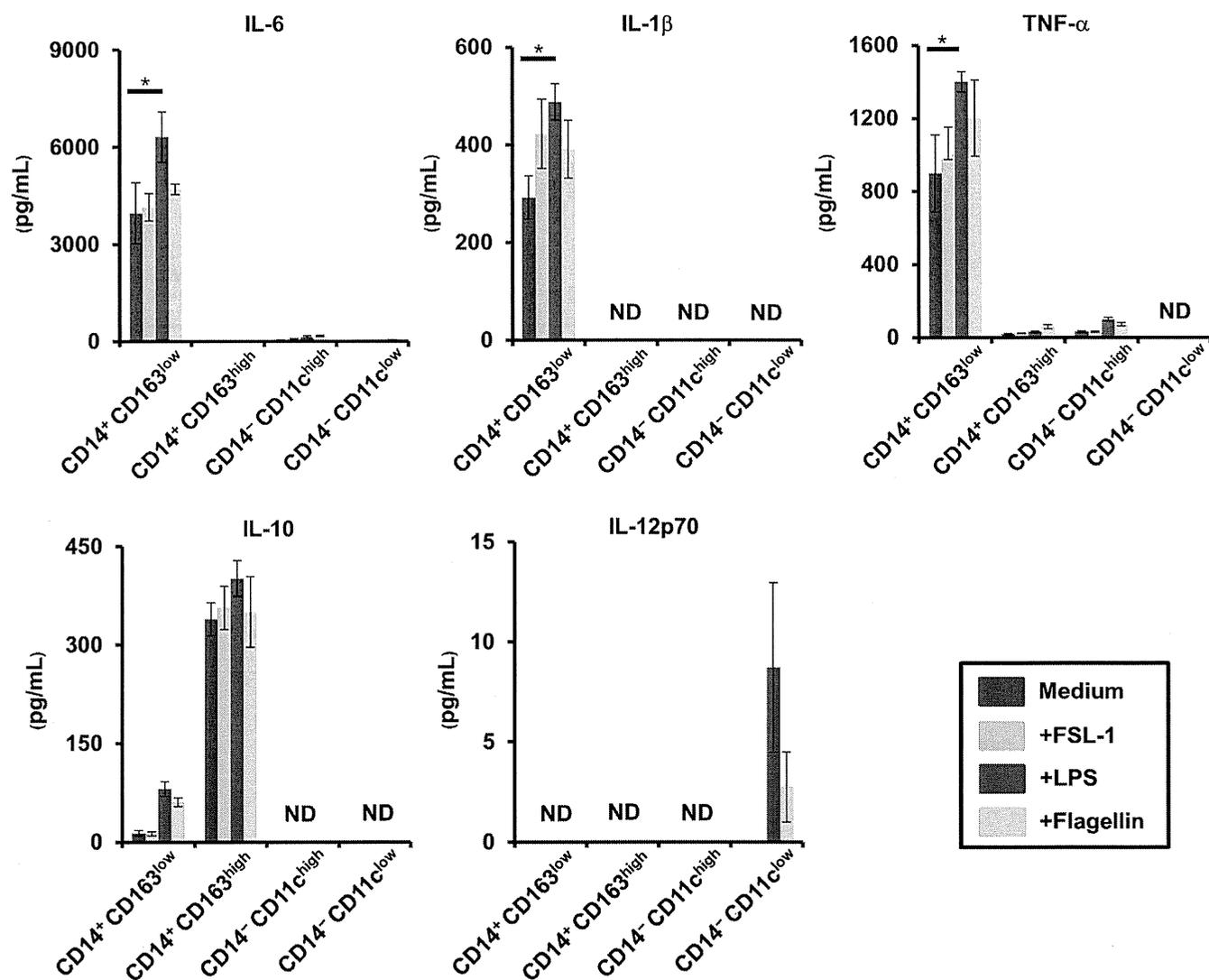


Figure 5. Cytokine production of LPC subsets. LPC subsets were analyzed for productions of IL-6, IL-1 β , TNF- α , IL-10, and IL-12p70 in response to FSL-1, LPS, or flagellin by enzyme-linked immunosorbent assay. Results are mean \pm SD from 5 independent experiments. ND, not detected. * P < .01.

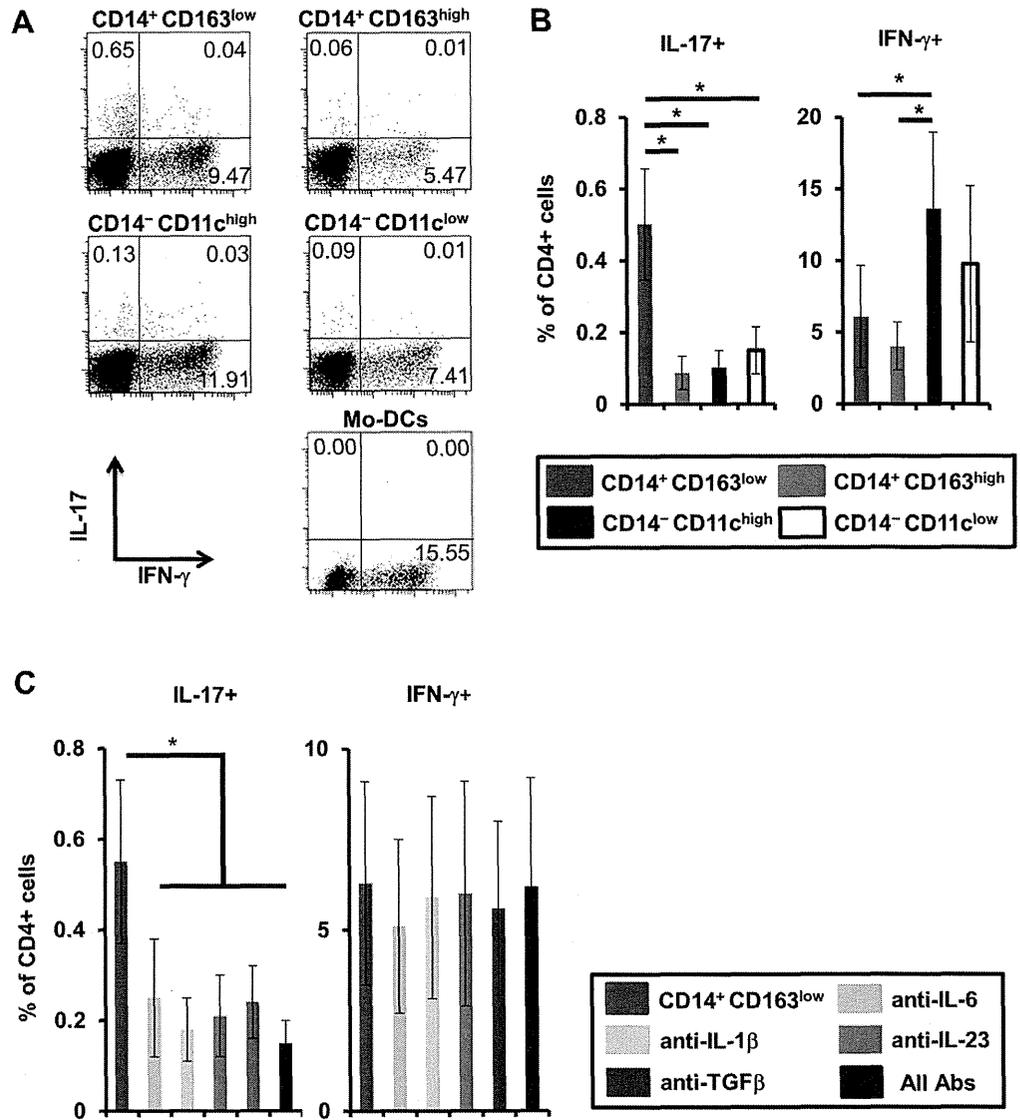


Figure 6. Functional properties of T-cell differentiation in LPC subsets. (A) Intracellular cytokine staining and flow cytometry performed to evaluate IFN gamma and IL-17 expression by naive T cells after 5 days of co-culture with allogeneic LPC subsets. Co-culture with monocyte-derived DCs was used as a control. Representative results of 8–14 independent experiments are shown. (B) Percentages of IL-17–producing cells and IFN gamma–producing cells among the CD4⁺ cells after co-culture are shown. Results are mean \pm SD from 7 independent experiments. **P* < .01. (C) Intracellular cytokine (IL-17 and IFN gamma) staining of naive T cells after co-culture with allogeneic CD14⁺ CD163^{low} subsets in the presence or absence of the indicated blocking antibodies. Percentages of IL-17–producing cells and IFN gamma–producing cells among the CD4⁺ cells are shown. Results are mean \pm SD from 5 independent experiments. **P* < .01.

to induce Th17 cells. Naïve CD4⁺ T cells co-cultured with CD14⁻ CD11c^{high} cells produced high amounts of IFN gamma (13.78% \pm 7.02%), indicating that CD14⁻ CD11c^{high} cells are DCs with a strong ability to induce Th1 cells. CD14⁻ CD11c^{low} cells induced moderate levels of Th1 cells (9.81% \pm 6.75%) and very few, if any, Th17 cells (0.15% \pm 0.06%). The CD14⁺ CD163^{high} cells showed the lowest ability to induce both Th1 and Th17 cells (4.07% \pm 1.61% and 0.08% \pm 0.04%, respectively). Overall, the 4 LPC subsets had differential activities for inducing Th1 and Th17 cells, with the CD14⁺ CD163^{low} cells highly inducing Th17 cells.

To analyze the impacts of IL-6, IL-1 β , IL-23, and TGF- β on CD14⁺ CD163^{low} cell-dependent induction of Th17 cells, we blocked the effect of these cytokines using neutralizing antibodies. Th17 cell differentiation by CD14⁺ CD163^{low} cells was severely impaired in the presence of the blocking antibodies, and Th1 cell differentiation was not affected (Figure 6C).

Functional Analysis of CD14⁺ CD163^{low} Cells in CD

Because CD14⁺ CD163^{low} cells facilitated Th17 cell differentiation, we next analyzed these cells in CD patients. Among HLA-DR^{high} Lin⁻ cells, CD14⁺ CD163^{low} cells were observed almost equally in the noninflamed region of CD (CDn; 28.1% \pm 7.1%) and the inflamed region of CD (CDi; 27.2% \pm 7.8%) compared with normal colon from colon cancer patients (NC; 25.5 \pm 9.4%) (Figure 7A and B). We also compared the cytokine gene expression of CD14⁺ CD163^{low} cells between NC, CDn, and CDi. CD14⁺ CD163^{low} cells in CDi expressed the highest levels of proinflammatory genes, such as *IL-6*, *IL-23p19*, *TNF*, and *IL-12p35*, as well as anti-inflammatory genes, such as *IL-10* and *TGF β* . CD14⁺ CD163^{low} cells in CDn also expressed higher *IL-6*, *IL-23p19*, and *TNF* and lower *IL-10* compared with those in NC. *TGF β* expression did not differ between CD14⁺ CD163^{low} cells in NC and CDn (Figure 7C). We next compared the abilities of

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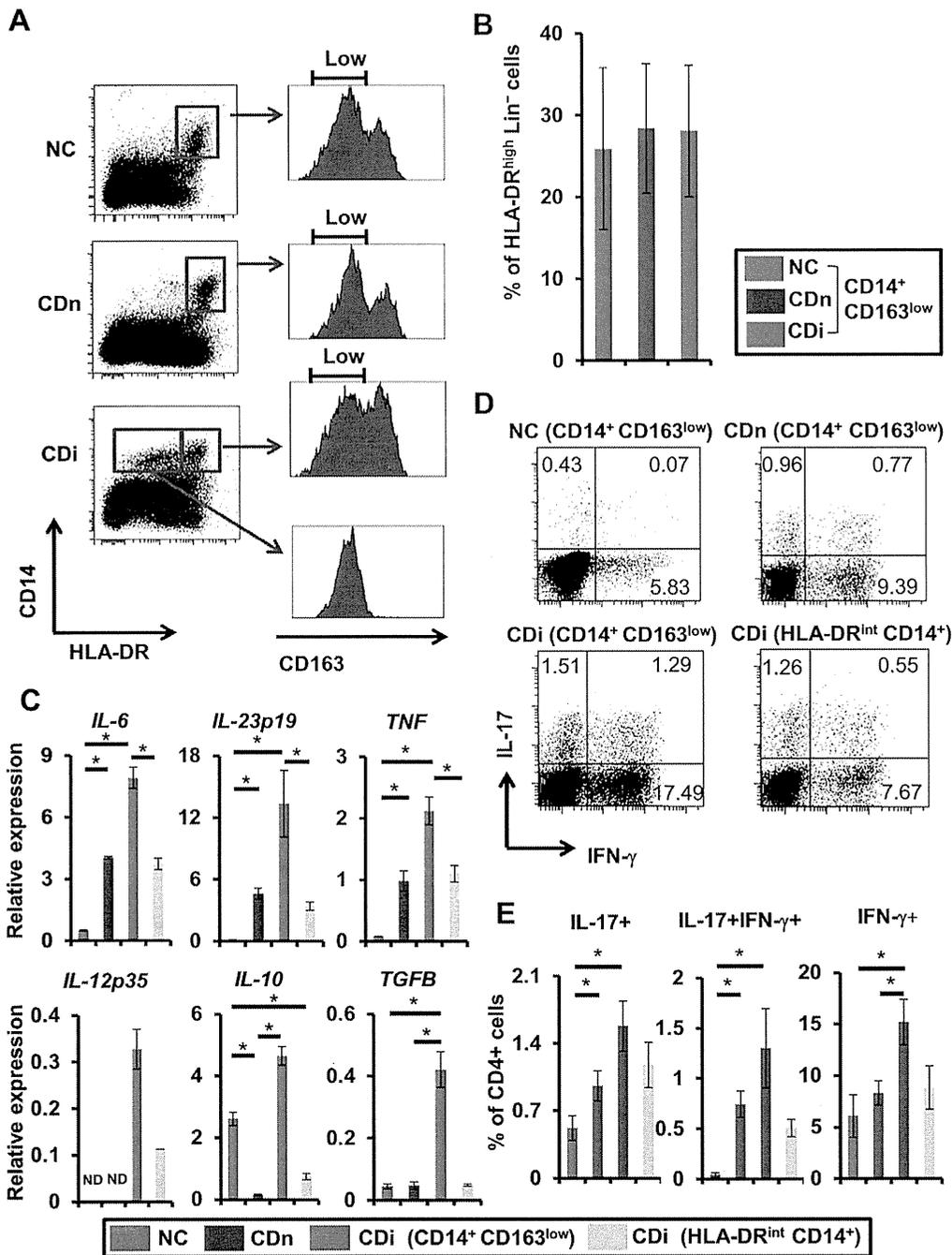


Figure 7. CD14⁺ CD163^{low} cells in Crohn's disease. (A) HLA-DR^{high} CD14⁺ cells were gated and analyzed for CD163 expression in Lin⁻ cells from NC (n = 30), CDn (n = 22), or CDi (n = 19). Representative results are shown. (B) Percentages of CD14⁺ CD163^{low} cells from NC, CDn, and CDi in HLA-DR^{high} Lin⁻ cells are shown. (C) Expressions of various cytokine mRNAs in CD14⁺ CD163^{low} cells from NC, CDn, and CDi, and in HLA-DR^{int} CD14⁺ cells from CDi. Results are mean ± SD from 5 independent experiments. ND, not detected. *P < .01. (D) Intracellular cytokine (IL-17 and IFN gamma) staining of naive T cells after co-culture with CD14⁺ CD163^{low} cells from NC, CDn, and CDi, and with HLA-DR^{int} CD14⁺ cells from CDi. Representative results of 5 independent experiments are shown. (E) Percentages of IL-17–producing cells, IL-17/IFN gamma double-producing cells, and IFN gamma–producing cells in CD4⁺ cells after co-culture. Results are mean ± SD from 5 independent experiments. *P < .01.

CD14⁺ CD163^{low} cells to induce Th1 and Th17 cell differentiation in NC, CDn, and CDi. As mentioned, CD14⁺ CD163^{low} cells in NC induced approximately 0.5% Th17 cells and few IFN gamma/IL-17 double-producing CD4⁺ T cells (Th1/17 cells: 0.03% ± 0.01%) (Figure 7D). Compared with those in NC, CD14⁺ CD163^{low} cells in CDn induced similar numbers of Th1 cells (8.31% ± 1.18%), but larger numbers of Th17 (0.96% ± 0.16%) and Th1/17 cells (0.74% ± 0.13%). CD14⁺ CD163^{low} cells in CDi induced the largest numbers of Th17 cells (1.59% ± 0.26%), Th1/17 cells (1.3% ± 0.39%), and Th1 cells (15.23% ± 2.21%) (Figure 7D and E).

Interestingly, we found a unique population of HLA-DR intermediate (int) CD14⁺ cells in CDi. This subset had expression patterns of surface molecules examined similar to CD14⁺ CD163^{low} cells (Supplementary Figure 3) and high expressions of *IL-6*, *IL-23p19*, and *TNF*, although these levels were lower than those of CD14⁺ CD163^{low} cells in CDi; these cells also exhibited low *IL-10* expression compared with those in NC (Figure 7C). HLA-DR^{int} CD14⁺ cells induced high levels of Th17 cells (1.18% ± 0.24%) and Th1/17 cells (0.51% ± 0.09%), and moderate levels of Th1 cells (8.83% ± 2.1%) (Figure 7D and E).

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Overall, we found that CD14⁺ CD163^{low} cells in CDn exhibited high inflammatory cytokine production and strongly induced Th17 cells, and that this ability was further enhanced in CD14⁺ CD163^{low} cells in CDi. In addition, a unique subset of HLA-DR^{int} CD14⁺ cells in CDi showed strong ability to produce inflammatory cytokines and to induce Th17 cells.

Discussion

In this study, we characterized differential functions of 4 LPC subsets in the human colon. Within the HLA-DR^{high} Lin⁻ population, CD14⁺ CD163^{low} cells mediated Th17 cell differentiation. In CD patients, this subset had strong capacities for cytokine production and Th17 cell induction. It was recently reported that the CD14^{high} population in human ileal mucosa includes HLA-DR^{low} CD209^{low} CD163^{low}, HLA-DR^{high} CD209^{low} CD163^{low}, and HLA-DR^{high} CD209^{high} CD163^{high} cells.²⁸ In terms of the surface molecule expression patterns in these subsets, HLA-DR^{high} CD209^{low} CD163^{low} cells and HLA-DR^{high} CD209^{high} CD163^{high} cells in that report correspond approximately to the CD14⁺ CD163^{low} cells and CD14⁺ CD163^{high} cells, respectively, in the present study.

In mice, subsets of intestinal myeloid cells are already well characterized and are distinguished using combinations of surface markers, such as CD11b, CD11c, CD103, or CX3CR1.^{16,21,24,29,35} In contrast, the appropriate surface markers for distinguishing myeloid cell subsets in humans remain unknown. In particular, CX3CR1 expression was hardly detected in human intestinal LPCs (unpublished results). In the present study, we found that LPCs in the HLA-DR^{high} Lin⁻ population could be divided into CD14⁺ CD163^{low}, CD14⁺ CD163^{high}, CD14⁻ CD11c^{high}, and CD14⁻ CD11c^{low} subsets.

Mouse intestinal subpopulations, such as CX3CR1^{int} and CX3CR1^{high} cells, are present selectively in the intestine, not in MLN and spleen, under steady state.^{25,29,36} Mouse CX3CR1^{int} cells, possessing macrophage-like and DC-like activities, highly express CD11c, major histocompatibility complex class II, and the co-stimulatory molecules CD86 and CD80. Consistent with murine CX3CR1^{int} cell expression of both macrophage (F4/80) and DC (DEC205) markers,^{29,37,38} here we found that CD14⁺ CD163^{low} cells expressed both macrophage- and DC-related markers. Gene expression profile analysis also indicates that CD14⁺ CD163^{low} cells express genes that mediate macrophage- and DC-related functions. In addition, CD14⁺ CD163^{low} cells exhibited macrophage-like morphology, like the case in intestinal CX3CR1^{int} cells in mice.^{17,28} These findings suggest that human CD14⁺ CD163^{low} cells might be equivalent to CX3CR1^{int} cells in mice, which are derived from blood monocytes. CD14⁺ CD163^{low} cells are monocyte-like lineage of cells that gain an ability of DC-related functions after migrating to the intestine. Similarly, a previous study suggests that CD14^{high} macrophages in normal and CD

ileum are similar to CX3CR1^{int} cells in mice.²⁸ We found that CD14⁺ CD163^{low} cells produced IL-1 β , IL-6, TNF- α , and IL-23p19, as well as the anti-inflammatory cytokine IL-10. A recent study in mice suggests that CX3CR1^{int} cells produce not only IL-6 and TNF- α , but also low levels of IL-10.²³ In terms of cytokine production patterns, CD14⁺ CD163^{low} cells in humans are similar to CX3CR1^{int} cells in mice, as found in the phenotypic analyses mentioned previously, further suggesting that CD14⁺ CD163^{low} cells are a human counterpart to mouse CX3CR1^{int} cells.

There have been several reports analyzing human DC subsets. CD1c⁺ cells in the intestinal lamina propria express higher levels of activation markers (CD40, CD83, CD86, HLA-DR) than those in blood and produce IL-23 at steady state.³⁹ The present study indicates that these CD1c⁺ LPCs are further subdivided into CD14⁺ CD163^{low}, CD14⁺ CD163^{high} and CD14⁻ CD11c^{high} cells, and that a main producer of IL-23 is CD14⁺ CD163^{low} cells. Lung CD1c⁺ DCs within the CD14⁻ CD11c⁺ population activate CD8⁺ T cells with induction of CD103 expression via TGF- β .⁴⁰ Given the similar expression pattern of surface molecules, the lung CD1c⁺ CD14⁻ CD11c⁺ DCs might correspond to the CD14⁻ CD11c^{high} subset in the present study. CD141^{high} DCs, a functional homolog of mouse CD103⁺ DCs, synthesize TNF- α , but not IL-12p70, in response to TLR3 stimulation.⁴¹ CD141^{high} cells are present within the CD14⁻ CD11c^{low} population, which produces IL-12p70 in response to LPS in the present study. In this regard, CD141⁻ population within the CD14⁻ CD11c^{low} population might be a IL-12p70 producer. Slan DCs expressing 6-sulfo LacNAc drive Th17/Th1 cell differentiation with production of IL-1 β , IL-23, IL-12, and IL-6 in psoriasis patients.⁴² The slan DCs do not express CD14 and CD163. CD14⁺ CD163^{low} cells expressed 6-sulfo LacNAc, but had no production of IL-12p70. Given the differential profiles of surface marker expression and cytokine production, CD14⁺ CD163^{low} cells are considered to be a distinct population from slan DCs observed in psoriasis patients.

Th17 cell-associated pro-inflammatory cytokines, such as IL-17 and IL-23, play key roles in several mouse autoimmune disease models and are thought to be involved in the pathogenesis of human immune disorders.⁴³⁻⁴⁷ Several reports have identified innate immune cells driving Th17 differentiation. In mice, CX3CR1^{int} cells in the intestinal LP induce Th17 cells,^{24,25} and TLR5⁺ lamina propria DCs induce Th17 cell differentiation.³⁵ IRF4-dependent CD11b⁺ DCs play a key role on Th17 induction.^{48,49} Human ovarian tumor cells and tumor-associated antigen-presenting cells reportedly mediate Th17 cell generation at tumor sites.⁵⁰ Additionally, a human Th17-inducing inflammatory DC subset derived from blood monocytes was recently identified in synovial fluid of rheumatoid arthritis patients and inflammatory tumor ascites.⁵¹ Human DCs inducing Th17 cell differentiation in the inflammatory condition have been identified; however, it remains unclear which human intestinal

LPC subsets are responsible for Th17 induction at steady state. In the present study, we showed that CD14⁺ CD163^{low} cells secreted IL-6 and IL-1 β and differentiated naïve T cells into Th17 cells. CD14⁺ CD163^{low} cells are the main DCs that induce Th17-mediated immunity in the intestine.

Several recent studies have shown that IBD patients exhibit massive infiltration of Th17 cells in inflamed gastrointestinal mucosa, as well as increased serum IL-17 levels.^{52,53} Polymorphisms in the gene encoding the IL-23 receptor are associated with CD.⁵ In CD patients, TREM-1⁺ macrophages are reportedly increased in intestinal mucosa and contribute to intestinal inflammation.⁵⁴ Spontaneous production of IL-1 β and TNF- α is observed in CD172a⁺ HLA-DR⁺ cells, which accumulate into MLN and the intestinal mucosa of CD patients.⁵⁵ In the present study, we showed that the proportion of CD14⁺ CD163^{low} cells was not altered between NC and CDn. However, Th17 cell differentiation by CD14⁺ CD163^{low} cells was significantly higher in CDn than in NC. Abnormal activity of CD14⁺ CD163^{low} cells is evident, even in the non-inflamed intestine of CD patients. In addition, the CD14⁺ CD163^{low} cells in CDi had extremely enhanced ability to induce Th17 cells. These results indicated that CD14⁺ CD163^{low} cells might be involved in the progression of CD via Th17 immunity. Compared with NC and CDn, CDi involved huge numbers of HLA-DR^{intc} CD14⁺ cells. In this regard, HLA-DR^{intc} CD14⁺ cells might migrate from the bloodstream to the intestine in inflammatory environments, as has been reported for mouse Ly6c^{high} CX3CR1^{intc} monocytes, which migrate into the intestine during intestinal inflammation.²⁸

In conclusion, here we have identified CD14⁺ CD163^{low} cells as a subset that produces IL-6 and IL-1 β , and can induce Th17 cells in human intestinal lamina propria. Further characterization of human CD14⁺ CD163^{low} cells showed them to be the putative equivalents of mouse CX3CR1^{intc} cells. Based on *TLR* expression and Th17 immunity, CD14⁺ CD163^{low} cells might play key roles in CD. Our findings suggest that CD14⁺ CD163^{low} cells could be a new diagnostic and therapeutic target for CD patients.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2013.08.049>.

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

Supplementary Material

Antibodies for Flow Cytometry

The following antibodies were used: phycoerythrin (PE)-conjugated anti-CD3 (HIT3a; BD Biosciences), anti-CD19 (SJ25C1; BD Biosciences), anti-CD20 (2H7; BD Biosciences), anti-CD56 (B159; BD Biosciences), fluorescein isothiocyanate-conjugated anti-CD14 (M ϕ P9; BD Biosciences), allophycocyanin-conjugated anti-CD11c (B-ly6; BD Biosciences), PE-Cy7-conjugated anti-HLA-DR (L243; Biolegend, San Diego, CA), peridinin chlorophyll protein complex-Cy5.5-conjugated anti-CD163 (GHI61; Biolegend), fluorescein isothiocyanate-conjugated anti-lineage (BD Biosciences), PE-Cy5-conjugated anti-CD86 (2331; BD Biosciences), anti-CD80 (L307.4; BD Biosciences), anti-CD83 (HB15e; BD Biosciences), anti-CD40 (5C3; BD Biosciences), PE-conjugated anti-CD64 (10.1; BD Biosciences), anti-CD103 (Ber-ACT8; Biolegend), anti-CD123 (7G3; BD Biosciences), anti-CD209 (DCN46; BD Biosciences), anti-CD11b (ICRF44; Biolegend), anti-CD141 (M80; Biolegend), anti-CD16 (3G8; BD Biosciences), anti-CD172a (SE5A5; Biolegend), anti-CD206 (15-2; Biolegend), anti-slcn (DD-1; Miltenyi Biotec, Bergisch Gladbach, Germany), Brilliant Violet-conjugated anti-CD1c (L161; Biolegend), peridinin chlorophyll protein complex-Cy5.5-conjugated anti-CD4 (SK3; BD Biosciences), PE-conjugated anti-IL17A (N49-653; BD Biosciences), allophycocyanin-conjugated anti-IL-4 (MP4-25D2; BD Biosciences), and fluorescein isothiocyanate-conjugated anti-IFN-gamma (B27; BD Biosciences).

Intracellular Cytokine Analysis

CD4⁺ T cells were stimulated for 4 hours in the presence of 5 μ M calcium ionosphere (Sigma), 50 ng/mL phorbol myristate acetate (Sigma), and GolgiStop (BD Biosciences). Intracellular cytokine staining was subsequently performed with fixation and permeabilization buffers.

Preparation of Monocyte-Derived DCs

Peripheral CD14⁺ monocytes were isolated from peripheral blood monocytes using CD14 microbeads (Miltenyi Biotec) following the manufacturer's instructions. For in vitro monocyte-derived DC differentiation, CD14⁺ monocytes were cultured for 5 days in RPMI

1640 containing 10% fetal bovine serum, with 100 ng/mL recombinant human granulocyte macrophage colony-stimulating factor (Primmune, Kobe, Japan) and 100 U/mL recombinant human IL-4 (Primmune).

Microarray Analysis

Gene expression analysis using SurePrint G3 Human Gene Expression v2 8x60K Microarray (G4845A) (Agilent Technologies) was performed as one color experiments by the Dragon Genomics Center (TaKaRa Bio Inc., Shiga, Japan). Total RNA from CD163^{high} (CD14⁺ CD163^{high}), CD163^{low} (CD14⁺ CD163^{low}), DN (CD14⁻ CD11c^{low}) and CD11c (CD14⁻ CD11c^{high}) cells, which were derived from the sorted HLA-DR^{high} Lin⁻ LPC subsets in noninvaded colonic tissue samples from individual colorectal cancer patients (n = 10) was extracted using RNeasy kit (Qiagen) following manufacturer's instructions. Twenty nanograms total RNA was reverse transcribed into double strand complementary DNAs by using an Ovation Pico WTA System V2 (NuGen) following manufacturer's protocol. Resulting complementary DNAs were subsequently used for in vitro transcription by DNA polymerase and labeled with cyanine-3 using a Genomic DNA Enzymatic Labeling Kit (Agilent Technologies) according to the manufacturer's protocol. After labeling, the labeled 2 μ g of each complementary DNA sample was then hybridized on SurePrint G3 Human Gene Expression v2 8x60K Microarray at 65°C for 17 hours with rotation in the dark. Hybridization was performed using a Gene Expression Hybridization Kit (Agilent Technologies) following the manufacturer's instructions. After washing in GE washing buffer, slides were scanned with an Agilent Microarray Scanner G2505C. Feature extraction software (version 10.10.1.1) was used to convert images into gene expression data. Raw data were imported into GeneSpring GX 11.0 (Agilent Technologies) for database management and quality control. This normalized data without the threshold raw signal and the global normalization algorithm was used for identifying differentially expressed genes. Raw data have been accepted in Gene Expression Omnibus, a public repository for microarray data, aimed at storing Minimum Information About Microarray Experiments Access to data concerning this study is found under Gene Expression Omnibus experiment accession number GSE49066.

ORIGINAL RESEARCH

Intraperitoneal injection of in vitro expanded V γ 9V δ 2 T cells together with zoledronate for the treatment of malignant ascites due to gastric cancer

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Keywords

Gastric cancer, malignant ascites, peritoneal dissemination, V γ 9V δ 2 T-cell, zoledronate

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Abstract

Malignant ascites caused by peritoneal dissemination of gastric cancer is chemotherapy-resistant and associated with poor prognosis. We conducted a pilot study to evaluate the safety of weekly intraperitoneal injections of in vitro expanded V γ 9V δ 2 T cells together with zoledronate for the treatment of such malignant ascites. Patient peripheral blood mononuclear cells were stimulated with zoledronate (5 μ mol/L) and interleukin-2 (1000 IU/mL). After 14 days culture, V γ 9V δ 2 T-cells were harvested and administered intraperitoneally in four weekly infusions. The day before T-cell injection, patients received zoledronate (1 mg) to sensitize their tumor cells to V γ 9V δ 2 T-cell recognition. Seven patients were enrolled in this study. The number of V γ 9V δ 2 T-cells in each injection ranged from 0.6 to 69.8 $\times 10^8$ (median 59.0 $\times 10^8$). There were no severe adverse events related to the therapy. Intraperitoneal injection of V γ 9V δ 2 T cells allows them access to the tumor cells in the peritoneal cavity. The number of tumor cells in the ascites was significantly reduced even after the first round of therapy and remained substantially lower over the course of treatment. IFN- γ was detected in the ascites on treatment. Computed tomography revealed a significant reduction in volume of ascites in two of seven patients. Thus, injection of these antitumor V γ 9V δ 2 T-cells can result in local control of malignant ascites in patients for whom no standard therapy apart from paracentesis is available. Adoptively transferred V γ 9V δ 2 T-cells do indeed recognize tumor cells and exert antitumor effector activity in vivo, when they access to the tumor cells.

Introduction

Human T cells carrying $\gamma\delta$ T-cell receptors account for 1–5% of peripheral blood T-cells [1, 2], the majority expressing the V γ 9V δ 2 receptor [3] that recognizes phosphoantigens [4, 5]. Recently, much attention has been paid to V γ 9V δ 2 T-cell-based cancer immunotherapy because these cells can secrete cytokines and exert potent cytotoxicity against a wide range of cancer cells [4]. Direct in vivo activation of V γ 9V δ 2 T cells by nitrogen-containing bisphosphonates (NBPs) in cancer patients [6, 7] as well as adoptive transfer of ex vivo expanded

V γ 9V δ 2 T cells have been investigated in several clinical trials [8, 9].

We have established a large-scale ex vivo expansion protocol for V γ 9V δ 2 T cells using zoledronate and interleukin-2 (IL-2) [10, 11]. We found that such cultured T cells retained cytokine secretion capacity and mediated cytotoxicity against a variety of cancer cell lines [10]. On the basis of these findings, we conducted a clinical study in patients with advanced or recurrent non-small cell lung cancer resistant to standard therapy [12, 13]. The adoptive transfer of autologous V γ 9V δ 2 T cells was well-tolerated. Some clinical benefit was observed in some

patients in whom V γ 9V δ 2 T-cells were able to survive and expand, and in whom plasma IFN- γ levels were elevated (but without statistical significance) [13]. However, it remained to be determined whether transferred V γ 9V δ 2 T cells infiltrated into the tumor, recognized tumor cells and exerted antitumor effector functions *in vivo*. Therefore, we conducted a clinical study of intraperitoneal (i.p.) V γ 9V δ 2 T-cell transfer therapy for the treatment of malignant ascites caused by peritoneal dissemination of gastric cancer.

Peritoneal dissemination is frequently observed in cases of advanced gastric cancer and occurs as a consequence of direct invasion and/or metastasis [14]. The presence of malignant ascites is a severe end-stage manifestation of the disease accompanied by several symptoms including nausea, appetite loss, abdominal tenderness and pain, fatigue and dyspnea, loss of proteins, and electrolyte disorders [15]. Recently, systemic chemotherapy with paclitaxel or S-1 plus cisplatin has improved the treatment of unresectable or recurrent gastric cancer [16–18]; i.p. chemotherapy [19] and immunotherapy, such as with catumaxomab [20], are also encouraging for the treatment of malignant ascites. However, to date, no options have been established for the management of peritoneal dissemination of ascites for patients' refractory to these treatments [15]. In such cases, only palliative therapies, such as paracentesis and diuretics, are available and the prognosis is extremely poor, with a median survival time of 3–4 months [21–23].

Therefore, we undertake adoptive cell therapy by injecting autologous V γ 9V δ 2 T cells expanded *ex vivo* with zoledronate and IL-2 into the peritoneal cavity of patients with malignant ascites. Direct injection of these cells into the peritoneal cavity allows them access to the tumor cells, bypassing the difficulties of transferred V γ 9V δ 2 T-cell recruitment into solid tumor. Because zoledronate leads to intracellular accumulation of isopentenyl pyrophosphate (IPP)/triphosphoric acid I-adenosine-50-yl ester 3-(3-methylbut-3-enyl) ester (ApppI) in tumor cells that are then recognized by V γ 9V δ 2 T cells by blocking the mevalonate pathway [24, 25], the injection of zoledronate preceded the infusion of expanded V γ 9V δ 2 T-cells (Fig. 1). Using this approach, we asked the crucial question of whether zoledronate-expanded V γ 9V δ 2 T-cells can recognize and kill tumor cells *in vivo*.

Materials and Methods

Study design and patient selection

This was a one-way, open-label, pilot study in patients with symptomatic malignant ascites secondary to gastric adenocarcinoma requiring symptomatic therapeutic

paracentesis. The primary objective of this study was to investigate the safety of i.p. injection of autologous V γ 9V δ 2 T-cells. The secondary objectives were to obtain immunological proof-of-concept of antitumor activity of V γ 9V δ 2 T-cells and to evaluate its clinical benefit. The research protocol was approved by the Ethical Committee of our institution (IRB-ID: P2010019-11Z), and registered at the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) (Unique trial number: UMIN000004130) on August 30, 2010. Written informed consent was obtained from each patient before they entered the study. The study was performed in accordance with the Declaration of Helsinki.

To be included, patients aged ≥ 20 years had to have histologically or cytologically proven gastric cancer, malignant ascites, an expected survival of at least 3 months, an Eastern Cooperative Oncology Group performance status (PS) of 0–2, normal kidney, liver, and bone marrow function, and be resistant to standard therapies. Patients positive for anti-adult T-cell leukemia-associated antigen or anti-human immunodeficiency virus antibody, other primary cancers, uncontrolled infection, active enterocolitis, severe heart disease, severe drug allergy, cryoglobulinemia, or autoimmune disease, were excluded from the study. Those receiving systemic steroid therapy or who were pregnant or lactating were also excluded. Small-scale 10-day V γ 9V δ 2 T-cell culture screening tests were first performed to establish the reactivity of each patient's V γ 9V δ 2 T cells prior to entry into the study. Proliferation was assessed as V γ 9V δ 2 T-cell count on day 10 of culture/V γ 9V δ 2 T-cell count at the initiation of culture. This value had to exceed 100 for the patient to be included in the study.

When the preliminary test fulfilled the criteria described above, leukapheresis was performed to isolate autologous peripheral blood mononuclear cells (PBMCs) and harvest plasma. Small-scale culture tests and leukapheresis were performed prior to chemotherapy; PBMCs and plasma were cryopreserved and stored before use. Patients received standard chemotherapy first; V γ 9V δ 2 T-cell culture was initiated immediately when patients became resistant to the chemotherapy (Table 1). After 14 days culture, V γ 9V δ 2 T-cells were harvested and administered i.p. to the patient. Four infusions were carried out weekly. The day before V γ 9V δ 2 T-cell injection, patients received 1 mg of zoledronate (Novartis, Basel, Switzerland) (Fig. 1A). To ensure safety, zoledronate was administered intravenously (i.v.) on day 0 and i.p. via a catheter on days 7, 14, and 21 (Fig. 1B). Before each zoledronate or V γ 9V δ 2 T-cell infusion and 1 day after cell infusion, blood (10 mL) was collected and ascites fluid (50 mL) was drained from the peritoneal cavity via the indwelling catheter for immunological analysis. When the

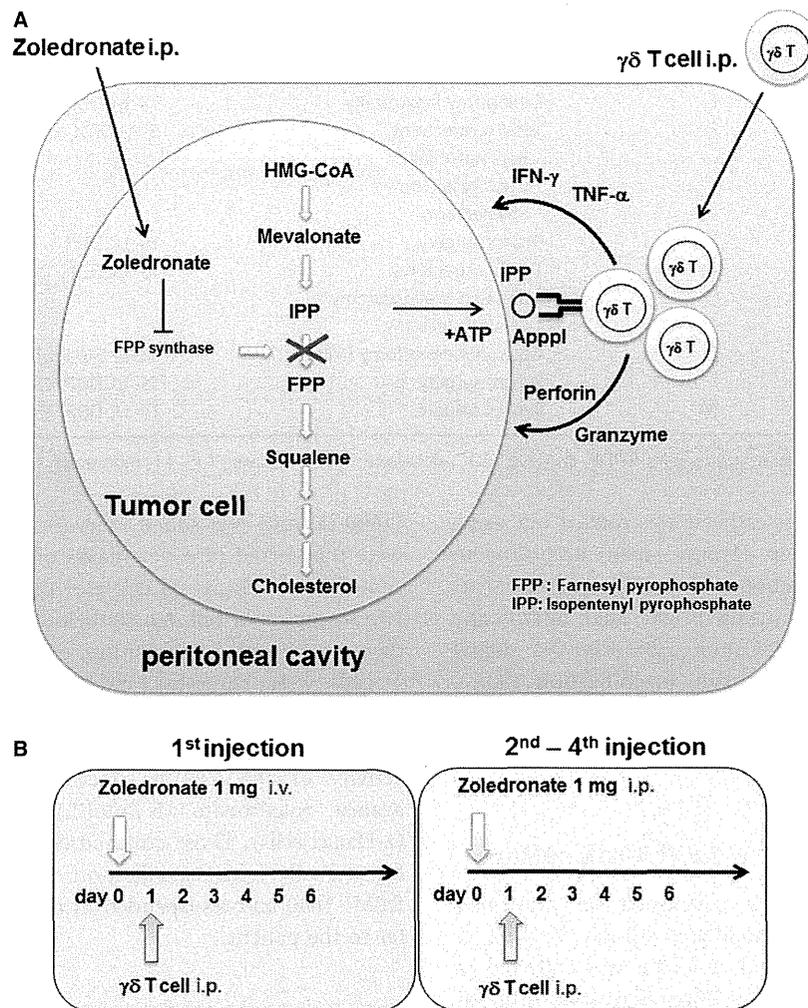


Figure 1. (A) Intraperitoneal administration of zoledronate sensitizes tumor cells to V γ 9V δ 2 T-cell recognition. Isopentenyl pyrophosphate (IPP) is an intermediate metabolite in the mevalonate–cholesterol pathway, recognized by V γ 9V δ 2 T-cells. Zoledronate inhibits farnesyl pyrophosphate (FPP) synthase, thereby causing the accumulation of IPP and triphosphoric acid l-adenosine-50-yl ester 3-(3-methylbut-3-enyl) ester (Apppl) in the tumor cells. When V γ 9V δ 2 T-cells are injected into the peritoneal cavity, they can recognize IPP and respond to tumor cells. (B) The study scheme of weekly i.p. V γ 9V δ 2 T-cell injection. In the first series of injections, zoledronate (1 mg) was administered i.v. on day 0, followed by i.p. V γ 9V δ 2 T-cell injection on day 1. Zoledronate was i.p. injected via a catheter from the second to fourth injection.

patient experienced clinical benefit without significant toxicity, additional V γ 9V δ 2 T-cell infusions were permitted.

Safety assessment, antitumor effects, and quality of life

Medical history, physical examination, PS, vital signs, chest X ray, electrocardiogram, and routine laboratory monitoring (including biochemistry, hematology, urinalysis, and tumor markers) were recorded at baseline. Thereafter, physical examination, PS, vital signs, and routine laboratory monitoring, as well as any adverse events, were assessed at each visit. Abdominal examination was

performed by the investigator to assess ascites signs, such as abdominal distension, dullness to percussion, shifting dullness, fluid thrill, and bulging flanks. Subjective symptoms related to ascites, such as anorexia, nausea, vomiting, abdominal pain, abdominal swelling, dyspnea, fatigue, and heartburn, were also recorded. Adverse events were graded according to National Cancer Institute-Common Terminology Criteria for Adverse Events version 4.0. Clinical responses were assessed by computed tomography performed at baseline and after the fourth infusion. To evaluate the antitumor effects of the treatment on peritoneal metastasis, the amount of malignant ascites and peritoneal cytology was also taken into account [26]. Cytology of ascites or peritoneal lavage fluid collected

Table 1. Summary of patients' background

Patient ID	Age	Gender	Surgery	Chemotherapy
2305	69	F	Exploratory laparotomy	TS-1/CDDP, TS-1/DOC, CPT-11/CDDP, UFT
2307	66	F	Total gastrectomy Roux-en-Y jejunostomy Cholecystectomy Splenectomy	5-FU/MTX, UFT, TS-1/CDDP, DOC
2319	58	F	Bypass surgery	TS-1/CDDP
2325	62	M	Total gastrectomy Roux-en-Y jejunostomy Cholecystectomy	TS-1/CDDP, TS-1/DOC
2334	39	F	Gastroduodenostomy (Billroth I)	DOC, 5-FU+MTX, UFT, TS-1/CDDP
2336	47	F	Bypass surgery	TS-1/CDDP, TS-1/PTX, CPT-11, PTX
2328	55	M	Bypass surgery	TS-1/CDDP, TS-1/DOC

TS-1, tegafur, gimeracil, and oteracil potassium; CDDP, cisplatin; DOC, docetaxel; PTX, paclitaxel; CPT-11, irinotecan; UFT, tegafur-uracil.

through a peritoneal access catheter was carried out using Diff-Quik staining (Sysmex, Hyogo, Japan) according to the manufacturer's instructions. The cellular components of ascites were evaluated using bright field microscopy (OLYMPUS BX41 with a Canon EOS Kiss X4 digital camera, OLYMPUS, Tokyo, Japan, magnification 200 \times). The safety assessment and clinical responses were determined by an independent data-monitoring committee after completion of the study.

Isolation of PBMC and V γ 9V δ 2 T-cell culture

V γ 9V δ 2 T-cell culture was performed as previously described [10, 27, 28]. For small-scale 10-day V γ 9V δ 2 T-cell culture tests, whole blood (7.5 mL) was collected in BD Vacutainer Cell Preparation Tubes with sodium heparin (Becton-Dickinson, Franklin Lakes, NJ) and directly centrifuged to isolate PBMC. To prepare V γ 9V δ 2 T-cells for the therapy, patients underwent leukapheresis to isolate PBMC and harvest plasma using Fresenius AS.TEC204 with C4Y white blood cell set (FRESENIUS KABI, Bad Homburg, Germany). Sodium citrate (ACD-A solution; TERUMO, Tokyo, Japan) was used as the anticoagulant. PBMC and plasma were isolated by density gradient centrifugation using Lymphoprep (AXIS-SHIELD Poc AS, Oslo, Norway). Leukapheresis yielded more than 1×10^9 PBMC and 100 mL plasma, both of which were cryopreserved until use. Depending on the data from small-scale 10-day V γ 9V δ 2 T-cell culture tests, the number of PBMCs to set up large-scale V γ 9V δ 2 T-cell cultures was estimated in order to obtain more than 1×10^9 V γ 9V δ 2 T-cells for each injection. PBMC were stimulated with 5 μ mol/L zoledronate in AlyS203 V γ 9V δ 2 medium (Cell Science and Technology Institute, Sendai, Japan) containing 1000 IU/mL human recombinant IL-2 (ProleukinTM; Chiron, Amsterdam, The Netherlands), and 10% autologous plasma. Fresh medium containing IL-2

(1000 IU/mL) was added every 2–3 days and the cultures were transferred into new flasks or culture bags as necessitated by the degree of cell growth. Cultures were split in two to maintain cell density below 1×10^6 /mL. Fourteen days after in vitro stimulation, ex vivo expanded V γ 9V δ 2 T cells were harvested and screened for their sterility (negative for endotoxin, bacteria, fungus, and mycoplasma contamination) and purity (>60%). The cytotoxic activity of the V γ 9V δ 2 T-cell cultures was evaluated against zoledronate (5 μ mol/L)-pretreated Daudi cells (z-Daudi cells). Those cultured cells which were approved for use after this examination were washed twice with RPMI-1640 and resuspended in normal saline to administer to the patient.

Flow cytometry, immunofluorescence, and cytology

The following monoclonal antibodies (mAbs) were used for flow cytometry: FITC-labeled anti-CD3, -TCRV γ 9, and -HLA-ABC, PE-labeled anti-TCR pan $\alpha\beta$, -NKG2D and mouse IgG₁ isotype, PC5-labeled anti-CD3, -CD8, -CD27, -CD56, and mouse IgG₁ isotype, ECD-labeled anti-CD4, -CD45, -CD45RA, and mouse IgG₁ isotype (Beckman Coulter, Immunotech, Marseille, France), PE-labeled anti-CD69 and -TCRV δ 2 (BD Bioscience Pharmingen, San Diego, CA), APC-labeled anti-EpCAM (Miltenyi Biotec, Bergisch Gladbach, Germany), and Pacific Blue-labeled anti-CD45 (BioLegend, San Diego, CA). Fixable Viability Dye eFluor 450 and 780 (eBioscience, San Diego, CA) were used to exclude dead cells. The cells were stained with antibodies and analyzed on a Cytomics FC 500 (Beckman Coulter) or Gallios (Beckman Coulter). The data were processed using Kaluza software (Beckman Coulter). Ascites cells were harvested by centrifugation and stained with mAbs described above. Tumor-cell load in ascites fluid (mL) was determined by

quantification of EpCAM⁺ tumor cells in ascites fluid/peritoneal lavage. The cells were also resuspended in PBS and examined by confocal microscopy, FV10i (Olympus, Tokyo, Japan).

Cytotoxicity assay

The cytotoxic activity of V γ 9V δ 2 T-cells was examined by flow cytometry as described previously, with minor modifications [11, 29]. Daudi cells were obtained from the RIKEN BRC Cell Bank (Ibaraki, Japan) and grown in RPMI-1640 medium (Wako, Osaka, Japan) containing 10% fetal calf serum, streptomycin (100 μ g/mL), and penicillin (100 U/mL). Daudi cells were preincubated with 5 μ mol/L zoledronate overnight, resuspended in Diluent C (Sigma, St Louis, MO), and incubated for 2 min with 2 μ mol/L freshly prepared PKH-26 (Sigma) at room temperature. Daudi cells without zoledronate pretreatment were also used as target cells. After extensive washing, target cells were cocultured with effector cells at the indicated E/T ratio. After 1.5 h of *in vitro* incubation, cells in 0.1 mL of binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2 mmol/L CaCl₂, pH 7.4) were incubated with 5 μ L of Annexin-V-FITC (BD Bioscience Pharmingen) and 20 μ g/mL of 7-aminoactinomycin D (7-AAD) (Sigma). Data analysis was performed first by gating on PKH-26-positive target cells followed by the analysis of Annexin-V-FITC- and 7-AAD-positive subpopulations. The percentage cytotoxicity in the PKH-26-gated cell population was calculated by subtracting the value of nonspecific Annexin-V-FITC- or 7-AAD-positive target cells, measured in appropriate controls without effector cells.

EpCAM⁺ cells were enriched from ascites fluid using CD326 (EpCAM) Tumor Cell Enrichment and Detection Kit, human (Miltenyi Biotec) and stained with PKH-26 (Sigma). The labeled cells were plated on 35-mm glass bottom dish (Matsunami Glass Inc., Osaka, Japan) in RPMI-1640 medium containing 10% fetal calf serum with 5 μ mol/L zoledronate. V γ 9V δ 2 T cells from same patient were expanded and labeled with 0.5 μ mol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oregon) according to the manufacturer's instructions. After EpCAM⁺ cells adhered to the bottom of the dish, CFSE-labeled V γ 9V δ 2 T-cells were added and cocultured under the confocal microscopy FV10i observation.

CD107 translocation assay

Daudi cells were preincubated overnight with indicated concentration of zoledronate (0, 1, 5, 10, 50, and 100 μ mol/L, Novartis, Basel, Switzerland) to accumulate IPP or 10 μ mol/L pravastatin sodium (Cayman Chemical,

Ann Arbor, MI) to inhibit IPP synthesis. Zoledronate- or pravastatin-treated Daudi cells were used as stimulator cells and incubated with the same number of V γ 9V δ 2 T-cells (5×10^5) for 2 h at 37°C in the presence of GolgiStop (BD bioscience), and anti-CD107a/b mAbs (BD bioscience). V γ 9V δ 2 T cells were also stimulated with 20 ng/mL PMA/2 μ g/mL ionomycin (both from Sigma-Aldrich). Cells were then washed in PBS supplemented with 2% FCS, 1 mmol/L EDTA, and stained for 30 min at 4°C with anti-CD3 and -TCRV γ 9. CD107 translocation was measured by flow cytometry.

Cytokine measurement

Cytokines, including IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12(p70), TNF- α , TNF- β , and IFN- γ in the plasma and ascites fluid were measured using FlowCytomix human Th1/Th2 11-plex kits (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions.

Results

Patients' characteristics

A total of seven patients underwent adoptive V γ 9V δ 2 T-cell immunotherapy. The characteristics of the enrolled patients are summarized in Table 1. There were two men and five women with a median age of 58 years (range, 39–69). All patients had previously received surgery and chemotherapy. They were resistant to current possible standard chemotherapy, followed by 4-week washout period; then adoptive V γ 9V δ 2 T-cell immunotherapy was reassessed. Malignant lesions were clinically restricted to the peritoneum in two cases (patients 2305 and 2319), while others had metastasized to lymph nodes, ovary, bladder, skin, and bones.

Adoptive transfer of V γ 9V δ 2 T cells

Aliquots of patients' PBMC were thawed and large-scale V γ 9V δ 2 T-cell expansion cultures initiated 14 days prior to each injection. The number and percentage of V γ 9V δ 2 T cells administered differed between individual patients and between separate infusions (Table 2). In most cases, the expansion of V γ 9V δ 2 T cells from cryopreserved PBMCs was successful and $>50 \times 10^8$ V γ 9V δ 2 T cells were prepared for injections. However, the number of harvested V γ 9V δ 2 T cells from the first large-scale culture was less than expected in patients 2305 and 2334, even though the number of cryopreserved PBMCs used was predetermined by the small-scale culture test. In these cases, more cryopreserved PBMCs were used for the next