



Unique CD14⁺ intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN- γ axis

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Intestinal macrophages play a central role in regulation of immune responses against commensal bacteria. In general, intestinal macrophages lack the expression of innate-immune receptor CD14 and do not produce proinflammatory cytokines against commensal bacteria. In this study, we identified what we believe to be a unique macrophage subset in human intestine. This subset expressed both macrophage (CD14, CD33, CD68) and DC markers (CD205, CD209) and produced larger amounts of proinflammatory cytokines, such as IL-23, TNF- α , and IL-6, than typical intestinal resident macrophages (CD14⁻CD33⁺ macrophages). In patients with Crohn disease (CD), the number of these CD14⁺ macrophages were significantly increased compared with normal control subjects. In addition to increased numbers of cells, these cells also produced larger amounts of IL-23 and TNF- α compared with those in normal controls or patients with ulcerative colitis. In addition, the CD14⁺ macrophages contributed to IFN- γ production rather than IL-17 production by lamina propria mononuclear cells (LPMCs) dependent on IL-23 and TNF- α . Furthermore, the IFN- γ produced by LPMCs triggered further abnormal macrophage differentiation with an IL-23–hyperproducing phenotype. Collectively, these data suggest that this IL-23/IFN- γ –positive feedback loop induced by abnormal intestinal macrophages contributes to the pathogenesis of chronic intestinal inflammation in patients with CD.

Introduction

Although the precise etiologies of inflammatory bowel diseases (IBDs), including Crohn disease (CD) and ulcerative colitis (UC), remain unclear, several reports have indicated that dysfunction of the mucosal immune system plays important roles in its pathogenesis (1, 2). It has been suggested that skewed Th1 immune responses, represented by IFN- γ , TNF- α , and IL-2, in the inflamed mucosa, play a pivotal role in the pathogenesis of CD (3, 4). Recently, it has become evident that abnormal innate-immune responses to commensal bacteria are responsible for the pathogenesis of CD (5).

Macrophages, the major population of tissue-resident mononuclear phagocytes, play key roles in bacterial recognition and elimination as well as in the polarization of innate and adaptive immunities. Besides these classical antibacterial immune roles, it has recently become evident that macrophages also play important roles in homeostasis maintenance, for example, inflammation dampening via the production of antiinflammatory cytokines such as IL-10 and TGF- β , debris scavenging, angiogenesis, and wound repair (6–8). Since the intestinal mucosa of the gut is always exposed to numerous commensal bacteria, it is considered that the gut may possess regulatory mechanisms preventing excessive inflammatory responses against commensal bacteria. Indeed, it was previously reported that human intestinal macrophages do not express innate response receptors (9, 10), and although these

cells retain their phagocytic and bacteriocidal functions, they do not produce proinflammatory cytokines in response to several inflammatory stimuli, including microbial components (11). In addition, a recent study also revealed that intestinal macrophage expressed several antiinflammatory molecules, including IL-10, and induced the differentiation of Foxp3⁺ Treg by a mechanism dependent on IL-10 and retinoic acid. Moreover, such intestinal macrophage suppresses the intestinal DC-derived Th1 and Th17 immunity dependent on or independent of Treg induction (12). Thus, recent studies have suggested that macrophages located in the intestinal mucosa play important roles in the maintenance of intestinal homeostasis by protecting the host from foreign pathogens and negatively regulating excess immune responses to commensals (13). On the other hand, disorders in such antiinflammatory functions of intestinal macrophages may cause abnormal immune responses to commensals and lead to the development of chronic intestinal inflammation, such as IBD (14–19). In fact, intestinal macrophages contributed to the development of Th1- and Th17-mediated chronic colitis via the production of both IL-12 and IL-23 in response to commensal bacteria in IL-10-deficient mice, an animal model of CD (20). In the present study, we focused on the functions of human intestinal macrophages to clarify their role in the pathogenesis of CD.

Results

Presence of unique proinflammatory CD14⁺ macrophages in the intestinal lamina propria. To identify the role of intestinal macrophage in the pathogenesis of human IBD, we first analyzed the macrophage population in the human intestine. Although many previous reports have indicated that CD14 is downregulated in intestinal

Nonstandard abbreviations used: CD, Crohn disease; CM, conditioned media; IBD, inflammatory bowel disease; LP, lamina propria; LPMC, LP mononuclear cell; PB, peripheral blood; UC, ulcerative colitis.

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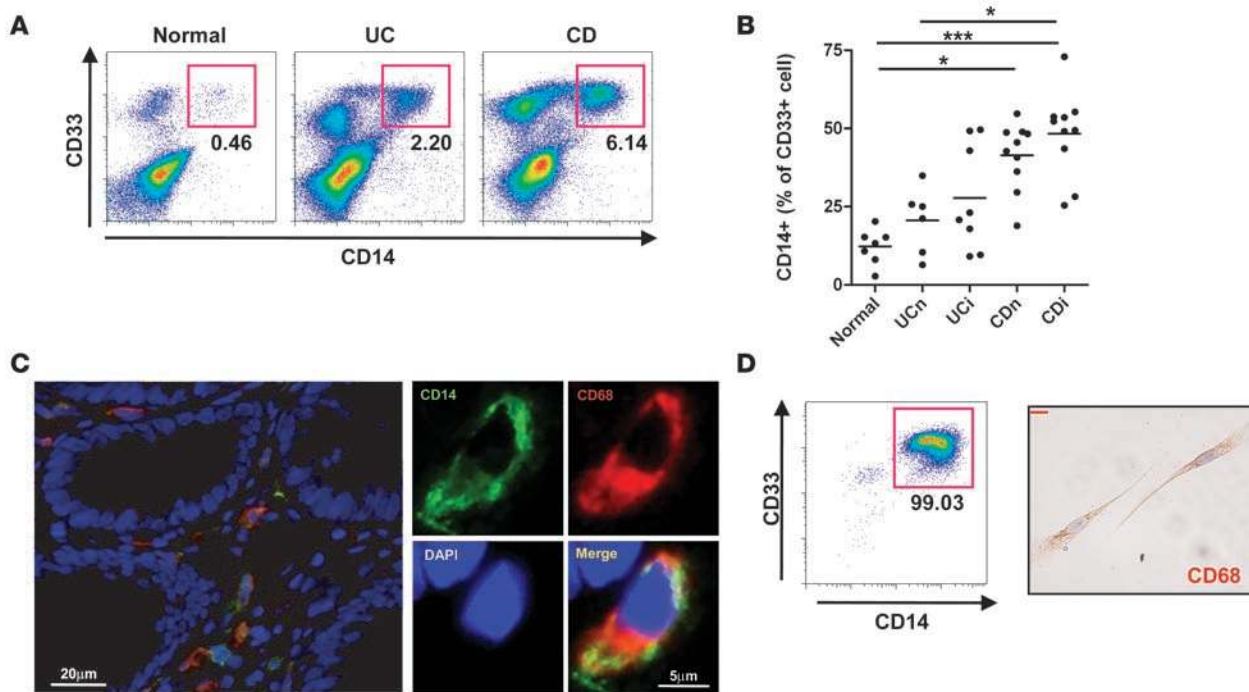


Figure 1

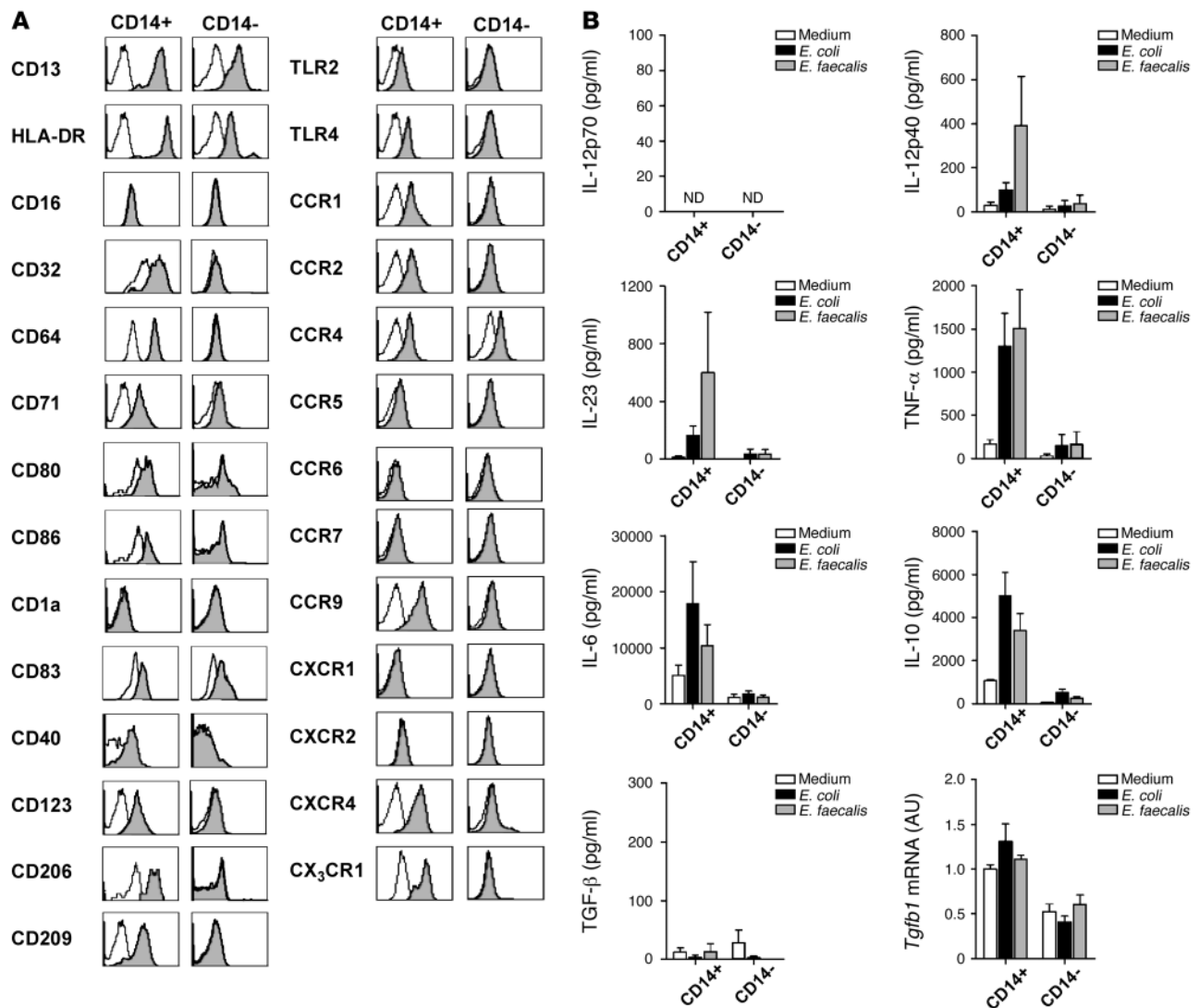
CD14-expressing cells were increased in the intestinal mucosa of patients with IBD. (A) LP macrophages of normal intestinal tissue specimens and of patients with active IBD were analyzed by FACS for CD14 and CD33 cell-surface expression. Numbers indicate the percentage of CD14⁺ cells present in human tissue. (B) Percentage of CD14⁺ intestinal macrophages among CD33⁺ cells from normal control subjects, noninflamed mucosa of patients with UC (UCn), inflamed mucosa of patients with UC (UCi), noninflamed mucosa of patients with CD (CDn), and inflamed mucosa of patients with CD (CDi). **P* < 0.05, ****P* < 0.001. (C) Fluorescence microscopy of human intestine from CD patients stained with anti-CD14 (green), anti-CD68 (red), and DAPI (blue). CD14⁺CD68⁺ macrophages were present in the intestinal LP. CD14⁺CD68⁺ macrophages were also observed. (D) Sorted CD14⁺CD33⁺ intestinal macrophages were analyzed for the expression of CD14 and CD33, and these cells were reseeded and analyzed for CD68. Numbers indicate the percentage of CD14⁺ cells per sorted cells. Scale bar: 20 μm.

macrophages (9, 11), a small number of CD14⁺ cells positive for the intestinal macrophage marker CD33 were present in normal human intestine (Figure 1A). Moreover, these CD14⁺ cells were significantly increased in the patients with IBDs, especially in the patient with CD (Figure 1, A and B). On the other hand, there were no significant differences in the number of CD14⁺ cells between noninflamed and inflamed mucosa in individual patients (Figure 1B). To clarify whether these cells were a new subset of intestinal macrophages or newly recruited monocytes, morphology and macrophage marker expression were assessed on both tissue localized and purified CD14⁺ cells from the intestinal mucosa of patients with CD. Immunohistochemical analysis revealed that the CD14⁺ cells also expressed macrophage marker CD68 (Figure 1C), and the purified CD14⁺ cells were adherent and showed spindle-like typical macrophage morphology (Figure 1D).

Next, we analyzed the phenotype of these CD14⁺CD33⁺ cells. These CD14⁺CD33⁺ cells expressed CD13, HLA-DR, Fc receptors (CD32, CD64), transferrin receptor (CD71), mannose receptor (CD206), and IL-3 receptor (CD123) but did not express the CD16 or DC markers CD1a, CD1c, and DC-LAMP (CD208). However, this subset expressed some DC markers such as DEC-205 (CD205) and DC-SIGN (CD209) and the costimulatory molecules CD80, CD86, CD40, and TLRs (Figure 2A and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI34610DS1). In addition, CD14⁺CD33⁺ cells expressed several

chemokine receptors such as CCR1, -2, -4, -9, CXCR4, and CX₃CR1 (Figure 2A). In terms of the expression of surface markers, there were no marked differences among the CD14⁺CD33⁺ cells from normal control subjects and 2 types of IBD patients, at least among those subjects we tested (data not shown). On the other hand, consistent with previous reports, the CD14⁺CD33⁺ subset did not express most macrophage and DC markers (Figure 2A). Thus, these CD14⁺ cells are thought to be a unique macrophage subset in intestine, which has both macrophage and DC markers.

Because this CD14⁺ unique myeloid cell subset was increased in IBD patients, there is a possibility that these cells contributed to the intestinal inflammation. To clarify this, CD14⁺CD33⁺ and CD14⁺CD33⁻ cells were isolated from the lamina propria (LP) of CD patients, and the cytokine productive function after stimulation with commensal bacteria *Escherichia coli* and *Enterococcus faecalis* was assessed. CD14⁺CD33⁺ cells produced larger amounts of pro-inflammatory cytokines IL-12/IL-23p40, IL-23, TNF-α, and IL-6, but not IL-12p70, in response to bacterial stimuli. In contrast, CD14⁺CD33⁻ cells produced only limited amounts of these pro-inflammatory cytokines (Figure 2B). Even in the production of anti-inflammatory cytokines, CD14⁺CD33⁺ cells produced a larger amount of IL-10 than CD14⁺CD33⁻ cells, and both subsets could produce only limited amount of TGF-β. These results suggest that CD14⁺CD33⁺ intestinal macrophage subsets act as a proinflammatory subset in the pathogenesis of human IBD.

**Figure 2**

CD14⁺CD33⁺ cells in the human intestinal LP revealed unique phenotypes and produced larger amounts of proinflammatory cytokines than CD14⁻CD33⁺ intestinal macrophages. (A) Flow cytometry for the surface phenotypes of intestinal CD14⁺CD33⁺ and CD14⁻CD33⁺ cells. The shaded histogram shows the profiles of the indicated Ab staining and the open histogram shows staining with isotype controls. The data shown are representative of 5 independent experiments on normal control subjects or noninflamed mucosa of CD patients. (B) Proinflammatory cytokine production by *E. coli* or *E. faecalis* heat-killed antigen-stimulated CD14⁺CD33⁺ or CD14⁻CD33⁺ intestinal macrophages from the inflamed mucosa of CD patients. Control stimulation used is cell culture medium alone. N.D., not detected. Data represent mean \pm SEM from at least 3 independent experiments.

CD14⁺ intestinal macrophages in patients with CD produce a large amount of IL-23 and TNF- α in response to commensal bacteria. Since the number of CD14⁺ macrophages was significantly increased in intestinal tissues from IBD patients, especially in CD patients, we next examined whether these CD14⁺ cells in CD patients were only increased in numbers or exhibited functional differences compared with normal controls and UC patients. We first analyzed the expression of IL-12-related genes by isolated CD14⁺ macrophages from normal control subjects or patients with IBD. As a result, the levels of IL-12/IL-23p40, IL-23p19, and IL-27p28, but not IL-12p35, were significantly increased in CD14⁺ macrophages from CD patients compared with those from normal individuals and patients with UC (Figure 3A). Moreover, in response to commensal bacteria stimulation, CD14⁺ intestinal macrophages from

CD patients produced abundant levels of IL-23 and TNF- α , but not IL-12p70, compared with those from normal individuals and UC patients (Figure 3, B and C). CD14⁺ macrophages from the CD patient also produced IL-6, but the level was lower than that in UC patients (Figure 3B). Thus, the CD14⁺ intestinal macrophages in CD patients are distinct from those in normal and UC patients, being hyperproducers of IL-23 but not IL-12.

CD14⁺ intestinal macrophages in CD patients are a main source of commensal-induced IL-23 by LPMCs. It has become clear that CD14⁺ macrophages from patients with CD produced abundant levels of IL-23. Next, we tried to identify the role of these IL-23-producing CD14⁺ intestinal macrophages in intestinal inflammation. To clarify the role of these macrophage subsets in the intestinal inflammation, whole LPMCs in a mixed culture system were used for evaluation

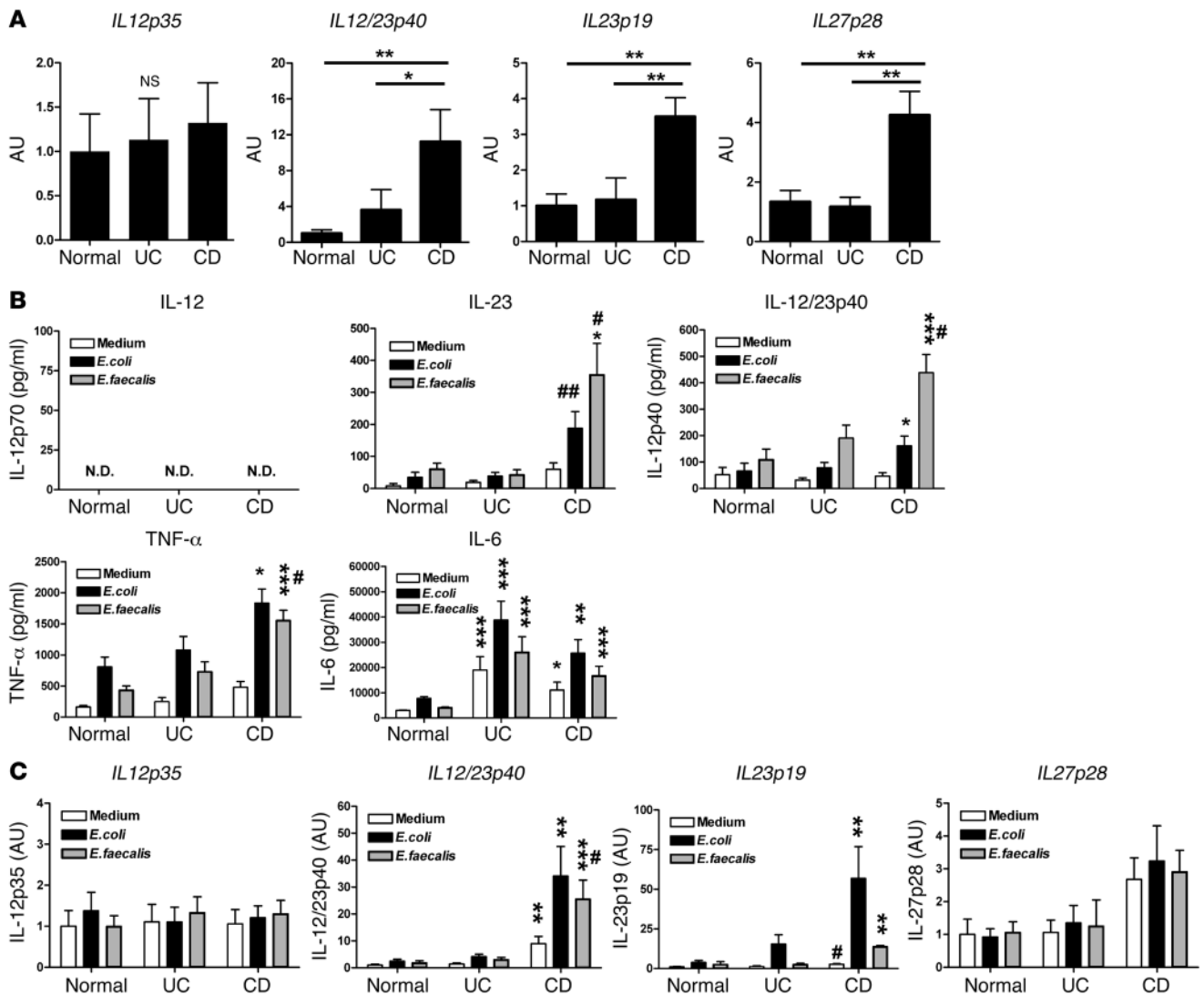


Figure 3
 CD14⁺ intestinal macrophages from patients with CD produced abundant levels of IL-23 and TNF- α in response to commensal bacteria antigen stimulation. (A) Quantitative RT-PCR of basal mRNA expression levels in isolated CD14⁺ macrophages from normal and IBD patients. (B) Cytokine production by CD14⁺ intestinal macrophages stimulated by heat-killed *E. coli* or *E. faecalis* (1×10^8 CFU/ml) for 24 hours. (C) Quantitative RT-PCR of IL-12-related cytokines by CD14⁺ intestinal macrophages stimulated by heat-killed *E. coli* or *E. faecalis* (1×10^8 CFU/ml) for 24 hours. All CD14⁺ macrophages used in this experiment are from inflamed mucosa of IBD patients and noninflamed mucosa of normal control subjects. Data are expressed as mean \pm SEM of individual patients or controls (normal, $n = 9$; UC, $n = 9$; CD, $n = 13$). Statistical analysis was performed using Kruskal-Wallis 1-way ANOVA and the Tukey-Kramer test for multiple comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus normal control subjects; # $P < 0.05$, ## $P < 0.01$ versus UC.

of macrophage-lymphocyte interaction. Intestinal LPMCs were isolated from inflamed mucosa of UC and CD patients or normal colon, respectively, and cultured with or without commensal bacteria stimulation. As shown in Figure 4A, commensal bacteria *E. faecalis* strongly induced the production of IL-23, TNF- α , and IL-6, but not IL-12p70, from LPMCs from patients with CD. Interestingly, TNF- α , which strongly contributes to the intestinal inflammation of CD, was constitutively produced to a greater extent by LPMCs from CD patients. In addition, not only pro-inflammatory cytokines TNF- α and IL-6, which are mainly produced by innate-immune cells, but T cell-related cytokines, such as IFN- γ , were also significantly elevated in LPMCs from CD patients

both before and after bacteria stimulation (Figure 4A). Surprisingly, although the Th17-related cytokine IL-23 was significantly induced in LPMCs from patients with CD, IL-17 production was not induced by LPMCs even after bacteria stimulation. We further checked the mRNA transcription promoted by commensal stimulation. Consistent with the results of protein secretion, commensal bacteria significantly upregulated the expression of *IL12/IL23p40*, *IL23p19*, and *IFNG* mRNA; however, stimulation did not induce *IL12p35* and *IL17* mRNA in LPMCs from CD patients (Figure 4B). In contrast to *IL17*, the other Th17-related cytokines *IL22* and *CCL20* were significantly induced by LPMCs from CD patients after commensal stimulation (Figure 4B). To further confirm the

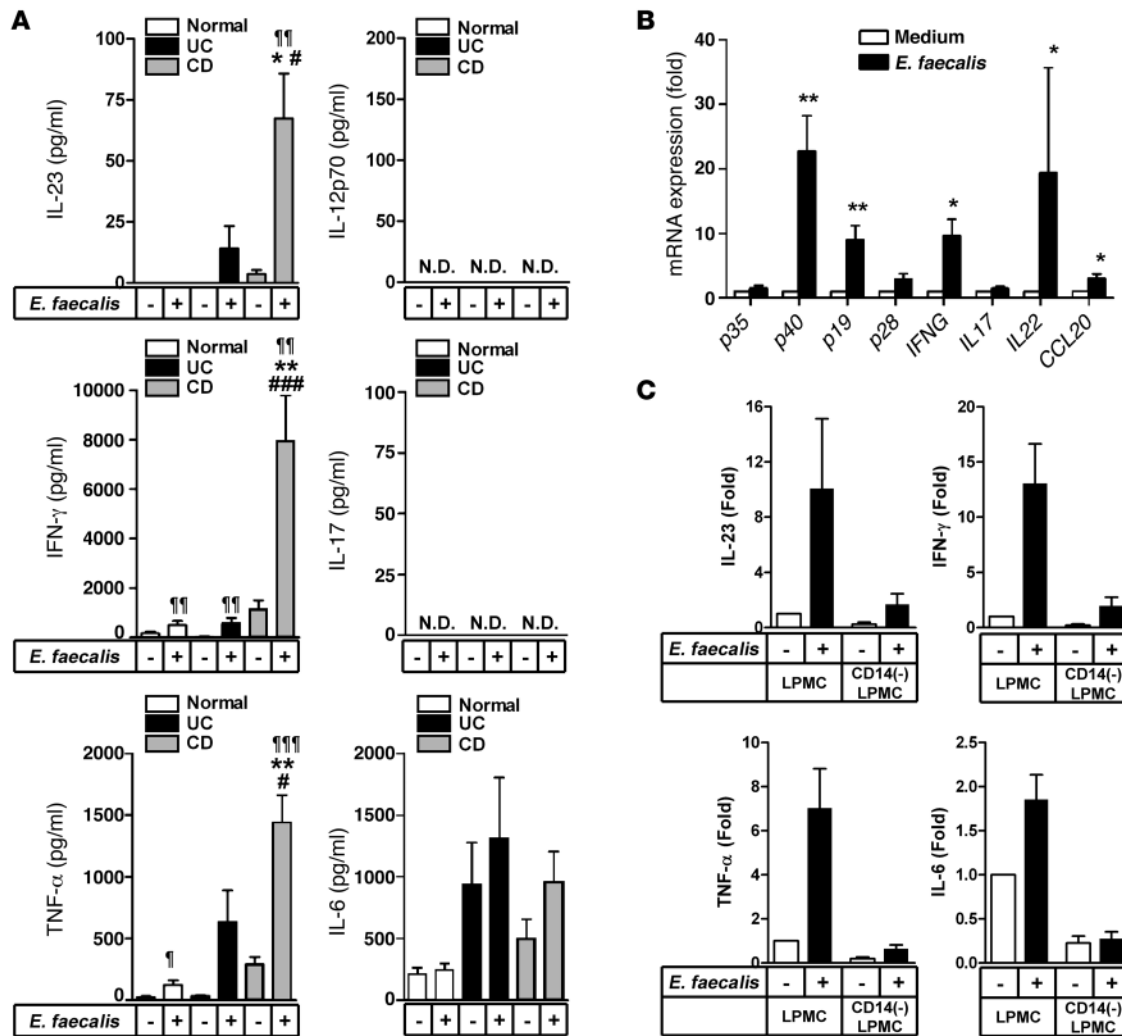


Figure 4

Commensal bacteria induced IFN- γ but not IL-17 by LPMCs via IL-23 produced by CD14⁺ intestinal macrophages. (A) Th1 and Th17 cytokine production by LPMCs (1×10^6 cells/ml) treated with heat-killed *E. faecalis* antigen for 24 hours. Data represent mean \pm SEM (normal control, $n = 5$; UC, $n = 8$; CD, $n = 8$). LPMCs of IBD patients were isolated from inflamed mucosa both in UC and CD. Statistical analysis was performed using Kruskal-Wallis 1-way ANOVA and the Tukey-Kramer test for multiple comparisons. * $P < 0.05$, ** $P < 0.01$ versus normal control; # $P < 0.05$, ### $P < 0.001$ versus UC; ¶ $P < 0.05$, ¶¶ $P < 0.01$, ¶¶¶ $P < 0.001$ versus unstimulated controls. (B) Th1- and Th17-related cytokine mRNA expression after commensal bacteria stimulation (12 hours) by LPMCs from inflamed mucosa of patients with CD. Data represent mean \pm SEM of at least 6 individuals. p35, *IL12p35*; p40, *IL12/IL23p40*; p19, *IL23p19*; p28, *IL27p28*. Statistical analysis was performed using Wilcoxon test. * $P < 0.05$, ** $P < 0.01$. (C) Basal and commensal-induced cytokine production by LPMCs or CD14⁺ cells depleted LPMCs from inflamed mucosa of patients with CD. Data represent mean \pm SEM from 6 independent experiments.

CD14⁺ cells as the major source of IL-23, the CD14⁺ cells were depleted from LPMCs, and then CD14⁻ LPMCs were stimulated with *E. faecalis*. As expected, the production of proinflammatory cytokines IL-23, TNF- α , and IL-6 was dramatically reduced in CD14⁻ LPMCs compared with whole LPMCs (Figure 4C). Because this reduction of cytokines by LPMCs was not due to the nonspecific cell damage caused by CD14⁺ cell depletion (Supplemental Figure 4), it seems likely that these phenomena were caused by the lack of CD14⁺ macrophages from LPMCs. Moreover, IFN- γ production was also dramatically decreased in the CD14⁻ LPMCs (Figure 4C). Because CD4⁺ T cells are a major source of IFN- γ in the inflamed mucosa of CD patients (3, 4) and CD14⁺ cells could not produce IFN- γ (data not shown), IFN- γ was thought to be pro-

duced by T cells as a result of the interaction with bacteria-activated innate-immune cells, such as macrophages, in the LPMCs.

Collectively, these results suggest that CD14⁺ macrophages were the major source of IL-23 in the LP of CD patients and might have contributed to the promotion of IFN- γ production from LP T cells.

CD14⁺ intestinal macrophages in CD patients promote IFN- γ , rather than IL-17, by LP T cells via an IL-23- and TNF- α -dependent manner. To unravel the role of IL-23, which is produced by CD14⁺ intestinal macrophages, we examined the effect of IL-23 on the intestinal inflammatory response using LPMC cultures. Consistent with the results of commensal bacteria stimulation, recombinant IL-23 (rIL-23) significantly induced IFN- γ , but not IL-17, production by LPMCs. Indeed, the amounts of IFN- γ were dramatically higher in

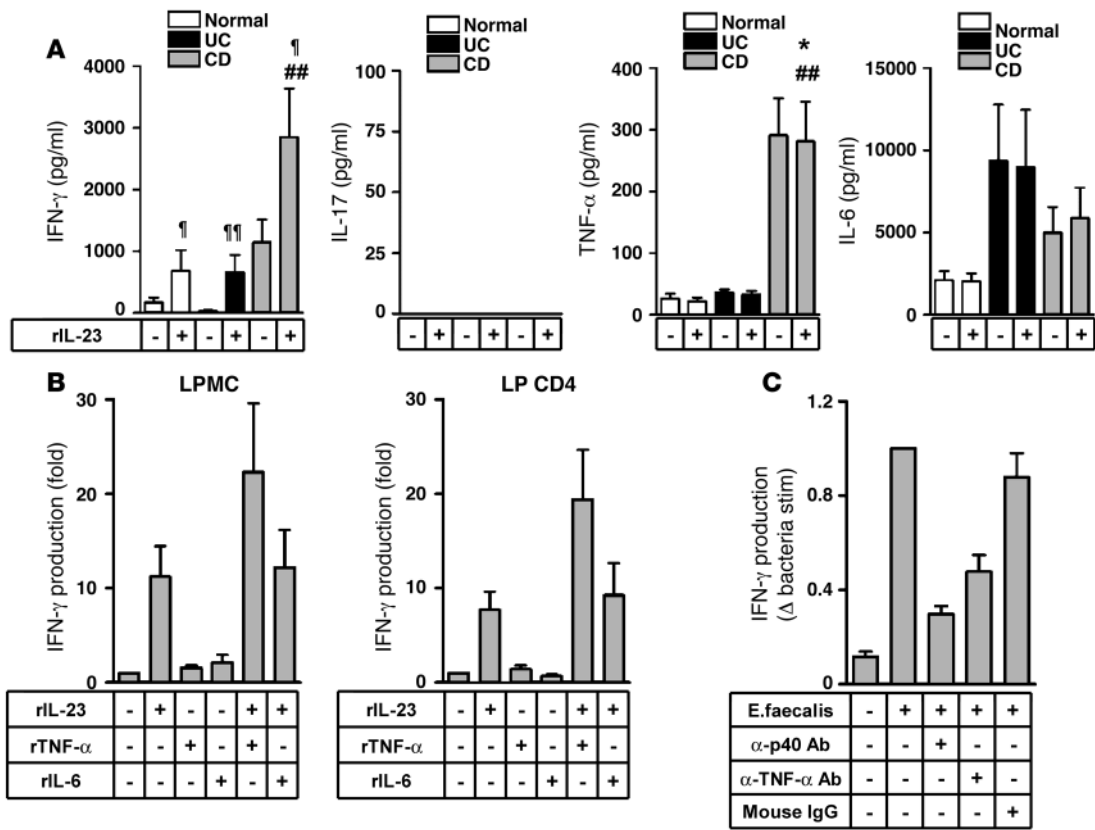


Figure 5

Intestinal macrophage-derived IL-23 induced IFN- γ production by LPMCs, and LP CD4⁺ T cells synergize with TNF- α in patients with CD. (A) IL-23–induced proinflammatory cytokine production by LPMCs from normal control subject or inflamed mucosa of patients with IBD. Data represent mean \pm SEM (normal control, $n = 5$; UC, $n = 8$; CD, $n = 8$). * $P < 0.05$ versus normal control; ## $P < 0.01$ versus UC; ¶ $P < 0.05$, ¶¶ $P < 0.01$ versus unstimulated controls. (B) Synergistic effect of TNF- α and IL-6 on the IL-23–induced IFN- γ production by LPMCs and LP CD4⁺ T cells from inflamed mucosa of patients with CD. Data represent mean \pm SEM from 4 individuals. (C) Analysis of the suppressive effect of anti-p40 or anti-TNF- α Abs on the commensal bacteria-induced IFN- γ production by LPMCs from inflamed mucosa of CD patients. α -p40 Ab, α -IL-12/IL-23p40 Ab. Data represent mean \pm SEM from at least 4 individuals. Statistical analysis was performed using Kruskal-Wallis 1-way ANOVA and the Tukey-Kramer test for multiple comparisons.

patients with CD than in normal control subjects or patients with UC (Figure 5A). While rIL-23 significantly promoted the production of IFN- γ by LPMCs, the amount of commensal-promoted IFN- γ was 2-fold larger than that of rIL-23–promoted IFN- γ , especially in CD patients (Figure 4A and Figure 5A). To explain this difference, we focused on the possibility that other proinflammatory cytokines exhibit synergistic effects with IL-23 on IFN- γ induction by LPMCs. Commensal bacteria stimulation induced not only IL-23 but also TNF- α and IL-6, while IL-23 alone did not induce such proinflammatory cytokines. Hence, there is a possibility that IL-23 and TNF- α or IL-6 can act synergistically on IFN- γ induction. As shown in Figure 5B, both TNF- α and IL-6 did not induce IFN- γ production by LPMCs and LP T cells. However, TNF- α , but not IL-6, synergistically induced IFN- γ from LPMCs and LP CD4⁺ T cells with IL-23. Indeed, commensal bacteria-induced IFN- γ by LPMCs was suppressed by neutralizing IL-12/IL-23p40 and TNF- α (Figure 5C). Thus, bacteria-induced TNF- α may act in cooperation with IL-23 on IFN- γ induction in the intestinal inflammatory site in CD patients. Collectively, CD14⁺ macrophages are a major producer of IL-23 and TNF- α in the intestinal LP of CD patients. Such IL-23 and TNF- α synergistically induce the production of IFN- γ by LP T cells.

The intestinal inflammatory microenvironment in CD patients promotes abnormal differentiation of intestinal macrophage. As we have demonstrated so far, an abnormal macrophage subset might contribute to the pathogenesis of intestinal inflammation of CD patients via IL-23 overproduction. In the next part of this study, we tried to identify how such abnormal intestinal macrophage differentiation is triggered in CD patients. We hypothesized that local inflammatory microenvironments in CD patients might cause abnormal macrophage differentiation. To examine this hypothesis, conditioned media (CM) were prepared from whole-cell cultures of intestinal LPMCs from normal subjects and patients with IBD, without stimulation. Then, the effect of LPMC-CM was assessed using an in vitro macrophage differentiation system. Peripheral blood (PB) CD14⁺ monocytes were obtained from healthy donors and differentiated into macrophage by M-CSF with or without LPMC-CM. There was no significant difference in morphology (Figure 6A), but the expression of cell-surface markers was different among the differentiated cells (Figure 6B). M-CSF–induced macrophages expressed CD14 and CD33 but not CD209 and CD206. Alternatively, CM-derived macrophages expressed all of these markers regardless of the source of CM, and the phenotype was similar to

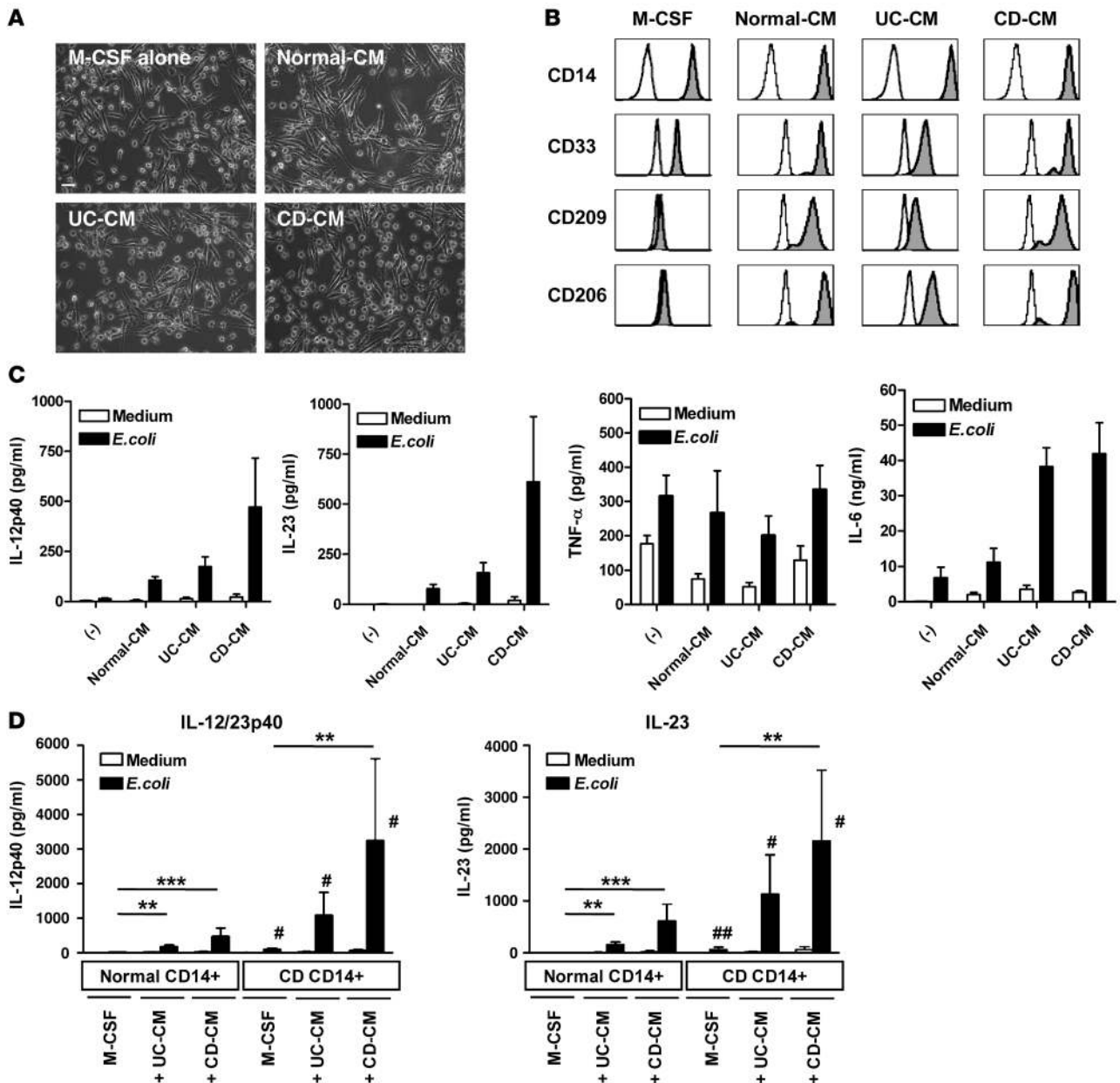


Figure 6

The intestinal inflammatory microenvironment affects macrophage differentiation and induces an IL-23–producing phenotype. **(A)** Morphological findings of in vitro–differentiated macrophages from peripheral CD14⁺ monocytes of normal controls with or without LPMC-CM. Scale bar: 20 μ m. **(B)** Flow cytometry for the surface phenotypes of LPMC-CM–induced in vitro–differentiated macrophages. The shaded histogram shows profiles of indicated Ab staining and the open histogram shows staining with isotype controls. The data shown are representative of 5 independent experiments. **(C)** Cytokine production by LPMC-CM–induced in vitro–differentiated macrophages stimulated with heat-killed *E. coli* for 24 hours. Data represent mean \pm SEM from 6 independent experiments. **(D)** Cytokine production by macrophages differentiated from normal and CD monocytes with or without UC- and CD-CM. Data represent mean \pm SEM from 5 independent experiments. All data used at least 3 different CM from individual patients and at least 3 different monocytes from individual patients and controls. Statistical analysis was performed using Kruskal-Wallis 1-way ANOVA and the Tukey-Kramer test for multiple comparisons. *******P* < 0.01, ********P* < 0.001 versus M-CSF induced macrophages; **#***P* < 0.01, **##***P* < 0.01 comparison between normal control monocytes and monocytes from CD patients.

that of intestinal macrophages. However, production of IL-23 and IL-12/IL-23p40 by these macrophages was significantly different and clearly higher in CD-CM–induced macrophages, as shown in Figure 6, C and D. Thus, it seems possible that CD-CM specifically affects monocyte differentiation, at least on the cytokine produc-

tion ability, and induces IL-23–hyperproducing macrophages. These results indicate that the inflammatory microenvironment of intestinal mucosa in patients with CD affected macrophage differentiation and altered their phenotype to abnormal macrophages with an IL-23–hyperproducing phenotype. Then, we tried

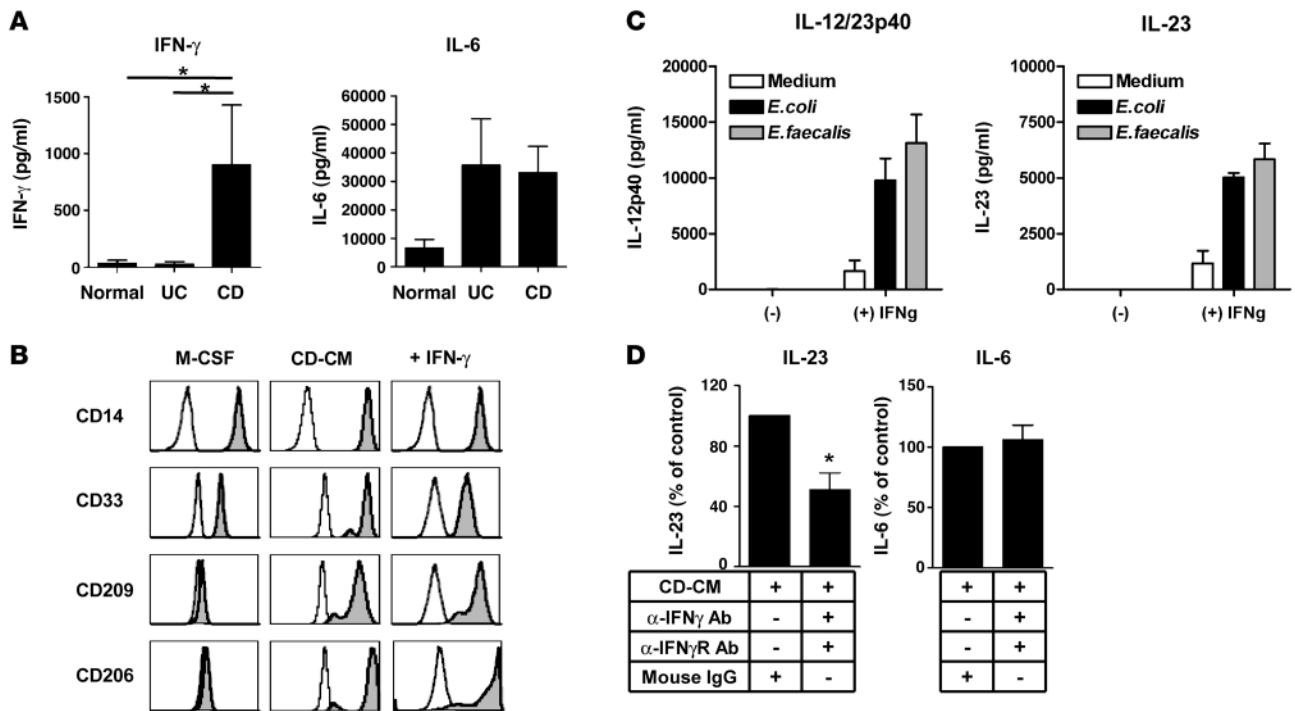


Figure 7 IFN- γ in CD-CM promotes IL-23-hyperproducing proinflammatory macrophage differentiation. (A) Quantification of IFN- γ and IL-6 in LPMC-CM. Data are shown as mean \pm SEM from 3 individual normal controls and 4 individual patients with IBD used for macrophage differentiation experiments. (B) Flow cytometry for the surface phenotypes of IFN- γ -induced in vitro-differentiated macrophages. The shaded histogram shows the profiles of the indicated Ab staining and the open histogram shows staining with isotype controls. (C) Production of IL-12/IL-23p40 and IL-23 by bacteria-stimulated macrophages differentiated with or without IFN- γ . Data represent mean \pm SEM from 3 independent experiments. (D) Effect of IFN- γ signal blocking using anti-IFN- γ Ab (α -IFN γ Ab) (1 μ g/ml) combination with anti-IFN- γ receptor 1 Ab (α -IFN γ R Ab) (10 μ g/ml) or same amount of those isotype controls (mouse IgG; mouse IgG_{2A} for α -IFN γ Ab, and mouse IgG₁ for α -IFN γ R Ab) from CD-CM on macrophage differentiation. Statistical analysis was performed using paired *t* test. Data represent mean \pm SEM from 5 independent experiments. **P* < 0.05 compared with controls.

to determine whether only inflammatory conditions affected macrophage differentiation or if CD patients have some abnormalities in monocytes. To address this issue, monocytes from patients with CD were used for in vitro macrophage differentiation with UC- or CD-CM. Compared with normal monocyte-derived macrophages, CD monocyte-derived macrophages produced more IL-23 in response to bacterial stimuli. These results suggest that CD monocytes are distinct from normal monocytes and are more susceptible to CD-CM and altered their phenotype into IL-23 hyperproducers (Figure 6D).

IFN- γ in CD-CM leads to abnormal macrophage differentiation with an IL-23-hyperproducing phenotype. As described above, it has become evident that intestinal microenvironments in patients with CD lead to abnormal macrophage differentiation with an IL-23-hyperproducing phenotype. However, it was still unclear which factors in CD-CMs cause abnormal macrophage differentiation. As shown in Figure 4, some proinflammatory cytokines, such as IFN- γ and TNF- α , were spontaneously produced by LPMCs after culture for 24 hours, especially in CD patients. Hence, we focused on the effect of such proinflammatory cytokines on macrophage differentiation. To identify these factors, the cytokines in CD-CM were analyzed. In fact, the amount of IFN- γ was highest in CD-CM among normal-, UC-, and CD-CM groups (Figure 7A). In contrast, other proinflammatory cytokines, such as IL-6, were elevated in both CD- and UC-CM (Figure 7A). Unexpectedly, in contrast to the results in Figure 4, all

LPMC-CMs used in this study did not contain a detectable amount of TNF- α (data not shown). This difference might occur due to differences in culture times (LPMC-CMs were prepared by 60-hour culture of LPMCs in the absence of stimulation, while the result in Figure 4A revealed the cytokine amounts at 24-hour culture). Therefore, we examined the effect of recombinant IFN- γ on macrophage differentiation. IFN- γ -derived macrophages were similar to intestinal CD14⁺ macrophages or CD-CM-derived macrophages in terms of the expression of some surface markers, and commensal bacteria stimulation induced the hyperproduction of IL-12/IL-23p40 and IL-23 (Figure 7, B and C). However, other proinflammatory cytokines, including TNF- α and IL-6, did not affect macrophage differentiation (data not shown). In addition, blocking of IFN- γ and its receptor in the culture of monocytes with CD-CM significantly, but not completely, attenuated such abnormal IL-23 production by macrophages (Figure 7D). These results indicate that IFN- γ in the CD-CM is a factor that promotes abnormal macrophage differentiation and leads to an IL-23-hyperproducing phenotype.

Discussion

It has been reported that human intestinal macrophages do not express typical macrophage innate-immune receptors, such as CD14 or TLRs, and that they exhibit antiinflammatory anergic phenotypes (9, 11). On the other hand, in IBD, it has been reported that abnormal proinflammatory macrophages, such as TREM-1⁺ mac-



rophages, are increased in intestinal mucosa and contributed to the intestinal inflammation (19). In addition to the prior report, it was also shown that CD14⁺ and TLR⁺ myeloid cells are also increased in the LP of patients with IBD (21, 22). Thus, such innate-immune receptor-positive myeloid cells may contribute to the pathogenesis of human IBD. In the present study, we identified what we believe to be unique CD14⁺ intestinal macrophages in the human intestinal LP. Consistent with the prior report, these cells expressed TREM-1 (Supplemental Figure 1). We also showed that the chemokine receptor expression pattern was quite different between CD14⁺CD33⁺ intestinal macrophages and CD14⁺CD33⁻ intestinal macrophages, which are considered to be the typical resident macrophages. These data suggest the possibility that these 2 intestinal macrophage subsets are derived from different subsets of monocyte, such as inflammatory or resident monocytes as previously reported (6, 23).

Moreover, the number of unique CD14⁺ macrophage subsets was dramatically increased not only in inflamed mucosa but also in non-inflamed mucosa with CD (Figure 1B). This result suggests that the increasing number of CD14⁺ macrophages in CD patients was not simply caused as a secondary event associated with increased inflammation in CD. Moreover, not only the numbers but also the functions of this subset were dramatically changed in CD patients. The CD14⁺ macrophage subset from CD patients produced abundant levels of IL-23 and TNF- α compared with normal control subjects and patients with UC in response to bacteria stimulation. However, it was unclear whether this enhanced IL-23 and TNF- α level produced by CD14⁺ macrophages in CD patients plays a causal role in the inflammation of CD patients or represents a secondary event associated with increased inflammation observed in CD, because the basal production of these proinflammatory cytokines by CD14⁺ macrophages was also higher in patients with CD. A similar unique macrophage subset has already been reported in mice. A subset of murine intestinal macrophages expressed both murine macrophage marker F4/80 and DC marker CD11c and directly induced the development of granuloma (24). In order for these unique intestinal macrophages subset in mice to develop granuloma formation, commensal bacteria-induced IL-23 is an essential factor. Based on these reports, the CD14⁺ intestinal macrophages identified herein in humans might be a counterpart of such unique intestinal macrophages subset in mice and contribute to the development of granulomas, a typical characteristic finding in patients with CD, owing to the production of excess IL-23 and TNF- α in response to commensal bacteria.

This abnormally large amount of IL-23 observed in CD patients was induced by specific commensal bacteria, such as *E. faecalis* and *E. coli*, which were determined as colitogenic bacteria in a murine model of CD (25). In addition, stimulation with pathogen-associated molecular patterns (PAMPs) alone could not induce IL-23. Furthermore, inhibition of phagocytosis suppressed IL-23 production (Supplemental Figure 2). These findings imply that the specific species of commensal bacteria, considered to be colitogenic bacteria, strongly induce IL-23 production via intracellular recognition pathways. A recent study has demonstrated that the intracellular bacteria recognition receptor NOD2 is important for the bacteria-induced IL-23 production by monocyte-derived DCs from patients with CD (26). However, a NOD2 ligand muramyl dipeptide (MDP) could not induce IL-23 by CD14⁺ intestinal macrophages (Supplemental Figure 2D).

Because it has become evident that the IL-23/Th17 axis is more important than the IL-12/Th1 axis in various autoimmune and inflammatory diseases (27–30), including animal models of IBD (31,

32), the role of the IL-23/Th17 axis in the pathogenesis of human CD has become increasingly attractive. In fact, recent studies have suggested the existence of an IL-23 receptor polymorphism that is associated with the pathogenesis of IBD (33). In the present study, it has become clear that unique CD14⁺ macrophages were the major source of IL-23 in the LP in response to commensal bacteria stimulation. Interestingly, these cells never produced IL-12p70. Moreover, such macrophages were not observed in PBMCs, and IL-23 did not show any effect on the production of IFN- γ or IL-17 by PBMCs or PB CD4⁺ T cells (data not shown). Because it was previously reported that IL-23 is important for local inflammation rather than systemic inflammation, while IL-12 shows the opposite effect (34), the unique macrophage subset that we identified might play a central role in local inflammation of the gut via IL-23, but not IL-12.

In the present study, we demonstrated that IL-23 and TNF- α but not IL-6 produced by CD14⁺ macrophages synergistically promoted IFN- γ production by LPMCs and LP CD4⁺ T cells. Unexpectedly, however, IL-17 was not induced by LPMCs after IL-23 or commensal bacteria stimulation. In contrast, IL-17 was detected from longer-duration culture supernatants of LPMCs (48 hours and 72 hours), but the amounts were quite low and were not significantly induced with commensal bacteria stimulation (Supplemental Figure 3). These results suggest the possibility that while IL-17 contributes more to the pathogenesis of CD patients in the later phase of inflammation than IFN- γ , commensal bacteria recognition by CD14⁺ intestinal macrophages predominantly enhances IFN- γ production rather than IL-17 production by LPMCs in patients with CD. On the other hand, we found an abundant amount of IL-17 was produced by purified LP CD4⁺ T cells from patients with CD, both with and without TCR engagement (data not shown). These results collectively suggest the possibility that although LP CD4⁺ T cells potentially produce both IFN- γ and IL-17, IL-17 production was suppressed in LPMCs, albeit by a largely unknown mechanism. However, we demonstrated that the other Th17-related cytokines IL-22 and CCL20 were significantly induced by LPMCs in CD patients after commensal stimulation. Thus, IL-23/Th17 immune responses were actually induced in CD patients after commensal bacteria stimulation. Since both IFN- γ and IL-17 producing cells, named Th17/Th1 cells, were identified in the patients with CD (35), there is a possibility that IL-23 induced the IFN- γ from Th17/Th1 cells rather than Th1 cells. Collectively, although IL-23 contributes to the induction of IL-17, IL-23 predominantly induces IFN- γ in the LP and leads to Th1- or Th17/Th1-mediated intestinal inflammation in CD. In agreement with our findings, although the IL-23/IL-17 axis is important in the pathogenesis of several animal models of colitis, IFN- γ production was strongly elevated (36, 37) and not only IL-17 but also IFN- γ production was markedly decreased when these mice were under IL-23-deficient conditions (38, 39). These results indicate that not only the IL-23/IL-17 axis but also the IL-23/IFN- γ axis is important for the pathogenesis of animal colitis models as well as pathogenesis of humans.

Meanwhile, it was also reported that the IL-23/IFN- γ axis was observed in both T cell dependent and independent colitis models (38). In these models, non-T cell-derived IFN- γ or IL-17 are important for colitis development. In fact, we have demonstrated that not only LP T cells but also LP CD3⁺CD56⁺ NK cells can produce IFN- γ in response to IL-23 (Supplemental Figure 5). A recent study has demonstrated that unique intestinal NK cell differentiation was accelerated in patients with CD, and such intestinal NK cells



produced larger amounts of IFN- γ (40). Thus, non-T cells, such as NK cells, in the intestine might be important for intestinal inflammation as well as T cells via IL-23/IFN- γ axis.

As described above, it has become evident that abnormal intestinal macrophages may contribute to intestinal inflammation in patients with CD via IL-23/IFN- γ axis. However, how this abnormal differentiation of macrophages occurs remains unknown. Alternatively, in the normal intestine of humans, intestinal macrophages lack the expression of the innate-immune receptor CD14; therefore, intestinal macrophages do not induce inflammatory responses against commensals. A previous study demonstrated that the downregulation of CD14 expression is dependent on TGF- β produced by intestinal stromal cells (11). The present study indicated that proinflammatory cytokines, such as IFN- γ , induced abnormal differentiation of intestinal macrophage with IL-23-producing intestinal macrophage phenotypes. Because IFN- γ suppresses the TGF- β /Smad signaling (41), IFN- γ was involved not only in IL-23 production but also in the retention of CD14 antigen expression on such abnormal macrophages. On the other hand, IL-6 production was enhanced both in UC- and CD-CM-induced macrophages. These results suggest that some common inflammatory mediators, which are present in both UC and CD LPMC-CMs, were responsible for the enhancement of IL-6 production by macrophages, while inflammatory mediators that predominate in CD, such as IFN- γ , might contribute to the enhanced IL-23 and IL-12/IL-23p40 production. In contrast to the cytokine producing ability, there were no significant differences in surface markers among macrophages induced by normal-, UC- and CD-CMs (Figure 6B). However, at present, we have only examined the expression of CD14, CD33, CD209, and CD206 and did not examine the other markers. Therefore, the possibility exists that the expression of other markers is different between these macrophages induced by normal-, UC-, and CD-CM. Further studies are required to clarify the markers that distinguish the CD14⁺ macrophages with the IL-23-hyperproducing ability. Moreover, we also demonstrated that monocytes in CD patients were distinct from those in normal controls and were more susceptible to CD-CM-induced abnormal macrophage differentiation, and the levels of IL-23 and IL-12p40 production by CD monocyte-derived abnormal macrophages were markedly higher than those in normal monocyte-derived abnormal macrophages. This implies a possibility that monocytes from CD patients exhibit high susceptibility to IFN- γ activations of the IL-23-hyperproducing phenotype. In contrast, although CD14⁺ intestinal macrophages in patients with CD revealed enhanced production of not only IL-23 but also TNF- α compared with those in normal and patients with UC, CD-CM did not elicit any effect on TNF- α production by macrophages derived from healthy monocytes (Figure 6D). At present we do not know whether CD-CM affects not only IL-23 but also TNF- α production on CD monocyte-derived macrophages. A number of unresolved issues still remain; however, these results indicate the possibility that some kind of intrinsic abnormality is imprinted in the monocyte of CD patients, which leads to enhanced TNF- α production by intestinal macrophages independent from the inflammatory microenvironment.

Our present study identifies what we believe to be unique macrophages that may play a central role in Th1- or Th17/Th1-skewed intestinal inflammation in human CD via IL-23 and TNF- α . Moreover, such inflammatory skewed intestinal microenvironments triggered further abnormal macrophage differentiation with IL-23 hyperproduction, which is dependent on IFN- γ (Supplemental Fig-

ure 6). Collectively, this IL-23/IFN- γ -positive feedback loop induced by abnormal intestinal macrophages contributes to the pathogenesis of chronic intestinal inflammation in patients with CD.

Methods

Tissue samples. Normal intestinal mucosa was obtained from macroscopically and microscopically unaffected areas of patients with colon cancer. Intestinal mucosa was also obtained from surgically resected specimens from patients with UC or CD, diagnosed on the basis of clinical, radiographic, endoscopic, and histological findings according to established criteria. In all samples from patients with CD or UC, the degree of inflammation was histologically moderate to severe. All experiments were approved by the institutional review board of Keio University School of Medicine and written informed consent was obtained from all patients.

Histological analysis. Tissue sections were treated according to well-established methods. Intestinal specimens were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries) and embedded in paraffin. For immunohistochemical staining, deparaffinized sections were heated at 100°C for 20 minutes in 10 mM sodium citrate buffer (pH 6.0) in a microwave oven. Sections were treated with 3% hydrogen peroxide (H₂O₂) (Wako Pure Chemical Industries) in 100% methanol and then incubated with normal rabbit serum (Nichirei Biosciences) for 15 minutes at room temperature to block nonspecific reactions. Thereafter, they were incubated with mouse anti-human CD14 Ab (Zymed Laboratories) at 4°C overnight. After washing with PBS, the sections were incubated with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). In the case of double labeling, slides were boiled for 15 minutes and treated in 3% H₂O₂/methanol for 10 minutes after completion of the first staining. This procedure completely blocked the antigenicity of the first primary and secondary antibody. Then, the same staining procedures were performed with the second primary antibody, mouse anti-human CD68 Ab (Dako Cytomation), and Alexa Fluor 568-conjugated secondary antibody. Sections were then washed in PBS, incubated with DAPI to stain nuclei, and examined and photographed using fluorescence microscopy (Nikon Eclipse 80i). In the case of DAB staining, Histofine anti-mouse Simplestain Max-PO (Nichirei) was used as the secondary antibody. Bound antibody was visualized with 3-3'-diaminobenzidine (DAB; Nichirei), and sections were counterstained with hematoxylin.

Preparation of LPMCs. LPMCs were isolated from intestinal specimens using modifications of previously described techniques (17). Briefly, dissected mucosa was incubated in calcium and magnesium-free HBSS (Sigma-Aldrich) containing 2.5% heat-inactivated fetal bovine serum (BioSource) and 1 mM dithiothreitol (Sigma-Aldrich) to remove mucus. The mucosa was then incubated twice in HBSS containing 1 mM EDTA (Sigma-Aldrich) for 45 minutes at 37°C. Tissues were collected and incubated in HBSS containing 1 mg/ml collagenase type 3 and 0.1 mg/ml DNase I (Worthington Biochemical) for 60 minutes at 37°C. The fraction was pelleted and resuspended in a 40% Percoll solution (Amersham Biosciences), then layered on 60% Percoll before centrifugation at 700 g for 20 minutes at room temperature. Viable LPMCs were recovered from the 40%–60% layer interface.

Isolation of PB monocytes or LP CD14⁺CD33⁺ or CD14⁺CD33⁺ macrophages. Peripheral CD14⁺ monocytes were isolated from PBMCs using CD14⁺ MACS (Miltenyi Biotec) according to the manufacturer's instructions. The percentage of monocytes isolated using this method was evaluated by flow cytometry and was routinely more than 98%. LP CD14⁺CD33⁺ macrophages were isolated from LPMCs using EasySep Human CD14⁺ (StemCell Technologies Inc.). CD14⁺CD33⁺ macrophages were isolated from LPMCs using MACS and EasySep (as CD14⁺CD3⁺CD56⁺CD33⁺ cells). The percentage of each subset of cells isolated using this method was evaluated by flow cytometry and was routinely more than 95%.



Flow cytometric analysis. Cell-surface fluorescence intensity was assessed using a FACSCalibur analyzer and analyzed using CellQuest software (BD Biosciences) or FlowJo (TreeStar). Dead cells were excluded with propidium iodide staining. Monoclonal antibodies for CD14, CD33, CD13, CD16, CD32, CD64, CD71, CD123, CD80, CD86, CD1a, CD83, CD40, CD206, CD209, HLA-DR, CCR1, CCR2, CCR7, CCR9, CXCR1, CXCR2, CXCR4, CD68, CD208, CD36, TREM-1, PD-L1, CD70, and CD103 were purchased from BD Biosciences. Abs for CD205, TLR2, TLR4, and PD-L2 were from eBioscience. Abs for CCR4, CCR5, and CCR6 were from R&D Systems. The CD1c Ab was from Ancell. CX₃CR1 Ab was from MBL.

Commensal bacteria heat-killed antigens. A gram-negative nonpathogenic commensal strain of *E. coli* (catalog no. 25922; ATCC) was cultured in Luria-Bertani (LB) medium, and a gram-positive commensal strain of *E. faecalis* (catalog no. 29212; ATCC) was cultured in brain-heart infusion (BHI) medium. Bacteria were harvested and washed twice with ice-cold PBS. Then, bacterial suspensions were heated at 80°C for 30 minutes, washed, resuspended in PBS, and stored at -80°C. Complete killing was confirmed by a 72-hour incubation at 37°C on plate medium.

Stimulation of macrophages by commensal bacteria antigens. Isolated macrophages were seeded on 96-well tissue culture plates (1 × 10⁶ cells/ml) in RPMI 1640 medium supplemented with 10% FBS, antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), 10 mM HEPES, and 50 µM 2-mercaptoethanol and stimulated with heat-killed bacteria (1 × 10⁸ CFU/ml) for 24 hours. Culture supernatants and total RNAs were collected and then stored at -80°C until the cytokine assay.

Stimulation of LPMCs. Isolated whole LPMCs were seeded on 96-well tissue culture plates (1 × 10⁶ cells/ml) and stimulated with 20 ng/ml recombinant human IL-23, TNF-α, and IL-6 (all from R&D Systems) or heat-killed bacteria (1 × 10⁸ CFU/ml) with or without 1 µg/ml anti-human IL-12/IL-23p40 Ab (eBiosciences), anti-human TNF-α Ab (R&D Systems), or the same amount of mouse IgG₁ (R&D Systems) for 24 hours. For measurement of secreted cytokines, culture supernatants were collected and stored at -80°C until the cytokine assay.

Preparation of LPMC-CM. Isolated LPMCs from the intestine of normal control subjects or the inflamed mucosa of IBD patients were cultured for 60 hours without any stimulation. Culture supernatants were collected, passed through a 0.22-µm filter, and then stored at -80°C until used.

In vitro macrophage differentiation. PB CD14⁺ monocytes were isolated from healthy donors or CD patients. CD14⁺ monocytes were cultured with 50 ng/ml recombinant human M-CSF (R&D Systems) for 6 days to obtain macrophages. Differentiated macrophages were harvested, washed to remove residual cytokines, and then plated on 96-well tissue culture plates (5 × 10⁵ cells/ml) in RPMI 1640 medium supplemented with 10% FBS, antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), 10 mM HEPES, 50 µM 2-mercaptoethanol, and 50 ng/ml M-CSF. After a 2-hour

preincubation, macrophages were stimulated with bacterial antigens. In some experiments, LPMC-CM (10% of final volume), recombinant IFN-γ (100 ng/ml), anti-IFN-γ Ab (1 µg/ml), and anti-IFN-γ receptor 1 Ab (10 µg/ml) were added during the macrophage differentiation process. For the differentiation experiments, mouse IgG₁ (for IFN-γ receptor 1 Ab) and mouse IgG_{2A} (for IFN-γ Ab) were used at the same concentrations as controls.

Quantitative real-time RT-PCR analysis. Total RNA was extracted using an RNeasy Micro kit (QIAGEN), and cDNA was synthesized using a Quantitect RT kit (QIAGEN) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using a TaqMan Universal PCR Master Mix (Applied Biosystems) and on-demand gene-specific primers, assessed using the DNA Engine Opticon 2 System (Bio-Rad), and analyzed with Opticon monitor software (MJ Research). The primers were as follows: *IL12p35* (Hs00168405), *IL12p40* (Hs00233688), *IL23p19* (Hs00372324), *IL27p28* (Hs00377366), *Tgfb1* (Hs00171257), *IFNG* (Hs00174143), *IL17* (Hs00174383), *IL22* (Hs00220924), *CCL20* (Hs00171125), *ACTB* (Hs99999903). All primers were purchased from Applied Biosystems. Relative quantification was achieved by normalizing to the values of the *Actb* gene.

Cytokine assay. The following kits were used for cytokine measurements and tests were performed according to the manufacturer's instructions: human IL-12p40 ELISA (BD Pharmingen), human IL-23 ELISA (BenderMed Systems), human IL-17 ELISA (R&D Systems), human TGF-β1 ELISA (R&D Systems), and human inflammation or Th1/Th2-II cytometric beads array (CBA) kit (BD Pharmingen).

Statistics. Statistical analysis was performed using GraphPad Prism software version 4.0 (GraphPad Software Inc.). Differences of *P* < 0.05 were considered to be significant. All data are expressed as mean ± SEM.

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