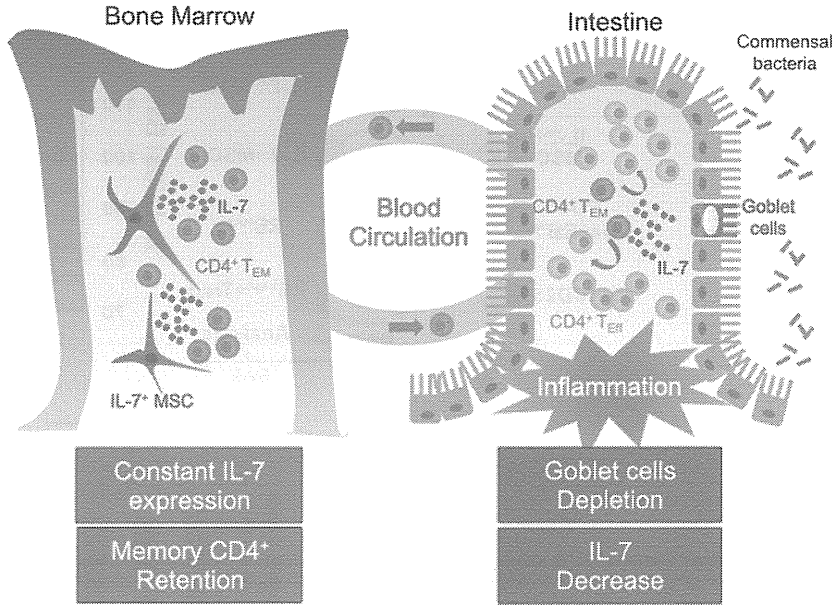

Figure 7 Model for the maintenance of colitogenic CD4 T_{EM} cells by bone marrow (BM) mesenchymal stem cell (MSC)-derived IL-7. BM MSC-derived IL-7 plays a role in the maintenance of colitogenic CD4 T_{EM} cells, and may be a clinical target for the treatment of inflammatory bowel diseases.

MSC are responsible for IL-7 production in the pathogenesis of human UC, and a strategy targeting IL-7 might be a feasible clinical approach for the treatment of UC. Furthermore, the current approach for the induction of remission (the acute stage of the disease) using autologous or allogeneic MSC in patients with intractable UC would be considered based on the present finding that MSC may play a pathological role in the maintenance of colitogenic memory T cells (remission stage). However, we would like to emphasise that the present results are consistent with the current concept of using MSC to treat human IBD in ongoing clinical trials, because we also confirmed that our cultured MSC suppressed the proliferation of CD4 T cells in the short-term culture system. Finally, it should be emphasised that the strategy of IL-7 blockade is at an immature stage at this time, because IL-7 is essential not only for colitogenic CD4 T cells but also protective memory CD4 T cells such as regulatory T cells. Therefore, further investigation in this field is warranted.

Overall, in support of previous evidence that BM is a reservoir organ for CD4 memory T cells, we demonstrated for the first time that BM MSC express IL-7 and comprise the key population that forms the niche for colitogenic memory CD4 T cells and causes the persistence of chronic colitis.

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Contributors YN helped to design the study, performed experiments, analysed the data, and wrote the paper; TK conceived and designed the study, analysed the data, and wrote the paper; MT performed experiments; SO, TN, RO and KT helped to design the study and MW supervised the study.

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ORIGINAL ARTICLE

Mucosal CXCR4$^+$ IgG plasma cells contribute to the pathogenesis of human ulcerative colitis through FcγR-mediated CD14 macrophage activation

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ABSTRACT

Background Chronic inflammation characterised by IgG-producing plasma cell infiltration of colonic mucosa is a histological hallmark of ulcerative colitis (UC); however, whether its function is pathogenic or protective remains unclear.

Objective To explore the contribution of intestinal IgG plasma cells to UC pathogenesis.

Methods We isolated lamina propria mononuclear cells (LPMCs) from intestinal mucosa of UC patients and analysed the characteristics of intestinal plasma cells (expression profiles of differentiation molecules and chemokine receptors). We investigated the involvement of IgG-immune complex (IC)-Fc gamma receptor (FcγR) signalling in intestinal inflammation by examining the cytokine production by LPMCs in response to IgG-IC stimulation.

Results IgG plasma cells that were markedly increased in number in the inflamed mucosa of UC patients showed a distinct expression profile ($CD19^+CD27^{low}$, $CCR10^{low}CXCR4^{high}$) compared with IgA plasma cells ($CD19^{+/-}CD27^{high}$, $CCR10^{high}CXCR4^{-/low}$). *In vitro* IgG-IC stimulation activated intestinal CD14 macrophages that were increased in number in the inflamed mucosa of UC patients via FcγRI and FcγRII, and induced the extensive production of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1β (IL-1β), comparable to the effect of commensal bacteria stimulation. Co-stimulation with IgG-IC and commensal bacteria increased TNF and IL-1β production more than stimulation with the latter alone. Furthermore, IgG-IC notably up-regulated the expression of TL1A, whereas commensal bacteria specifically induced IL-23.

Conclusions Collectively, these results demonstrate a novel aspect of UC pathogenesis in which unique IgG plasma cells infiltrate the inflamed mucosa via CXCR4, and critically influence UC pathogenesis by exacerbating mucosal inflammation through the activation of 'pathogenic' intestinal CD14 macrophages via IgG-IC-FcγR signalling.

Significance of this study

What is already known on this subject?

- Ulcerative colitis (UC) is characterised by infiltration of IgG-producing plasma cells into the inflamed colonic mucosa.
- IgG antibodies can perpetuate several chronic inflammatory disorders; FcγRs (receptors for IgG) are thought to be involved in the pathogenesis of such disorders, including UC.
- Unique intestinal CD14 macrophages play a central role in the pathogenesis of inflammatory bowel disease via excess production of TNF and IL-23 in response to commensal bacteria.

What are the new findings?

- IgG plasma cells in the inflamed mucosa of UC patients have a distinct chemokine receptor-expression profile ($CCR10^{low}CXCR4^{high}$) compared with IgA plasma cells ($CCR10^{high}CXCR4^{-/low}$).
- IgG-immune complex (IC) induces abundant production of pro-inflammatory cytokines such as TNF, IL-1β and TL1A by lamina propria mononuclear cells (LPMCs) from UC patients, demonstrating IgG-IC as another potent inducer of intestinal inflammation besides commensal bacteria.
- Intestinal CD14 macrophages express FcγRs, and are responsible for IgG-IC-FcγR signalling-induced pro-inflammatory cytokine production by LPMCs.

How might it impact on clinical practice in the foreseeable future?

- Our results provide a novel mechanistic insight into the disease process of human UC, highlighting the crucial roles of IgG plasma cells and CD14 macrophages in the intestinal inflammation and UC pathogenesis.
- Modulation of these IgG plasma cells or the signalling pathway might represent a promising therapeutic strategy for the treatment of UC.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic intestinal inflammatory disorder with two major types: ulcerative colitis (UC) and Crohn's disease (CD). Although the precise aetiologies remain unclear, genetic backgrounds, environmental factors, and immunological disorders are critical factors in the pathogenesis of IBD.[1 2] Histological observations of UC include infiltration of IgG-producing plasma cells into the inflamed

colonic mucosa[3 4]; however, the precise mechanism of IgG plasma cell infiltration, and the roles that IgG antibodies and IgG plasma cells play in the pathogenesis of UC are not fully understood, despite considerable progress made in recent studies.[5–7]

Plasma cells are the terminal stage of B cell differentiation, and are dedicated to the large-scale secretion of antibodies. Besides bone marrow, lamina propria (LP) is the main reservoir of plasma cells, and most intestinal plasma cells are IgA-producing cells, contributing to the maintenance of gut homeostasis. IgG antibodies are the most abundant serum immunoglobulins involved in the secondary immune response, and their numbers increase in response to infection, chronic inflammation, and autoimmune diseases. Indeed, accumulation of IgG plasma cells in inflamed tissues has been reported not only in UC but also in several autoimmune diseases such as rheumatoid arthritis (RA).[8] There is also increasing evidence that autoantibodies produced by IgG plasma cells can perpetuate several autoimmune diseases in humans.[9]

Fc gamma receptors (FcγRs) play a crucial role in immunity by linking IgG antibody-mediated responses with cellular effector and regulatory functions.[10] The three human FcγRs, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), differ in cell distribution, function, and affinity for IgG. FcγRI is a high-affinity receptor for IgG, whereas the low-affinity receptors FcγRII and FcγRIII preferentially bind IgG in the form of the immune complex (IC). FcγRs are critically involved in phagocytosis, IC clearance, degranulation, antibody-dependent cellular cytotoxicity (ADCC), and the release of pro-inflammatory cytokines. Genetic variants of these receptors have previously been identified as risk factors for several chronic inflammatory conditions such as RA, multiple sclerosis (MS) and systemic lupus erythematosus (SLE).[11] Recently, *FcγRIIA* was also identified as a susceptible gene of UC in Japanese and European descent populations[12 13]; FcγRs are therefore thought to play a crucial role in the pathogenesis of several chronic inflammatory disorders including UC. However, no research has focused on the involvement of IgG-FcγR signalling in the intestinal inflammation of patients with UC.

The present study therefore explored the contribution of intestinal IgG plasma cells to chronic intestinal inflammation in UC patients by analysing characteristic cellular features and investigating the involvement of IgG-IC-FcγR signalling in intestinal inflammation. Here we show that IgG plasma cells in the inflamed mucosa of UC patients have a unique immature and chemokine receptor expression phenotype, and suggest that IgG antibodies produced by these cells are key pathogenic effectors of colonic inflammation in UC.

MATERIALS AND 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. The degree of local mucosal inflammation was assessed macroscopically according to the level of superficial erosion, mucosal atrophy and epithelial destruction with ulcerations (–, normal mucosa; +, mild; ++, moderate; +++, severe inflammation). All experiments were conducted according to the Declaration of Helsinki principles, and approved by the institutional review boards of Keio University School of Medicine (Tokyo, Japan) and Yokohama Municipal Citizen's Hospital (Yokohama, Japan). Written informed consent was obtained from all patients (see online supplementary materials and methods for full details).

RESULTS

IgG plasma cells heavily infiltrate the inflamed mucosa of patients with UC

Immunohistochemistry confirmed the previously reported[14] massive influx of CD138 plasma cells into the inflamed mucosa of patients with IBD, particularly those with UC (figure 1A). To analyse the characteristics of intestinal plasma cells in detail, we isolated LP mononuclear cells (LPMCs) from the intestinal mucosa of IBD patients or the non-affected colonic mucosa of patients with colon cancer (non-IBD controls; Normal), and analysed them by flow cytometry. The $CD38^{high}$ phenotype is a common specific marker for human plasma cells and is applicable for the identification of intestinal plasma cells (see online supplementary figure S1A).[15] We observed no difference in the proportion of LPMCs that were $CD38^{high}$ between Normal and IBD patients (figure 1B). In the Normal group, the vast majority of intestinal plasma cells were IgA-producing (figure 1C); however, this proportion was drastically decreased and negatively correlated with the degree of local mucosal inflammation in patients with IBD (figure 1C,D), especially UC (figure 1E).

We also observed an increase of IgG plasma cells in the inflamed mucosa of patients with IBD, especially UC (figure 1F, see online supplementary figure S1B). Further analysis revealed that IgG plasma cells in UC were mainly IgG1-producing, whereas those in CD were mainly IgG1- or IgG2-producing (see online supplementary figure S1C,D), in line with previous findings.[16] These results show that IgG-producing plasma cells heavily infiltrate the inflamed mucosa of patients with IBD, especially UC, suggesting their involvement in the intestinal inflammation and pathogenesis of the disorder.

LP plasma cells in the inflamed mucosa of patients with UC have a unique immature phenotype

To reveal the characteristics of intestinal plasma cells in UC patients, we analysed the expression profiles of cell surface-differentiation molecules. We found that plasma cells in the Normal group and in the non-inflamed mucosa of IBD patients had a $CD19^{+/-}CD20^{-}CD27^{high}$ phenotype, whereas plasma cells in the inflamed mucosa of UC patients had a $CD19^{+}CD20^{-}CD27^{low}$ unique immature phenotype (figure 2A, C). These changes were also observed in both IgA and IgG (ie, surface IgA^{-})-producing plasma cells (figure 2B). These results are partly consistent with an earlier finding that the inflamed mucosa of UC patients contained CD19 immature plasma cells.[14]

For further characterisation, we isolated intestinal plasma cells from the LPMCs of Normal and UC patients (figure 2D, see online supplementary figure S2), and performed microarray gene-expression analysis. As shown in figure 2E, among the five major transcription factors involved in B cell to plasma cell differentiation,[17] plasma cells in UC patients expressed *BCL6* more highly than those of the Normal group; the expression of other transcription factors such as *PRDM1*, which encodes Blimp-1 and is necessary for plasma cell survival,[9] did not differ between the two groups. This result was confirmed by quantitative real-time RT-PCR analysis (figure 2F).

We also showed that plasma cells of the inflamed mucosa of CD patients had a $CD19^{+}CD20^{-}CD27^{high}$ phenotype, and that the expression level of *BCL6* was comparable to Normal plasma cells (figure 2A–C,F). As BCL6 functions as

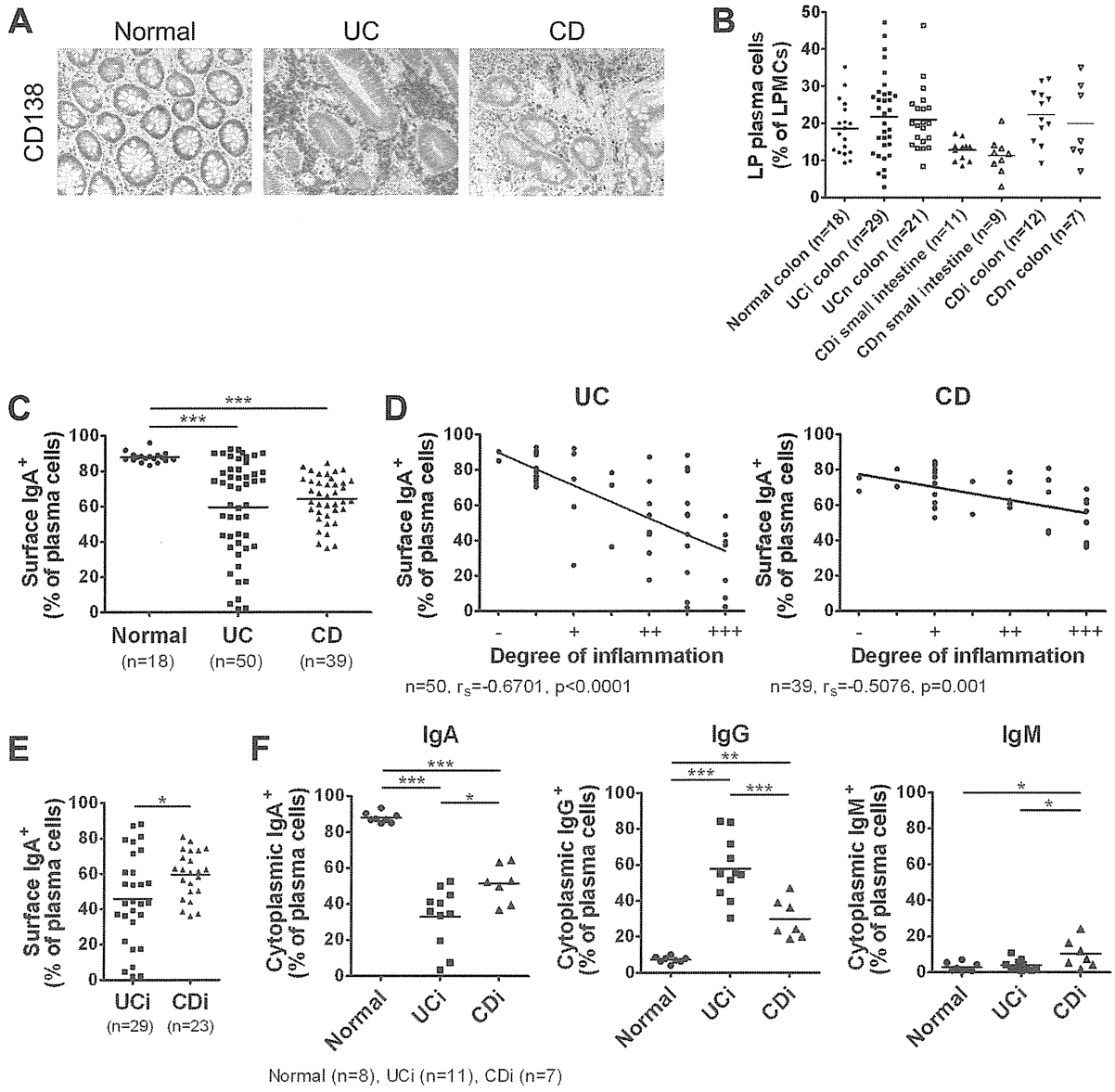

Figure 1 IgG plasma cells heavily infiltrate the inflamed mucosa of ulcerative colitis (UC) patients. (A) Immunostaining images of intestinal mucosa from non-inflammatory bowel disease (IBD) controls (Normal) and IBD patients stained with anti-CD138. Data are representative of three independent specimens. (B) Percentage of lamina propria (LP) $CD38^{high}$ plasma cells among lamina propria mononuclear cells (LPMCs). i, inflamed; n, non-inflamed. Horizontal bars indicate mean values. (C) Percentage of surface IgA^+ cells among LP $CD38^{high}$ plasma cells. (D) Negative correlations between the degree of local mucosal inflammation and percentage of surface IgA^+ cells among LP $CD38^{high}$ plasma cells. (E) Percentage of surface IgA^+ cells among LP $CD38^{high}$ plasma cells from inflamed (moderate-to-severe) mucosa of UC and Crohn's disease (CD) patients. (F) LP $CD38^{high}$ plasma cells from normal intestinal mucosa and inflamed mucosa of IBD patients were analysed by intracellular staining for cytoplasmic IgA, IgG and IgM expression. Shown are percentages of cytoplasmic Ig^+ cells among LP plasma cells. *p<0.05, **p<0.01, ***p<0.001.

a transcription factor that maintains the B cell phenotype and proliferation,[17] these data support the idea that plasma cells in the inflamed mucosa of UC patients have a unique immature phenotype that differs in part from that of CD patients.

LP IgG plasma cells in the inflamed mucosa of patients with UC have a different chemokine receptor-expression profile

Chemokines, together with tissue-specific adhesion molecules, coordinate the trafficking of plasma cells.[18] Therefore, to dissect the mechanism of IgG plasma cell accumulation in the inflamed mucosa of UC patients, we examined the expression profiles of chemokine receptors in intestinal plasma cells. Normal intestinal IgA plasma cells express the chemokine receptor CCR10, which is important for the homing mechanism to the mucosal tissues.[18] Among the panel of chemokine receptors tested, we found that the expression of *CXCR4* was higher in UC plasma cells than in Normal plasma cells, and that the former had relatively low *CCR10* expression (figure 3A). Thereafter, the expression profiles of these chemokine receptors were confirmed by flow cytometry.

We also examined the expression of CXCR3, which is involved in the homing to inflamed tissues and the development of several autoimmune diseases.[19] As shown in figure 3B, the Normal group plasma cells and those of non-inflamed mucosa of IBD patients had a $CCR10^{high}CXCR3^{+}CXCR4^{-/low}$

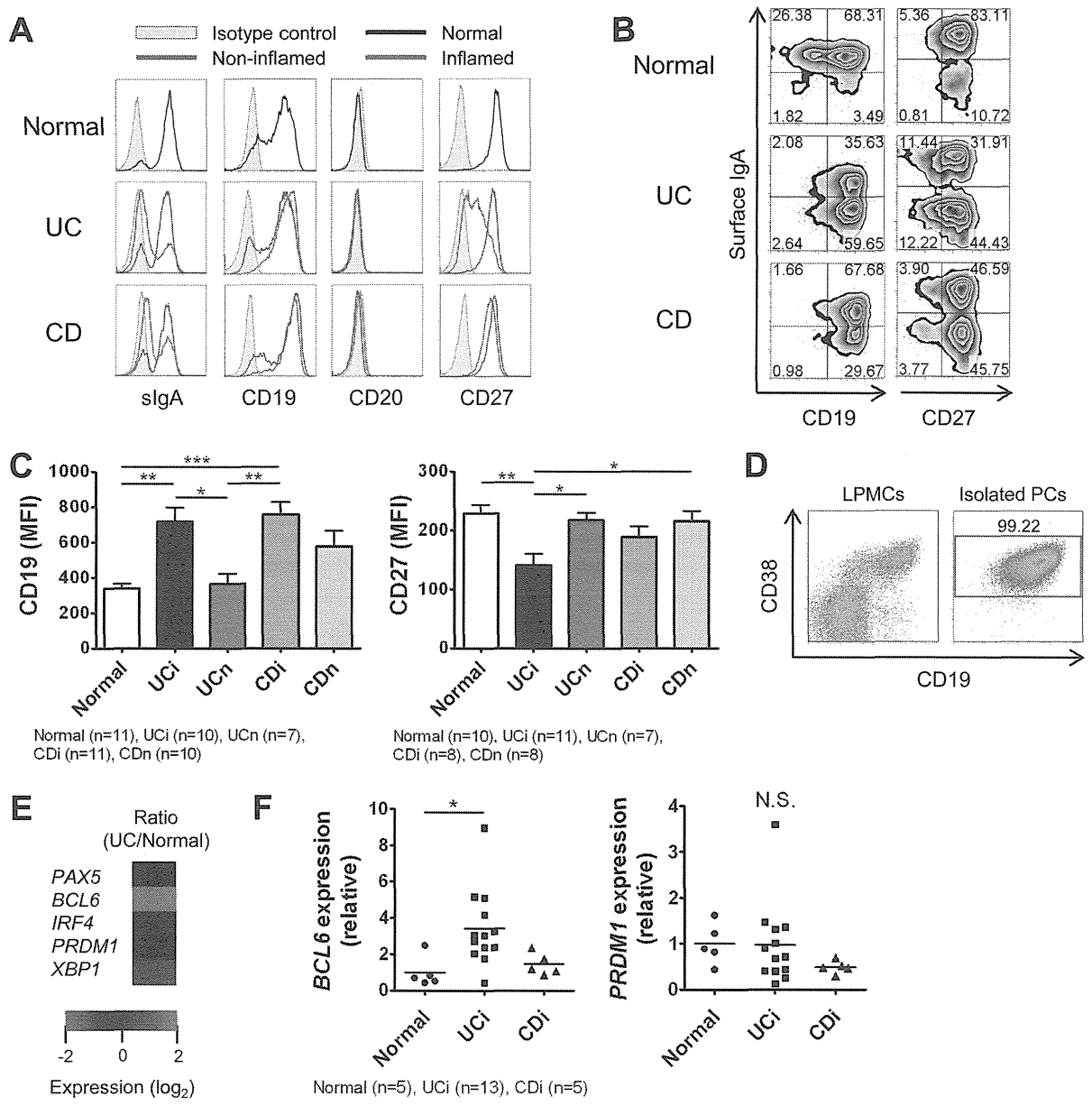

Figure 2 Lamina propria (LP) plasma cells in the inflamed mucosa of ulcerative colitis (UC) patients have a unique immature phenotype. (A) LP CD38^{high} plasma cells from normal intestinal mucosa and inflamed or non-inflamed mucosa of inflammatory bowel disease (IBD) patients were analysed by flow cytometry for IgA, CD19, CD20 and CD27 cell surface expression. CD, Crohn's disease. (B) LP CD38^{high} plasma cells from normal intestinal mucosa and inflamed mucosa of IBD patients were analysed by flow cytometry for IgA and CD19/CD27 cell surface expression. (C) Mean fluorescence intensities (MFI) of CD19 and CD27 expression levels on LP CD38^{high} plasma cells from normal controls and IBD patients. (D) LPMCs and isolated LP plasma cells (PCs) from inflamed mucosa of UC patients were analysed for CD38 and CD19 expression. (E) Microarray analysis of LP plasma cells isolated from normal intestinal mucosa and inflamed mucosa of UC patients, presented as a heat map of five major transcription factors involved in B cell to plasma cell differentiation (presented as a ratio of normalised intensity). (F) Quantitative real-time RT-PCR of basal mRNA expression levels in isolated LP plasma cells from normal intestinal mucosa and inflamed mucosa of IBD patients.*p<0.05, **p<0.01, ***p<0.001, N.S., not significant.

phenotype, whereas plasma cells of inflamed mucosa of UC patients had a CCR10^{low}CXCR3^{+}CXCR4^{high} phenotype. Notably, we showed that the up-regulation of CXCR4 and the down-regulation of CCR10 were more evident in IgG (ie, surface IgA^{-}) plasma cells than IgA plasma cells in the inflamed mucosa of UC patients (figure 3C).

A more detailed analysis showed that IgA and IgG plasma cells in the inflamed mucosa of UC patients showed different chemokine receptor-expression profiles: a CCR10^{high}CXCR4^{low} phenotype for IgA plasma cells, similar to Normal IgA plasma cells, and a CCR10^{low}CXCR4^{high} phenotype for IgG plasma cells (figure 3D,E). In the inflamed mucosa of CD patients, IgA and IgM plasma cells had a CCR10^{high}CXCR4$^{-/low}$ phenotype, whereas IgG plasma cells had a CCR10^{low}CXCR4^{high} phenotype (figure 3D). We used immunohistochemistry to confirm the infiltration of IgG^{+}CXCR4^{+} cells displaying a classical plasma cell morphology in the inflamed mucosa of UC patients (figure 3F). Overall, these results suggest that IgG, not IgA, plasma cells selectively infiltrate the inflamed mucosa of UC patients via CXCR4.

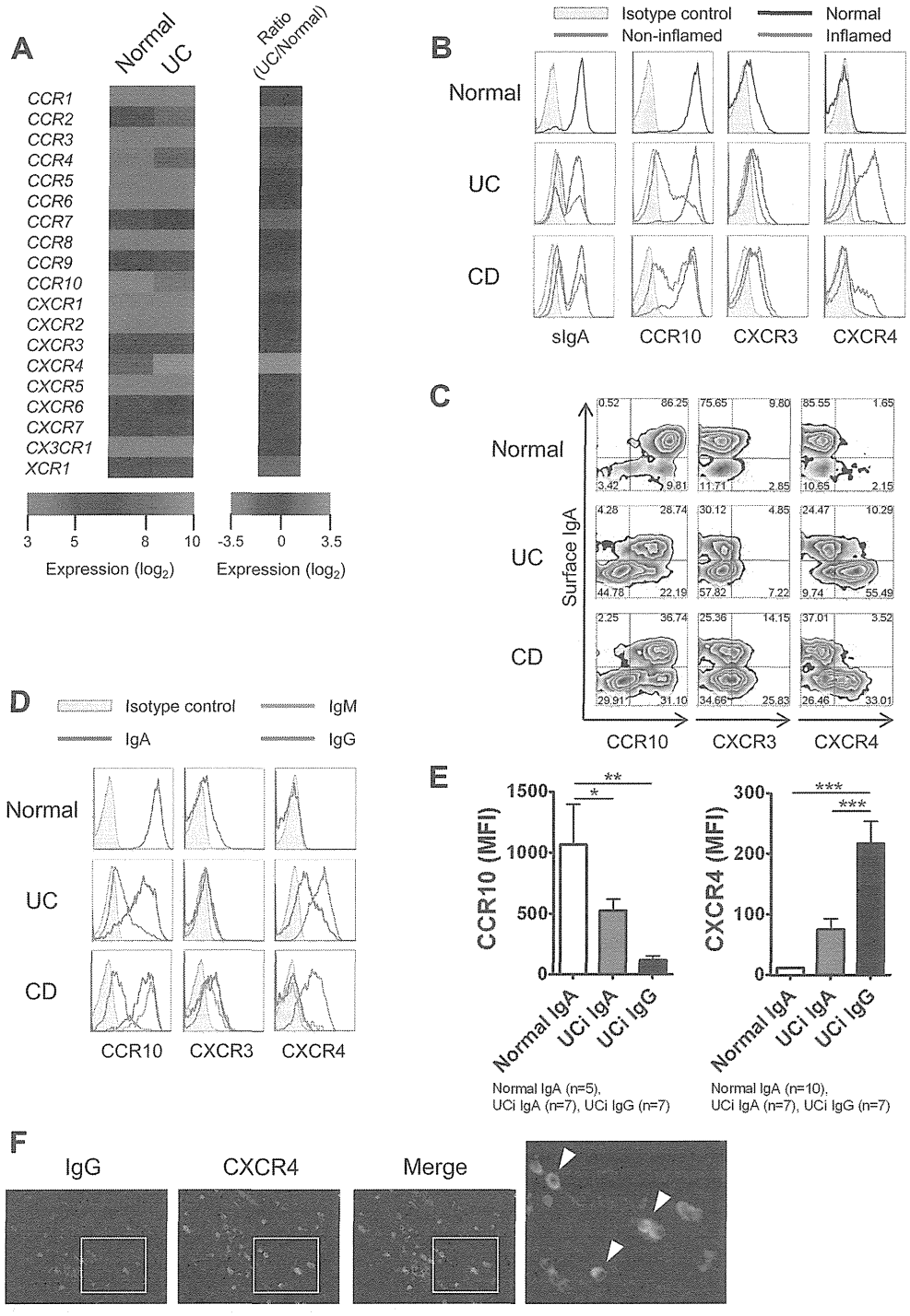

Figure 3 Lamina propria (LP) IgG plasma cells in the inflamed mucosa of ulcerative colitis (UC) patients have a different chemokine receptor-expression profile. (A) Microarray analysis of LP plasma cells isolated from normal intestinal mucosa and inflamed mucosa of UC patients, presented as a heat map of chemokine receptors (presented as a normalised intensity (log_2) and a ratio of normalised intensity). (B) LP $CD38^{high}$ plasma cells from normal intestinal mucosa and inflamed or non-inflamed mucosa of inflammatory bowel disease (IBD) patients were analysed by flow cytometry for IgA, CCR10, CXCR3 and CXCR4 cell surface expression. CD, Crohn's disease. (C) LP $CD38^{high}$ plasma cells from normal intestinal mucosa and inflamed mucosa of IBD patients were analysed by flow cytometry for IgA and CCR10/CXCR3/CXCR4 cell surface expression. (D) LP IgA, IgG and IgM plasma cells from normal intestinal mucosa and inflamed mucosa of IBD patients were analysed by flow cytometry for CCR10, CXCR3 and CXCR4 cell surface expression. IgA, surface IgA^+ $CD38^{high}$ plasma cells; IgM, surface IgM^+ $CD38^{high}$ plasma cells; IgG, surface IgA^- and surface IgM^- $CD38^{high}$ plasma cells. (E) Mean fluorescence intensities (MFI) of CCR10 and CXCR4 expression levels on LP $CD38^{high}$ plasma cells from normal controls and UC patients. *p<0.05, **p<0.01, ***p<0.001. (F) Immunofluorescent double staining of colonic mucosa from UC patients stained with anti-IgG (red) and anti-CXCR4 (green). Double-positive cells displaying a classical plasma cell morphology are observed in yellow (arrowheads). Data are representative of three independent specimens.

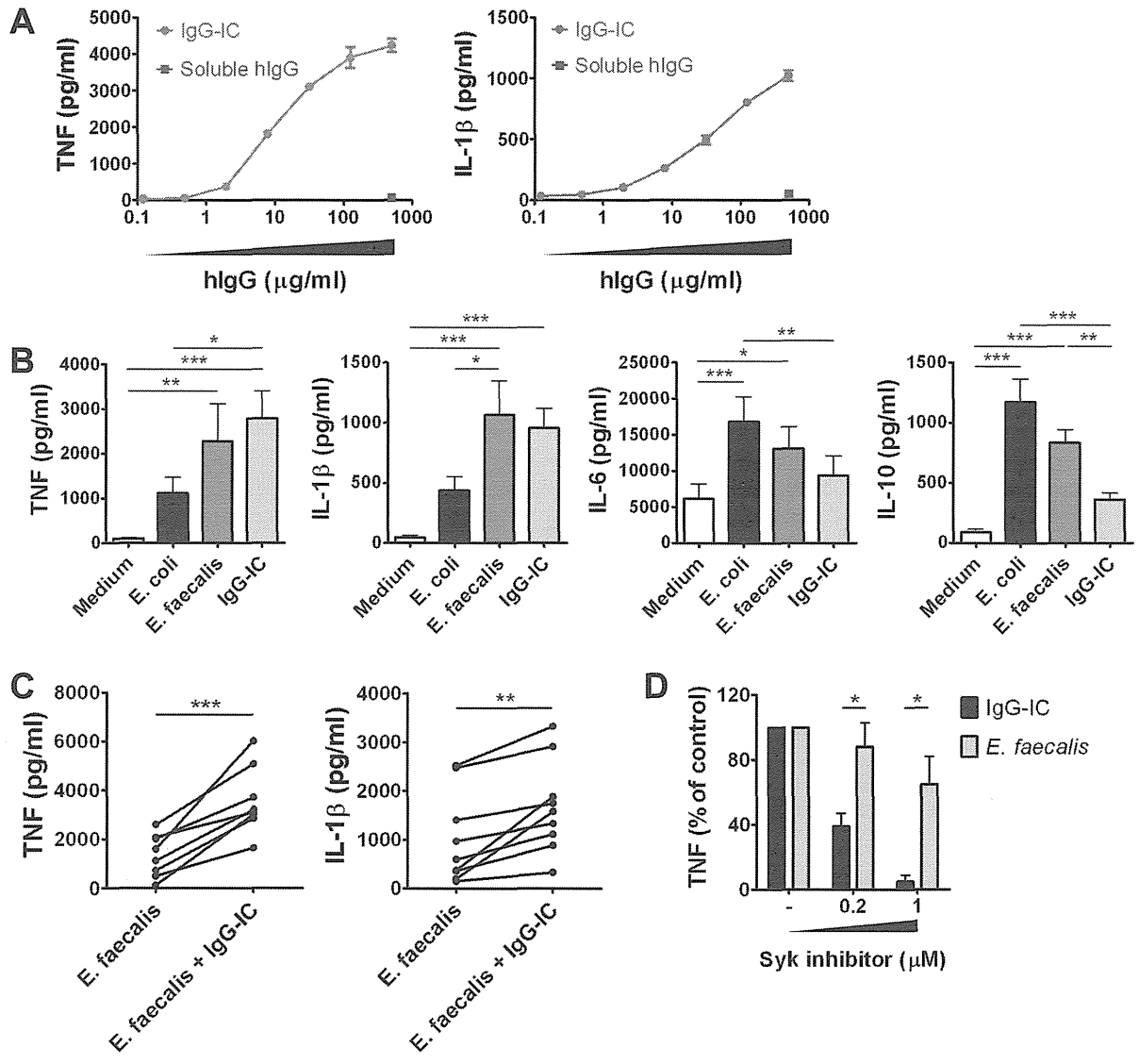

Figure 4 IgG-immune complex (IC) stimulation induces abundant pro-inflammatory cytokine production by lamina propria mononuclear cells (LPMCs) from ulcerative colitis (UC) patients. (A) Cytokine production by LPMCs from the inflamed mucosa of UC patients stimulated with IgG-IC or soluble human IgG (hIgG). Data represent means±SD and are representative of three independent experiments. (B) Cytokine production by LPMCs from the inflamed mucosa of UC patients stimulated with heat-killed commensal bacteria (*E coli* or *E faecalis*) or IgG-IC. Data represent means±SEM (n=11); statistical analysis was performed using repeated measures ANOVA and Tukey's multiple comparison test. (C) Cytokine production by LPMCs from the inflamed mucosa of UC patients stimulated with *E faecalis* alone or with IgG-IC (paired *t* test, n=9). (D) Effect of Syk inhibitor on IgG-IC or *E faecalis*-induced TNF production by LPMCs from the inflamed mucosa of UC patients. Data represent means±SEM (n=4) and statistical analysis was performed using paired *t* test. *p<0.05, **p<0.01, ***p<0.001.

IgG-IC stimulation induces abundant pro-inflammatory cytokine production by LPMCs from patients with UC

The mechanism by which IgG plasma cells contribute to the UC pathogenesis remains unclear. We therefore evaluated the potential involvement of IgG-IC-FcγR signalling in intestinal inflammation. Our hypothesis was that IgG antibodies produced by IgG plasma cells form an IgG-IC with their specific antigens, thereby activating a particular immune cell subset via FcγRs and exacerbating intestinal inflammation. To test this, we examined the cytokine production by LPMCs from the inflamed mucosa of UC patients in response to IgG-IC stimulation, using plate-immobilised IgG to mimic complexed IgG.[20] As expected, we found that IgG-IC stimulation, but not soluble human IgG, induced the production of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1β (IL-1β) by UC LPMCs in a dose-dependent manner (figure 4A).

The cytokine production induced by IgG-IC stimulation was compared with that induced by commensal bacteria (heat-killed *Escherichia coli* and *Enterococcus faecalis*) stimulation, which plays an important role in the pathogenesis of IBD.[1 2] Notably, we found that pro-inflammatory cytokine (TNF and IL-1β) production was stimulated to similar levels by IgG-IC and commensal bacteria (*E faecalis*), whereas the amount of other cytokines (IL-6 and IL-10) produced by IgG-IC was relatively low (figure 4B). In addition, IgG-IC stimulation could act additively with commensal bacterial stimulation, as they co-stimulated LPMCs to produce many more pro-inflammatory cytokines than commensal bacteria stimulation alone (figure 4C). Furthermore, FcγR signalling inhibitor (spleen tyrosine kinase (Syk) inhibitor) selectively inhibited IgG-IC-induced TNF production in a dose-dependent manner, demonstrating that IgG-IC stimulation-induced pro-inflammatory cytokine production is transmitted

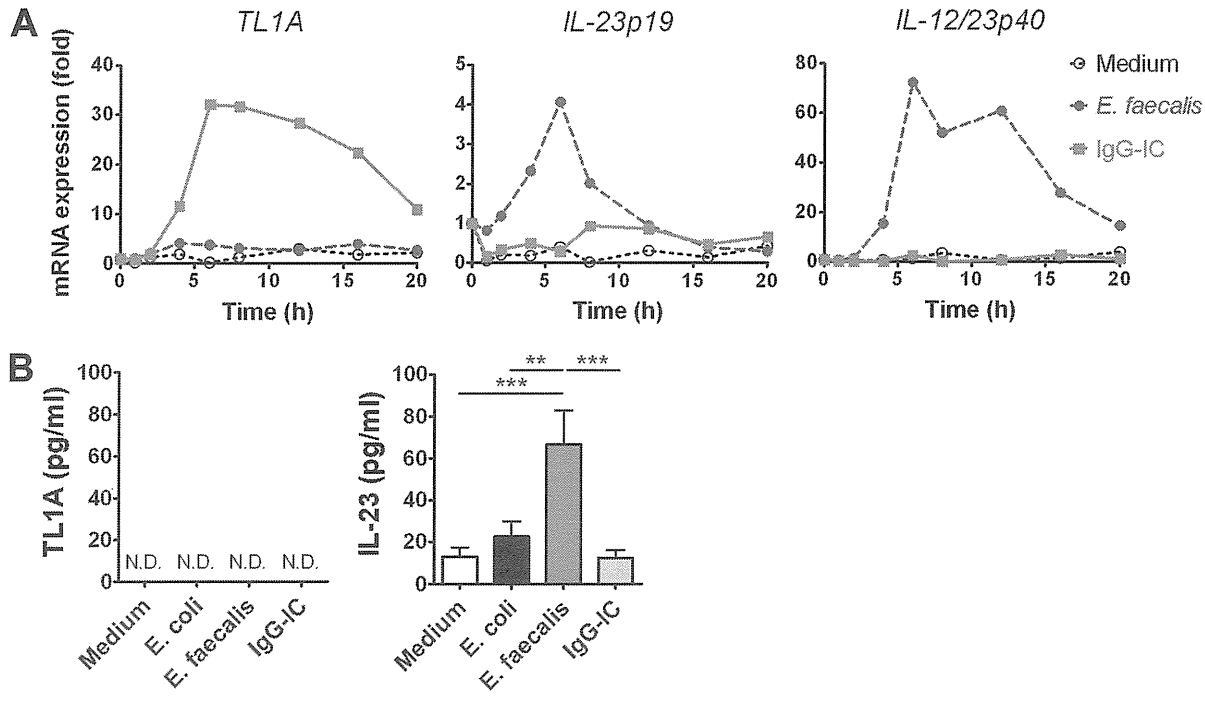

Figure 5 IgG-immune complex (IC) stimulation and commensal bacteria stimulation induce the production of different pro-inflammatory cytokines by lamina propria mononuclear cells (LPMCs) from ulcerative colitis (UC) patients. (A) mRNA expression of *TL1A*, *IL-23p19* and *IL-12/23p40* by LPMCs from the inflamed mucosa of UC patients after stimulation with heat-killed commensal bacteria (*E faecalis*, blue dotted line) or IgG-IC (red solid line). Data are representative of two independent experiments. (B) Cytokine production by LPMCs from the inflamed mucosa of UC patients stimulated with heat-killed commensal bacteria (*E coli* or *E faecalis*) or IgG-IC. Data represent means±SEM (n=11); statistical analysis was performed using repeated measures ANOVA and Tukey's multiple comparison test. **p<0.01, ***p<0.001, N.D., not detected.

through the activation of FcγR(s) and its downstream kinase Syk (figure 4D). These findings demonstrate that IgG-IC activates UC LPMCs through an independent signal transduction pathway and is another potent inducer of intestinal inflammation, comparable to commensal bacteria. We obtained similar results in CD patients (see online supplementary figure S3A).

IgG-IC stimulation and commensal bacteria stimulation induce the production of different pro-inflammatory cytokines by LPMCs from patients with UC

Although the amount of pro-inflammatory cytokines produced by both IgG-IC and commensal bacteria stimulation was similar, we postulated that the cytokine expression profiles must differ between these two stimulations. For example, it was previously reported that TL1A (TNFSF15) expression is strongly induced by IgG-IC, but not TLR ligand, stimulation in human monocytes and dendritic cells.[20]

We focused here on two key pro-inflammatory cytokines, TL1A and IL-23, which were reported to be largely involved in intestinal inflammation and the pathogenesis of IBD.[21] We stimulated UC LPMCs with commensal bacteria (*E faecalis*) or IgG-IC, and then assessed the mRNA expression of *TL1A*, *IL-23p19* and *IL-12/23p40*. As shown in figure 5A, the expression of *TL1A* was strongly induced by IgG-IC stimulation, whereas the expression of *IL-23p19* and *IL-12/23p40* was specifically induced by commensal bacteria stimulation. To determine whether induction of these cytokines led to protein expression, TL1A and IL-23 levels were measured in supernatants following stimulation. Although the production of IL-23 was detected in commensal bacteria (*E faecalis*)-stimulated samples, soluble TL1A production was not detected in all samples (figure 5B), suggesting that the expression of the membrane-bound form of TL1A was induced by IgG-IC stimulation.[22] Thus IgG-IC stimulation is thought to be involved in intestinal inflammation through distinct signal transduction pathways, resulting in differential cytokine induction from commensal bacteria stimulation.

Intestinal CD14 macrophages are responsible for IgG-IC-FcγR signalling-induced pro-inflammatory cytokine production by LPMCs from patients with UC

Finally, to determine which subset of immune cells is responsive to IgG-IC stimulation, LPMCs from the inflamed mucosa of UC patients were analysed for the expression of FcγRs by flow cytometry. As shown in figure 6A, some CD16 (FcγRIII) cells were detected in the $CD14^-$ population, which was considered to be composed of neutrophils. CD32 (FcγRII) cells were partially found to be CD14 cells, and were also detected in the $CD14^-$ population, which mainly comprised plasma cells (data not shown). CD64 (FcγRI) cells were largely CD14 cells. We therefore focused on these CD14CD33 cells as the subset that expressed FcγRI and FcγRII (figure 6B). Previously, we reported that this subset was composed of unique intestinal macrophages that are increased in number in the inflamed mucosa of IBD, and produce large quantities of TNF and IL-23 in response to commensal bacteria, contributing to the pathogenesis of IBD.[23]

To analyse whether these CD14 macrophages are responsible for the IgG-IC-FcγR signalling-induced pro-inflammatory cytokine production by UC LPMCs, we isolated or depleted CD14 cells from LPMCs, and stimulated them with IgG-IC. We found that isolated CD14 macrophages produced large quantities of TNF following IgG-IC stimulation (figure 6C), whereas LPMCs depleted of CD14 macrophages did not (figure 6D). These results demonstrate that CD14 macrophages are responsible for

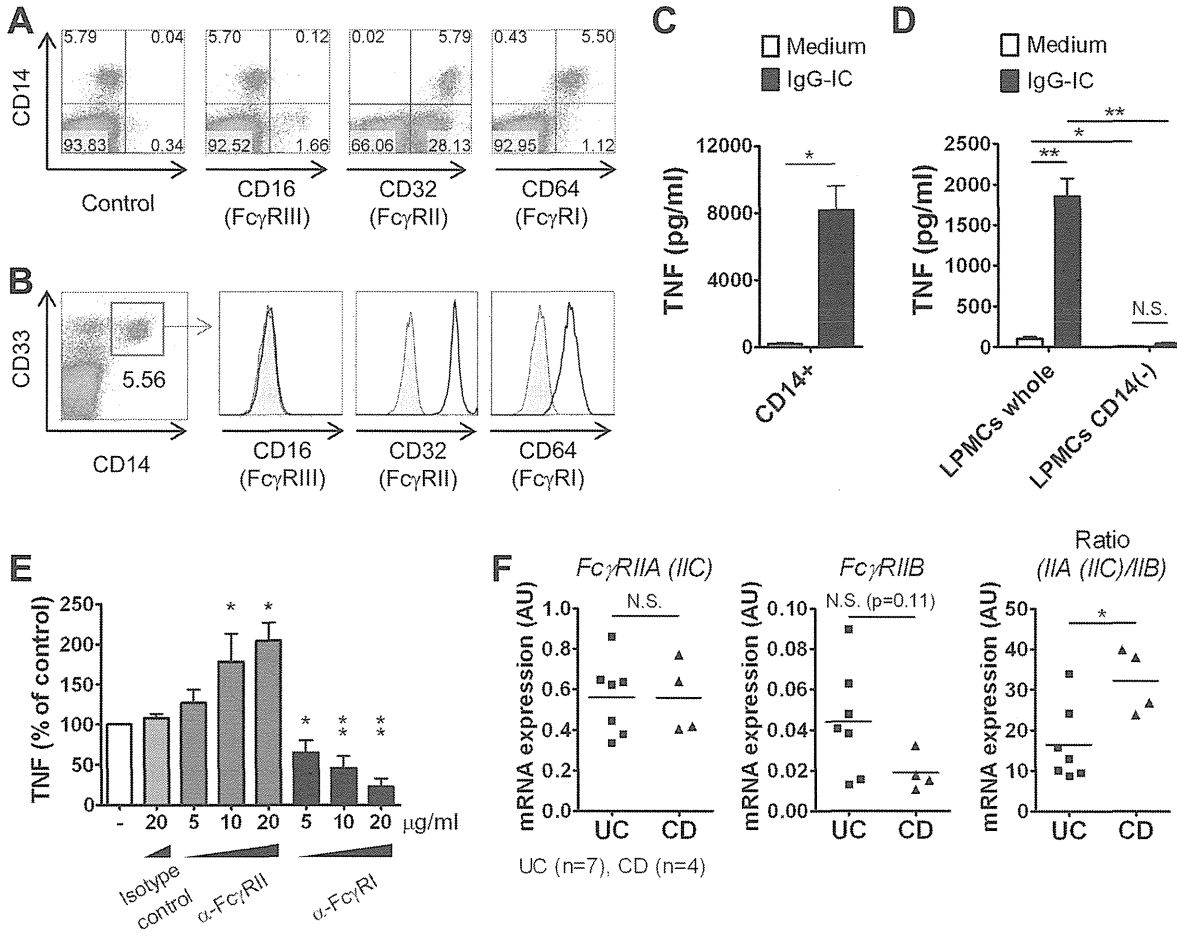

Figure 6 Intestinal CD14 macrophages are responsible for IgG-immune complex (IC)-FcγR signalling-induced pro-inflammatory cytokine production by lamina propria mononuclear cells (LPMCs) from ulcerative colitis (UC) patients. (A) LPMCs from the inflamed mucosa of UC patients were analysed by flow cytometry for CD14 and CD16 (FcγRIII)/CD32 (FcγRII)/CD64 (FcγRI) cell surface expression. Data are representative of three independent patients. (B) Flow cytometry for CD16 (FcγRIII)/CD32 (FcγRII)/CD64 (FcγRI) cell surface expression in CD14CD33 macrophages. The shaded histograms show staining with isotype controls. (C) IgG-IC-induced TNF production by isolated CD14 macrophages from the inflamed mucosa of UC patients (201.9±65.3 vs 8157.8±1463.5 pg/ml, n=3). (D) IgG-IC-induced TNF production by LPMCs or LPMCs depleted of CD14 cells from the inflamed mucosa of UC patients (n=5). (E) Effect of FcγRI or FcγRII blocking antibody on IgG-IC-induced TNF production by LPMCs from the inflamed mucosa of UC patients (n=3). Statistical analysis was performed using repeated measures ANOVA and Dunnett's multiple comparison test (vs isotype control). (F) Quantitative real-time RT-PCR of basal mRNA expression levels in isolated CD14 macrophages from the inflamed mucosa of inflammatory bowel disease patients. AU, arbitrary unit. *p<0.05, **p<0.01, N.S., not significant.

the IgG-IC-FcγR signalling-induced pro-inflammatory cytokine production by UC LPMCs. We also obtained similar results in CD patients (see online supplementary figure S3B,C).

To establish which FcγR(s) was involved in triggering pro-inflammatory cytokine production, LPMCs were pretreated with anti-FcγR blocking antibodies or related isotype control antibodies before IgG-IC stimulation. FcγRI blocking antibodies inhibited IgG-IC-induced TNF production in a dose-dependent manner (figure 6E). Unexpectedly, however, IgG-IC-induced TNF production was significantly increased by FcγRII blocking antibodies. As human FcγRII has three isoforms with opposite functions, and the balance between these receptors allows the IgG-IC to mediate opposing effects on cell function,[24] we confirmed the expression of these activating and inhibitory receptors on CD14 macrophages by quantitative real-time RT-PCR analysis. As shown in figure 6F, although the balance was different between UC and CD, intestinal CD14 macrophages from the inflamed mucosa of IBD patients expressed both activating (*FcγRIIA* and *FcγRIIC*) and inhibitory (*FcγRIIB*) isoforms. Therefore, the observed increase of cytokine production by treatment with FcγRII blocking antibodies appeared to be caused by blocking inhibitory FcγRIIB. These results demonstrate an involvement of FcγRI and FcγRII (at least inhibitory FcγRIIB) in the regulation of IgG-IC-mediated CD14 macrophage activation and cytokine production.

DISCUSSION

This study explored the contribution of intestinal IgG plasma cells to chronic inflammation in UC patients, and revealed a novel aspect and crucial roles for IgG antibodies and IgG plasma cells in its pathogenesis. We confirmed the known inflammatory condition-correlated massive influx of IgG plasma cells in the intestinal mucosa of IBD, and found that UC plasma cells had a unique immature phenotype. Notably, these characteristics were more evident or were only observed in UC, not CD, plasma cells. Thus, these results are possibly based on the differences in pathophysiological conditions between UC and CD, indicating that UC plasma cells are, at least partially, qualitatively different from CD plasma cells, and also suggesting their differential contribution to the pathogenesis of each disease.

We showed that chemokine receptor-expression profiles differed between IgG ($CCR10^{low}CXCR4^{high}$) and IgA ($CCR10^{high}CXCR4^{low}$) plasma cells in the inflamed mucosa of UC patients. These differences can explain the mechanism of selective infiltration of IgG plasma cells into the inflamed mucosa of UC patients. Although we did not analyse the expression of chemokines in the intestinal mucosa, previous studies reported the up-regulation of CXCR3 ligand chemokines (CXCL9/MIG, CXCL10/IP-10 and CXCL11/I-TAC)[25] and CXCR4 ligand chemokine (CXCL12/SDF-1α)[26] in the inflamed mucosa of IBD patients. We observed no difference in CXCR3 expression between IgA and IgG plasma cells in the inflamed mucosa of UC patients, which does not support the hypothesis that IgG plasma cells selectively infiltrate the inflamed mucosa via CXCR3. Therefore, we suggest that CXCR4 is crucially involved in the selective infiltration of IgG plasma cells into the inflamed mucosa of UC patients.

Recent studies have described the relationship between the CXCL12-CXCR4 axis and the pathophysiology of several autoimmune diseases such as RA, MS and SLE.[27–29] For example, Nanki *et al* reported that synovial tissue CD4 memory T cells highly express CXCR4, and that the CXCL12 concentration is relatively high in the synovial fluid of RA patients.[27] Dotan *et al* reported that the expression of CXCL12 is up-regulated in the intestinal mucosa (specifically more inflamed mucosa) of patients with IBD, especially UC, and showed a chemotactic effect of CXCL12 on LP T cells in IBD patients.[26] In addition, Mikami *et al* reported that CXCR4 expression on peripheral T cells was increased in patients with active UC, and showed that administration of a CXCR4 antagonist decreased the severity of dextran sodium sulphate (DSS)-induced mice colitis and the colonic inflammation of IL-10-knockout mice.[30] These reports suggest that the CXCL12-CXCR4 axis plays a crucial role in cell trafficking under inflammatory conditions, and might be a common pathway in IBD and other autoimmune diseases for the homing of T cells and IgG plasma cells to inflamed tissues.

Although the infiltration of IgG plasma cells into the inflamed mucosa is a well-known characteristic feature of UC, its contribution to the UC pathogenesis is controversial. It has been reported that colonic plasma cells in UC patients express multiple β-defensins, possibly contributing to host defence,[6] whereas intestinal plasma cells in UC patients were shown to express large amounts of matrix metalloproteinase-3, suggesting a crucial role in tissue destruction.[7] As the classical role of plasma cells is defined by antibody secretion, we previously showed that IgG autoantibodies against colonic epithelial cells produced by intestinal lymphocytes in UC patients bind the epithelial cell surface and mediate ADCC.[31] The present study evaluated the potential involvement of IgG-IC-FcγR signalling in intestinal inflammation and identified IgG-IC as another potent inducer of intestinal inflammation besides commensal bacteria. Moreover, among UC LPMCs, we identified CD14 macrophages as an immune cell subset responsible for IgG-IC-FcγR signalling-induced pro-inflammatory cytokine production. Intestinal CD14 macrophages were therefore found to be key pathogenic immune cells that respond not only to commensal bacteria but also to IgG-IC, further highlighting their crucial roles in intestinal inflammation and IBD pathogenesis.

The present study showed that commensal bacteria and IgG-IC activate intestinal CD14 macrophages through distinct signal transduction pathways and induce the expression of pro-inflammatory molecules with different profiles. Consistent with our results, it has been reported that both commensal bacteria and IgG-IC can initiate TL1A expression, yet commensal bacteria are less efficient in TL1A induction, and the continued activation of TL1A expression requires FcγR signalling in human CD14 monocytes.[32] Importantly, we and others previously reported that TL1A and IL-23 synergistically enhance T cell function.[21 22] Therefore, IgG-IC signalling induces not only the production of pro-inflammatory cytokines but also the expression of the T cell co-stimulatory molecule TL1A, and possibly exacerbates the disease through T cell-mediated inflammation. In addition, *TL1A* and *IL-23R* have been identified and confirmed as IBD susceptibility genes,[33 34] and *TL1A* is also implicated in severe UC.[35] Therefore, these distinct potent inducers of the inflammatory response might mutually accelerate intestinal inflammation and synergistically contribute to the establishment of the complicated pathophysiological conditions of IBD.

Using blocking antibodies, we revealed the involvement of activating FcγRI and inhibitory FcγRIIB in the regulation of IgG-IC-mediated CD14 macrophage activation. We could not clarify the involvement of activating FcγRIIA, as no isoform-specific blocking antibodies are commercially available. As mentioned above, *FcγRIIA* has been identified as a susceptible gene of UC, and it is suggested that individuals with the His131 variant of FcγRIIA, the susceptibility loci of UC, might have a higher capacity for IC, which could lead to hyperactivation of immune cells.[12] Therefore, although further studies focusing on the involvement of FcγRIIA and the effects of susceptibility loci in the regulation of IgG-IC-mediated CD14 macrophage activation are needed, our proposed concept agrees with this hypothesis and suggests a novel mechanism of IgG-IC-mediated intestinal inflammation.

Several previous reports have described the relationship between plasma cell infiltration and IBD clinical events. For example, basal plasmacytosis, defined as a dense infiltration of plasma cells extending into the lower third of the LP, is associated with a shorter time to relapse in UC patients.[36] In addition, in a mouse model of colitis, Kobayashi *et al* reported that passive administration of anti-bacterial IgG (anti-flagellin IgG) promotes the development of intestinal inflammation in DSS-induced colitis, suggesting that antibacterial IgG antibodies are themselves pathogenic.[37] Together, these reports suggest that intestinal plasma cells and IgG antibodies have the potential to exacerbate intestinal inflammation, consistent with our present results.

Several unresolved issues remain, one of which is the identification of antigens that react with IgG antibodies produced by intestinal IgG plasma cells to form IgG-IC. We presume that a number of heterogeneous antigens are involved, and that components of commensal bacteria and auto-antigens are potent candidates for such antigens. Previous reports support this hypothesis: marked elevation of IgG antibodies specific for bacterial antigens is a characteristic hallmark of human IBD[38]; and IgG auto-antibodies against colonic epithelial cells (including anti-human tropomyosin isoform 5 antibodies) are also observed in UC patients.[39]

The present study does not show direct evidence that IgG antibodies produced by intestinal IgG plasma cells can form IC with their specific antigens, and activate intestinal CD14 macrophages through FcγRs in vivo. A recent study reported that DSS-treated RORγt-deficient mice showed severe colonic inflammation, characterised by infiltration of IgG plasma cells, which was mitigated by treatment with intravenous IgG (IVIG).[40] As the anti-inflammatory effect of IVIG treatment is FcγRs-dependent, the authors hypothesised that the severe inflammation is a consequence of the formation of IC consisting

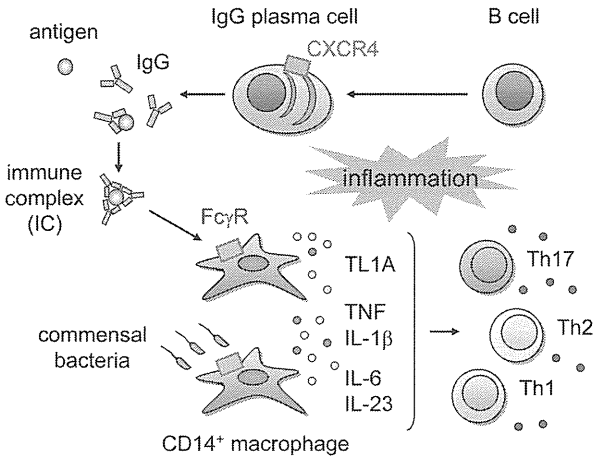

Figure 7 Schematic representation of the pathogenic role of intestinal CXCR4$^+$ IgG plasma cells in human ulcerative colitis (UC). Unique IgG plasma cells might infiltrate the inflamed mucosa via CXCR4, and IgG antibodies produced by IgG plasma cells form immune complex (IC) with their specific antigens, thereby activating intestinal CD14 macrophages via FcγR. IgG-IC-activated CD14 macrophages produce large quantities of TNF and IL-1β, and notably up-regulate the expression of TL1A. Commensal bacteria also activate CD14 macrophages and induce the production of TNF, IL-1β, IL-6 and IL-23. Collectively, these distinct potent inducers of inflammatory response might mutually accelerate intestinal inflammation and synergistically contribute to the establishment of the complicated pathophysiological conditions of UC.

of bacteria and specific IgG, which activates FcγR-expressing inflammatory cells. Collectively, these data suggest that IgG plasma cells accumulating in intestinal mucosa trigger IgG-IC-FcγR signalling-mediated intestinal inflammation in mice, and possibly in humans.

In summary, our results reveal a novel aspect of UC pathogenesis in which unique IgG plasma cells infiltrate the inflamed mucosa via CXCR4, and critically influence UC pathogenesis by exacerbating mucosal inflammation through the activation of 'pathogenic' intestinal CD14 macrophages via IgG-IC-FcγR signalling (figure 7). Modulation of these IgG plasma cells or the signalling pathway might therefore represent a promising therapeutic strategy for the treatment of UC.

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Contributors MU, TH (Hisamatsu): conceived and designed the study, wrote the manuscript. MU: conducted most of the experiments, analysed data. JM, DK, KY, MK, AS, KK: provided materials and performed experiments. MM, KM, TK: helped design the study and interpretation of data. TH (Hibi): directed and supervised the study.

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Microbe-dependent CD11b$^+$ IgA$^+$ plasma cells mediate robust early-phase intestinal IgA responses in mice

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Intestinal plasma cells predominantly produce immunoglobulin (Ig) A, however, their functional diversity remains poorly characterized. Here we show that murine intestinal IgA plasma cells can be newly classified into two populations on the basis of CD11b expression, which cannot be discriminated by currently known criteria such as general plasma cell markers, B cell origin and T cell dependence. CD11b$^+$ IgA$^+$ plasma cells require the lymphoid structure of Peyer's patches, produce more IgA than CD11b$^-$ IgA$^+$ plasma cells, proliferate vigorously, and require microbial stimulation and IL-10 for their development and maintenance. These features allow CD11b$^+$ IgA$^+$ plasma cells to mediate early-phase antigen-specific intestinal IgA responses induced by oral immunization with protein antigen. These findings reveal the functional diversity of IgA$^+$ plasma cells in the murine intestine.

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Immunoglobulin (Ig) A is an antibody found predominantly in the intestinal lumen, where it protects the host against pathogenic infections[1,2]. It also has an important role in the creation and maintenance of immunological homoeostasis by shaping homeostatic communities of commensal bacteria[3–5]. Indeed, some patients with IgA deficiency show marked susceptibility to infections with pathogens such as *Giardia lamblia*, *Campylobacter*, *Clostridium*, *Salmonella* and rotavirus; they also have increased incidences of intestinal immune diseases such as coeliac disease and inflammatory bowel diseases[6].

Peyer's patches (PPs) are the major sites for the initiation of antigen-specific intestinal IgA production, mainly in a T cell-dependent manner[7]. Intestinal IgA also originates from B1 cells. B1 cells differ from B2 cells in terms of origin, surface markers (for examples, B220, IgM, IgD, CD5, CD11b and CD23), growth properties and V_H repertoire[8–10]. B1 cells are predominantly present in the peritoneal cavity (PerC) and traffic into the intestinal compartment for the production of IgA against T cell-independent antigens such as DNA and phosphatidylcholine[11]. T cell independent antigen-specific IgA responses are also initiated in the isolated lymphoid follicles (ILFs), which are small clusters of B2 cells in the intestine[12].

Upon Ig class switching from μ to α, IgA^+ B cells acquire the expression of type 1 sphingosine-1-phosphate receptor, CCR9 and α4β7 integrin, allowing them to migrate out from the PPs or PerC and traffic to the intestinal lamina propria (iLP)[11,13,14]. In the iLP, they further differentiate into IgA-secreting plasma cells (PCs) under the influence of terminal differentiation factors (for example, IL-6)[15]. As these locally produced IgA antibodies are continuously transported and secreted by epithelial cells as a form of secretory IgA into the intestinal lumen, stably high levels of IgA production are required for the maintenance of sufficient amounts of IgA; this production is determined by the generation, survival and function of IgA PCs.

Several lines of evidence have demonstrated that the function and survival of PCs in the systemic compartments (for example, spleen and bone marrow (BM)) are not only determined by intrinsic factors but are regulated by the presence of environmental niches[16]. As with systemic PCs, differentiation of IgA PCs in the iLP is regulated by exogenous factors such as IgA-enhancing cytokines (for example, interleukin (IL)-5, IL-6, IL-10, IL-15, a proliferation-inducing ligand (APRIL) and B cell activating factor (BAFF))[7,15]. In addition, microbial stimulation is required for the full effects of intestinal IgA. Indeed, germ-free (GF) mice have decreased intestinal IgA responses with immature structures of PPs and ILFs[17,18]. Previous studies in mono-associated GF mice have indicated that only a small proportion of the total amount of intestinal IgA is reactive to monoassociated bacteria; microbe-dependent IgA production is therefore mediated by polyclonal stimulation through innate immune receptors such as toll-like receptors, rather than through B cell receptors specific for microbial antigens[19,20]. Accumulating evidence has revealed the molecular and cellular pathways of IgA production mediated by innate immunity, including the involvement of myeloid differentiation primary response gene 88 (MyD88) in the regulation of tumour necrosis factor/inducible nitric oxide synthase-producing DCs in the iLP[21] and follicular DCs in the PPs[22]. However, the effects of microbial stimulation on the regulation of differentiated IgA^+ PCs remain to be investigated. Here, we identified unique microbe-dependent subsets of IgA^+ PCs, which add a new level of complexity to the intestinal IgA system of mice.

Results

Microbe dependency of intestinal IgA^+ cells. To examine the immunological elements of intestinal IgA production associated with commensal bacteria, we initially compared the IgA^+ cells of specific pathogen-free (SPF) and GF mice. Flow cytometric analysis showed that $CD11b^+$ IgA^+ cells accounted for about 30% of IgA^+ cells, and we found a lack of $CD11b^+$ IgA^+ cells in the iLP of GF mice (Fig. 1a). Similarly, the numbers of intestinal $CD11b^+$ IgA^+ cells were reduced in both antibiotic-treated SPF mice and MyD88 KO mice (Fig. 1b–d). Immunohistological analysis indicated that $CD11b^+$ IgA^+ cells were dispersed throughout the iLP of wild-type (WT) mice (Fig. 1d), although their frequency appeared lower than expected from the flow cytometric data, probably because of difference in methodological sensitivity. These findings collectively suggest that $CD11b^+$ IgA^+ cells are unique subset that requires MyD88-dependent microbial stimulation for its development and maintenance.

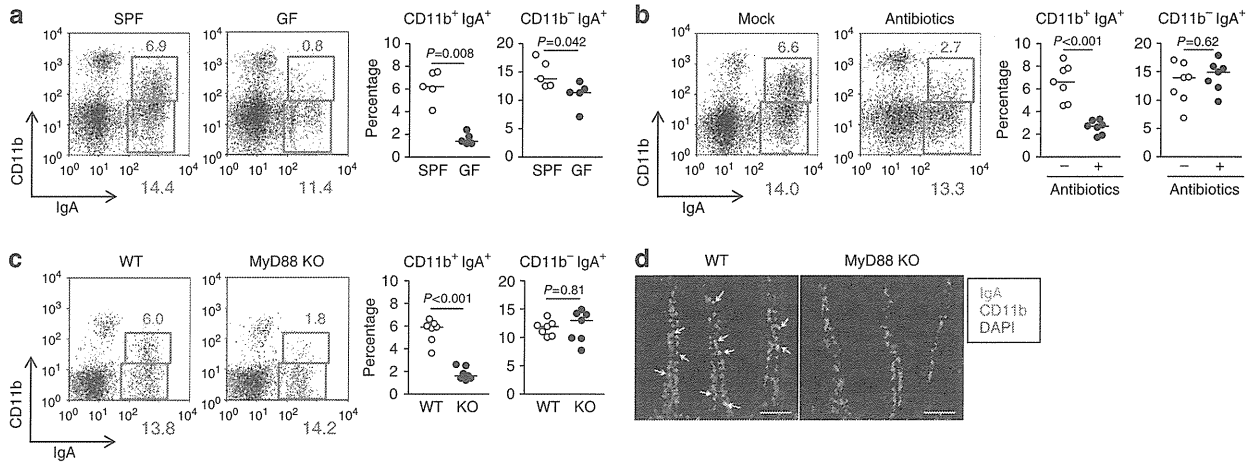

Figure 1 | Intestinal $CD11b^+$ IgA^+ cells require microbial stimulation. (**a**–**c**) Mononuclear cells were isolated from the small intestines of SPF or GF mice (**a**), mock- or antibiotic-treated SPF mice (**b**), or MyD88 WT or knockout (KO) mice (**c**) for analysis of IgA and CD11b expression by flow cytometry. Graphs show data from individual mice, and bars indicate median. Statistical analyses were performed with Mann–Whitney's *U*-test. (**d**) Specimens of small intestinal tissues of WT and MyD88 KO mice were stained for IgA and CD11b, and counterstained with 4′,6-diamidino-2-phenylindole. Data are representative of three independent experiments. Scale bars, 50 μm.

Intestinal CD11b$^+$ IgA$^+$ cells are PCs. We next aimed to characterize the CD11b$^+$ and CD11b$^-$ IgA$^+$ cells in the iLP. In addition to a gating strategy to exclude the possibility that the CD11b$^+$ IgA$^+$ cells detected by flow cytometry were doublets (Supplementary Fig. S1), we further performed a cytospin analysis and confirmed that both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells had homogeneous morphology that was the same as that of PCs (for example, large irregular nuclei with prominent nucleoli), whereas CD11b^{hi} IgA$^-$ cells were composed of different kinds of cells, including eosinophils and macrophages (Fig. 2a). We also confirmed that both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells did not express markers for macrophages (F4/80), DCs (CD11c) or eosinophils (CCR3) (Fig. 2b). Thus, CD11b$^+$ IgA$^+$ cells are neither doublets nor myeloid cells decorated by bound IgA on their surfaces.

CD11b$^+$ and CD11b$^-$ IgA$^+$ cells were identical in cell size and density, as determined by forward scatter (FSC) and side scatter (SSC), respectively, and by their surface expression patterns (CD19^{int}, B220$^-$, CD138$^+$, CD38^{hi} and CD40^{int}) (Fig. 2c). Although PCs in the systemic compartments (for example, the spleen) generally express little or no surface immunoglobulin[23], we previously confirmed that CD38$^+$ CD138$^+$ cells in the iLP express IgA both on the cell surface and in the intracellular compartment (Supplementary Fig. S2)[13]. These findings indicated that both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells could be classically categorized as PCs. This view was further supported by our finding that both populations expressed equal levels of Blimp1, a master transcription factor for PCs (Fig. 2c)[23].

The phenotypes of IgA$^+$ cells in the iLP differed from those of IgA$^+$ cells in the spleen. Splenic CD11b$^-$ IgA$^+$ cells exclusively had a memory phenotype (that is, B220$^+$, CD138$^-$, CD38^{int} and CD40^{hi}), whereas splenic CD11b$^+$ IgA$^+$ cells contained almost equal amounts of B220$^+$ CD138$^-$ CD38^{int} CD40^{hi} memory cells and B220$^-$ CD138$^+$ CD38^{hi} CD40^{low} PCs (Supplementary Fig. S3). These results indicated that CD11b$^+$ IgA$^+$ cells in the iLP were unique PCs that had an immunologically different status from splenic CD11b$^+$ IgA$^+$ cells.

Intestinal CD11b$^+$ IgA$^+$ PCs require PP lymphoid structure. CD11b$^+$ IgA$^+$ PCs expressed CD18 (Supplementary Fig. S4), which associates with CD11b and acts as a ligand for intercellular adhesion molecule-1 (ICAM-1)[24]. As ICAM-1 is an endothelial adhesion molecule that regulates cell trafficking[24,25], we considered that CD11b$^+$ IgA$^+$ PCs were recent emigrants from IgA-inductive tissues (for example, PPs and PerC) and had migrated into the iLP. To test this possibility, we employed FTY720 to inhibit the trafficking of IgA-committed B cells from PPs and PerC into the iLP. As we previously reported[11,13], FTY720 treatment reduced the numbers of intestinal IgA$^+$ PCs,

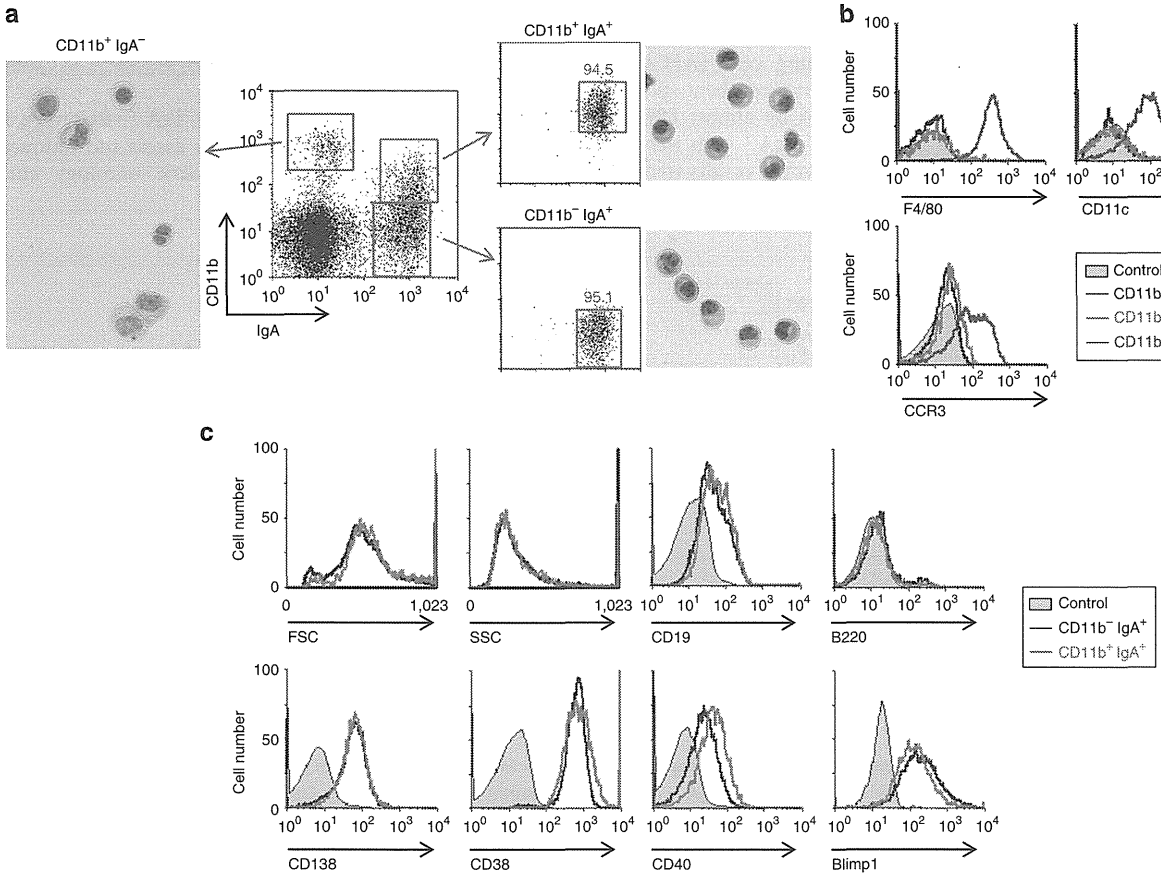

Figure 2 | Both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells in the intestine are categorized as plasma cells. (**a**) Cells were purified by cell sorting from the iLP, and their morphology was examined by haematoxylin and eosin staining after cytospin. Data are representative of three independent experiments. (**b**) Cells were isolated from the iLP for the analysis of F4/80, CD11c and CCR3 expression on CD11b$^-$ IgA$^+$, CD11b$^+$ IgA$^+$ and CD11b$^+$ IgA$^-$ cells. Grey indicates isotype control. Similar results were obtained from three separate experiments. (**c**) Cells were isolated from the iLP for comparisons between CD11b$^+$ and CD11b$^-$ IgA$^+$ cells in terms of cell size (FSC) and density (SSC), and expression of CD19, B220, CD138, CD38, CD40 and Blimp1. Grey indicates isotype control. Similar results were obtained from five separate experiments.

but the effect was not specific to CD11b$^+$ IgA$^+$ PCs (Fig. 3a). These data suggested that CD11b$^+$ IgA$^+$ PCs were not recent emigrants from IgA inductive tissues (for example, PPs and PerC).

The second possibility was that CD11b$^+$ IgA$^+$ PCs originated from B1 cells, because CD11b is a marker of peritoneal B1 cells[26]. To test this possibility, peritoneal CD11b$^+$ B1 cells were purified and adoptively transferred into severe combined immunodeficiency mice. As we reported previously[11], adoptively transferred CD11b$^+$ B1 cells migrated into the intestine, where they differentiated into IgA$^+$ PCs. Although we transferred B cells expressing CD11b, they lost their CD11b expression in the iLP (Supplementary Fig. S5). Although only a few cells were detected in the iLP under these experimental conditions, CD11b expression was likely to be reversible on B cells and was thus not to be a marker of PCs originating from peritoneal CD11b$^+$ B1 cells.

As a third possibility for discriminating between CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs, we examined the T cell dependency of their differentiation and IgA production. For this, we employed TCRβδ mice. Although TCRβ δ mice had decreased levels of intestinal IgA$^+$ cells, the ratio between CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs did not differ between the WT mice and the TCRβ δ mice (Fig. 3b).

We also examined the production of IgA against T cell dependent and T cell independent antigens by CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs. For the analysis of T cell dependent antigen, mice were orally immunized with ovalbumin (OVA) plus cholera toxin (CT). Following three oral immunizations, substantial amounts of OVA-specific IgA antibody-forming cells (AFCs) were detected in the iLP by enzyme-linked immunosorbent spot (ELISPOT) assay; this production was reduced by almost 50% when either the CD11b$^+$ IgA$^+$ or the CD11b$^-$ IgA$^+$ cells were removed before the ELISPOT assay (Fig. 3c). Similar results were obtained when we enumerated IgA AFCs against phosphorylcholine, a typical TI antigen, induced by commensal bacteria (Fig. 3c)[27]. These results collectively suggested that both CD11b$^+$ IgA$^+$ and CD11b$^-$ IgA$^+$ cells almost equally included IgA AFCs producing IgA antibodies specific for T cell dependent and T cell independent antigens.

Next, to examine the involvement of PPs, we established PP-null mice by *in utero* treatment with anti-IL-7Rα antibody[28] and found that PP-null mice had reduced numbers of CD11b$^+$ IgA$^+$ PCs in the iLP (Fig. 3d). In addition, CD11b was not expressed on IgA$^+$ B cells in the PPs (Fig. 3e). We treated mice with anti-IL-7Rα antibody only once *in utero* and confirmed that it did not affect the ILFs[28]. Although it is still possible that CD11b$^+$ IgA$^+$ PCs specifically require IL-7, the most plausible conclusion based on our current findings is that CD11b$^+$ IgA$^+$ B cells require the lymphoid structure of PPs, and CD11b$^-$ IgA$^+$ B cells acquire CD11b expression in the iLP.

As in antibiotic-treated and MyD88 KO mice (Fig. 1), the numbers of CD11b$^-$ IgA$^+$ PCs changed little in PP-null mice (Fig. 3d), suggesting that it is unlikely that CD11b$^+$ IgA$^+$ PCs differentiate back into CD11b$^-$ IgA$^+$ cells in the iLP. This view is further supported by the results of *in vitro* analysis. When purified CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs were separately cultured with different kinds of stimulants (for example, phorbol 12-myristate 13-acetate plus ionomycin, or lipopolysaccharide) little change was noted in CD11b expression (Supplementary Fig. S6). Although the origin of these cells remains to be firmly established, it is plausible that CD11b$^+$ IgA$^+$ PCs act as a separate lineage once they differentiate in the iLP.

High proliferation activity of CD11b$^+$ IgA$^+$ PCs. We next performed a gene microarray analysis to assess the uniqueness of CD11b$^+$ IgA$^+$ PCs in the iLP. Gene ontology enrich-

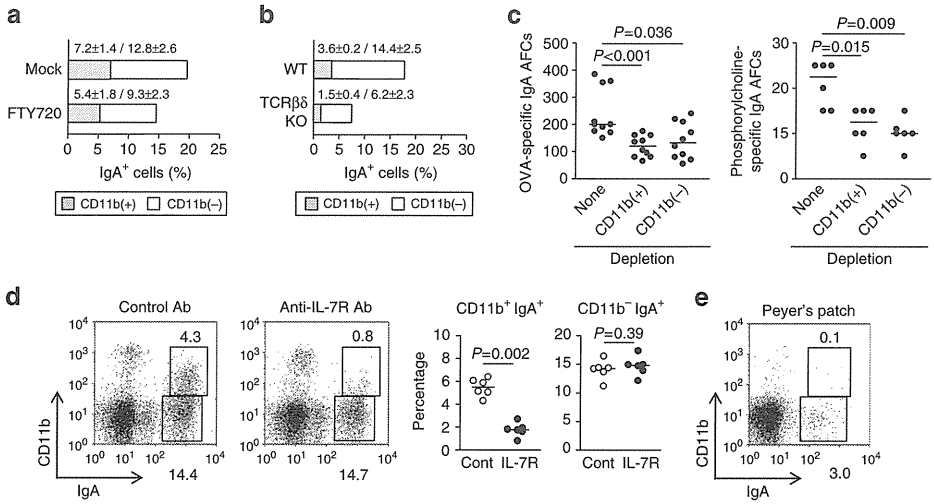

Figure 3 | CD11b$^+$ IgA$^+$ cells require the lymphoid structure of Peyer's patches. (**a**) Mice were treated with FTY720 every day for 5 days. The day after the final treatment, the proportions of CD11b$^+$ and CD11b$^-$ IgA$^+$ cells were measured by flow cytometry. Data are presented as means ± s.d. from four mice. Similar results were obtained from three separate experiments. (**b**) Proportions of CD11b$^+$and CD11b$^-$ IgA$^+$ cells in the iLP of WT and TCRβδ KO mice were measured by flow cytometry. Data are presented as means ± s.d. from four mice. Similar results were obtained from three separate experiments. (**c**) After three oral immunizations with OVA plus cholera toxin, cells were isolated from the iLP and used in an ELISPOT assay to enumerate OVA-specific IgA AFCs. In some groups of mice, CD11b$^+$ or CD11b$^-$ IgA$^+$ cells were depleted by cell sorting before application of ELISPOT assay. Phosphorylcholine-specific IgA AFCs were measured. Graphs show data from individual mice, and bars indicate median. Statistical analyses were performed with Mann–Whitney's *U*-test. (**d**) Mononuclear cells were isolated from the iLP of Peyer's patch (PP)-normal (control Ab) and -null (anti-IL-7Rα Ab) mice for analysis of IgA and CD11b expression by flow cytometry. Graphs show data from individual mice. Statistical analyses were performed with Mann–Whitney's *U*-test. (**e**) Mononuclear cells were isolated from PPs for analysis of CD11b$^+$ and CD11b$^-$ IgA$^+$ cells by flow cytometry. Similar results were obtained from three separate experiments.

ment score computation analysis showed that the activity of cell-cycle-associated pathways was higher in CD11b$^+$ IgA$^+$ PCs than in CD11b$^-$ IgA$^+$ PCs (Supplementary Table S1). Consistent with this finding, higher expression of cell-cycle-associated genes was noted in CD11b$^+$ IgA$^+$ PCs than in CD11b$^-$ IgA$^+$ PCs; these genes included members of the cell division cycle family (Fig. 4a and Supplementary Table S2). In line with this, these cells expressed higher levels of the proliferation marker Ki67 than did CD11b$^-$ IgA$^+$ PCs (Fig. 4a and Supplementary Table S2). Additionally, CD11b$^+$ IgA$^+$ PCs showed greater uptake of bromodeoxyuridine (BrdU) than did CD11b$^-$ IgA$^+$ PCs (Fig. 4b). CD11b$^+$ IgA$^+$ PCs were preferentially removed by treatment with cyclophosphamide (CPM), which selectively targets proliferating cells (Fig. 4c). These data collectively suggested that CD11b$^+$ IgA$^+$ PCs possessed greater proliferating activity than did CD11b$^-$ IgA$^+$ PCs in the iLP.

Microarray analysis further identified CD150 (also known as signalling lymphocytic activation molecule family member 1, SLAMF1)[29], β1 integrin and CD168 (also known as hyaluronan-mediated motility receptor)[30] as possible candidates uniquely expressed on CD11b$^+$ IgA$^+$ PCs (Supplementary Table S3). Flow cytometric analysis confirmed that CD11b$^+$ IgA$^+$ PCs expressed higher levels of CD150 than did CD11b$^-$ IgA$^+$ PCs, whereas CD11b$^+$ IgA$^+$ and CD11b$^-$ IgA$^+$ PCs identically expressed β1 integrin and no CD168 (Supplementary Fig. S7).

IL-10 is essential for intestinal CD11b$^+$ IgA$^+$ cells. We next aimed to identify key molecules for inducing and maintaining CD11b$^+$ IgA$^+$ PCs in the iLP. As CD11b$^+$ IgA$^+$ PC numbers were reduced in MyD88 mice (Fig. 1c), and MyD88 is expressed in not only hematopoietic cells, including B cells, but also non-hematopoietic cells, including epithelial cells[31], we performed BM chimeric experiments to determine whether MyD88 in non-hematopoietic cells, hematopoietic cells, or both, was required for the generation of CD11b$^+$ IgA$^+$ cells. Similar levels of CD11b$^+$ IgA$^+$ cells were observed in irradiated WT mice receiving WT or MyD88 BM cells and in irradiated MyD88 mice receiving WT BM cells (Supplementary Fig. S8), suggesting that MyD88-dependent molecules commonly expressed in both non-hematopoietic and hematopoietic cells are involved in the microbe-dependent induction of CD11b$^+$ IgA$^+$ PCs.

We then examined the involvement of cytokines known to enhance IgA responses. Among several IgA-enhancing cytokines (for example, IL-5, IL-6, IL-10 and APRIL/BAFF)[7,15], we found that neutralization of IL-10 resulted in preferential reduction in CD11b$^+$ IgA$^+$ PCs, whereas blocking of other cytokines induced a reduction in IgA$^+$ cell numbers regardless of CD11b expression (Fig. 5a). Additionally, CD11b$^+$ IgA$^+$ cell numbers were preferentially reduced in IL-10 KO mice (Fig. 5b). As normal differentiation into IgA$^+$ B cells was observed in the PPs and PerC of IL-10 KO mice (Supplementary Fig. S9), it is plausible that IL-10 targets the maintenance of CD11b$^+$ IgA$^+$ cells in the iLP, but not the induction of IgA$^+$ cells in inductive tissues such as PPs and PerC.

Early-phase robust IgA responses by proliferating IgA$^+$ PCs. To examine the immunological importance of proliferating IgA$^+$ PCs present mainly in CD11b$^+$ IgA$^+$ PCs, mice were orally immunized with OVA plus CT. In this assay, one group received CPM treatment during immunization and the second group received CPM treatment 4 days after the final immunization (Fig. 6a). Because of the high cell-proliferation activity, CPM treatment during oral immunization resulted in efficient killing of peanut agglutinin (PNA^{hi}) B220$^+$ GC B cells and thus a reduction in the numbers of IgA$^+$ IgM$^-$ plasmablasts in the PPs (Supplementary Fig. S10). Thus, treatment with CPM during oral immunization led to an ~90% reduction in the numbers of OVA-specific IgA AFCs (Fig. 6b); this was associated with almost complete disappearance of faecal IgA produced against OVA (Fig. 6c). On the other hand, when mice were treated with CPM 4 days after the final immunization to remove proliferating cells mainly present in CD11b$^+$ IgA$^+$ cells in the iLP, the reduction in numbers of OVA-specific IgA AFCs in the iLP was only about 50% (Fig. 6b). This finding was consistent with our current finding that CD11b$^+$ IgA$^+$ PCs accounted for half the number of OVA-specific IgA AFCs (Fig. 3c). Thus, CPM treatment after the last immunization preferentially depleted CD11b$^+$ IgA$^+$ cells, with little influence on CD11b$^-$

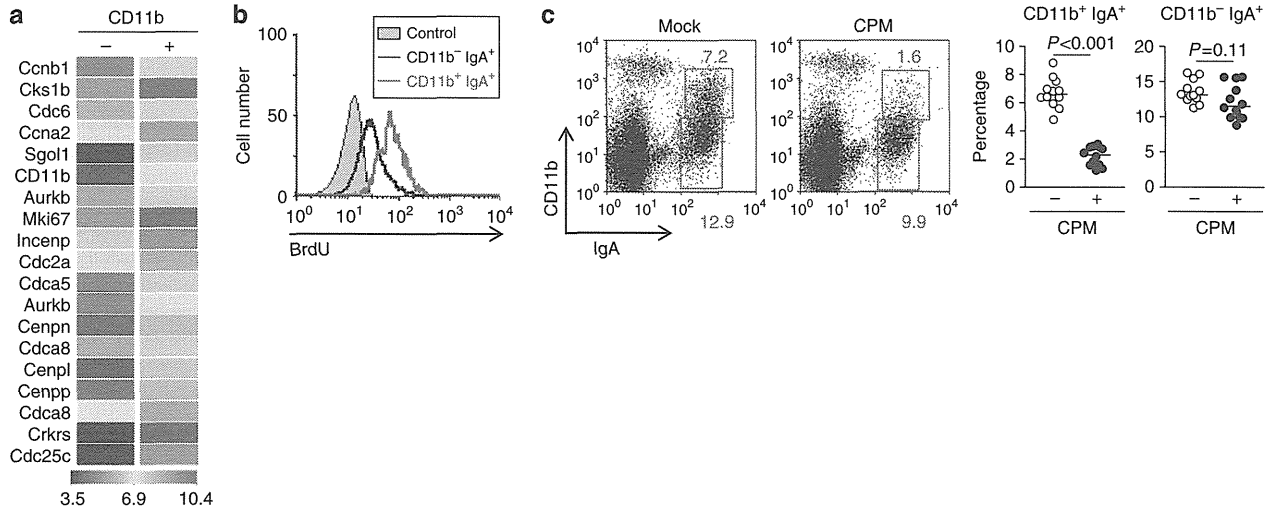

Figure 4 | CD11b$^+$ IgA$^+$ cells are proliferating cells. (**a**) mRNA was purified from small intestinal CD11b$^+$ and CD11b$^-$ IgA$^+$ cells and used for microarray analysis. Data related to the cell cycle and proliferation are shown. Data are representative of two independent experiments. (**b**) Mice were treated with BrdU, and uptake of BrdU by CD11b$^+$ and CD11b$^-$ IgA$^+$ cells was determined by flow cytometry. Data are representative of four independent experiments. (**c**) Cells were isolated from the intestinal lamina propria of mice receiving CPM to analyse CD11b$^+$ IgA$^+$ cells. Similar results were obtained from four separate experiments. Graphs show data from individual mice. Statistical analyses were performed with Mann–Whitney's *U*-test.

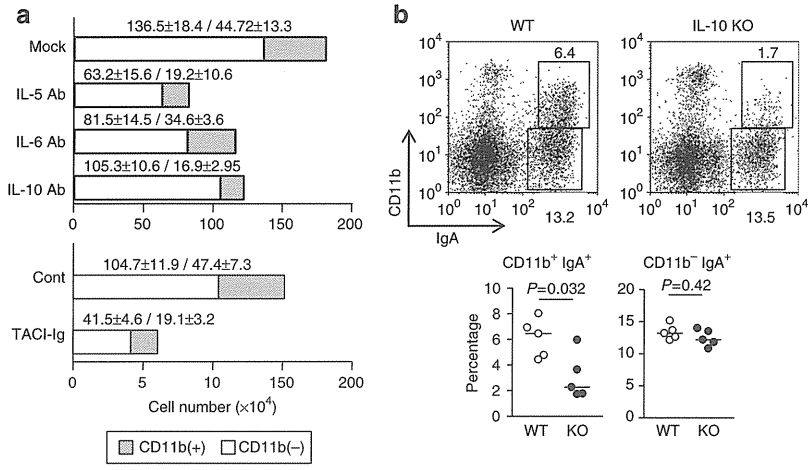

Figure 5 | Role of IL-10 in the maintenance of CD11b$^+$ IgA$^+$ cells in the iLP. (**a**) Mice were treated with antibodies to block IL-5, IL-6, IL-10 or antagonistic TACI-immunoglobulin (TACI-Ig) fusion protein. Mononuclear cells were isolated from the iLP and used for analysis of CD11b$^+$ and CD11b$^-$ IgA$^+$ cells by flow cytometry. Data are presented as means ± s.d. ($n=4$). (**b**) Mononuclear cells were isolated from the iLP of WT or IL-10 KO mice for analysis of IgA and CD11b expression by flow cytometry. Graphs show data from individual mice. Statistical analyses were performed with Mann–Whitney's *U*-test.

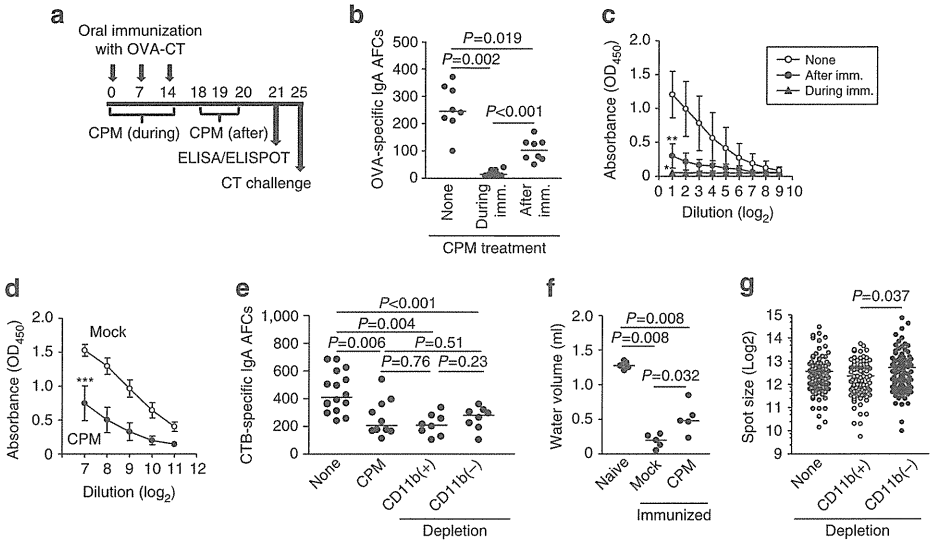

Figure 6 | Proliferating IgA$^+$ cells mediate early-phase IgA responses to oral antigen. (**a**) Experimental schedule for oral immunization and CPM treatment. Mice were orally immunized with OVA plus CT on days 0, 7 and 14. One group received CPM during oral immunization (days 0, 7 and 14) and another received CPM after the last immunization (days 18, 19 and 20). (**b**,**c**) One week after the final immunization (day 21), mononuclear cells were isolated from the iLP to quantify OVA-specific IgA-forming cells by ELISPOT (**b**). Simultaneously, faeces (**c**,**d**) were collected and were used for the detection of the (**c**) OVA-or (**d**) B subunit of CT (CTB)-specific IgA by enzyme-linked immunosorbent assay. Data are from individual mice and bars indicate median (**b**) and represent means ± s.d. ($n=10$) from two separate experiments (**c**,**d**). *$P<0.001$, **$P<0.01$, ***$P<0.05$ (two tailed unpaired *t*-test). (**e**) Mononuclear cells were isolated from the iLP of mock- or CPM-treated mice 1 week after the final immunization to quantify CTB-specific IgA-forming cells by ELISPOT. In some groups of mock-treated mice, CD11b$^+$ or CD11b$^-$ IgA$^+$ cells were depleted by cell sorting before application of ELISPOT assay. Graphs show data from individual mice, and bars indicate median. (**f**) On day 21, mice were orally challenged with 100 μg CT. After 15 h, the volume of intestinal fluid was measured. Graphs show data from individual mice, and bars indicate median. Similar results were obtained from two separate experiments. (**g**) Spot sizes of CTB-specific IgA AFCs were measured by Zeiss KS ELISPOT software. Graphs show data from individual mice, and bars indicate median. Statistical analyses were performed with Mann–Whitney's *U*-test (**e**–**g**).

IgA$^+$ cells. Of note, these mice showed ~90% reduction in OVA-specific IgA content in the faeces compared with mice not treated with CPM (Fig. 6c). We also confirmed that CPM treatment 4 days after final immunization induced a reduction in the production of IgA specific to the B subunit of CT (that is, CTB), which was associated with the halving of the abundance of CTB-specific IgA AFCs in the intestine (Fig. 6d,e). Like OVA-specific IgA responses (Figs. 3c and 6b), similar levels of reduction of CTB-specific IgA AFCs were noted when CD11b$^+$ IgA$^+$ cells were depleted before ELISPOT assay (Fig. 6e). These mice showed reduced resistance to oral challenge with CT and developed watery diarrhoea (Fig. 6f and Supplementary Fig. S11).

These findings led us to hypothesize that CD11b$^+$ IgA$^+$ PCs are capable of producing more IgA than are CD11b$^-$ IgA$^+$ PCs. To test this hypothesis, we measured the size of each spot in CTB-specific IgA AFCs in an ELISPOT assay. The cells in the CD11b$^+$ IgA$^+$ cell-enriched fraction (depletion of CD11b$^-$ IgA$^+$ cells) were bigger than those in the CD11b$^-$ IgA$^+$ cell-enriched fraction (depletion of CD11b$^+$ IgA$^+$ cells) (Fig. 6g). Furthermore, an adoptive transfer experiment demonstrated higher intestinal IgA production in severe combined immunodeficiency mice receiving CD11b$^+$ IgA$^+$ PCs than in those receiving CD11b$^-$ IgA$^+$ PCs (Supplementary Fig. S12), presumably because of both high IgA production and proliferating activity of CD11b$^+$ IgA$^+$ PCs. Although some possibilities (for example, proliferation and CD11b expression of IgA$^+$ cells might be changed during immunization) cannot be excluded, it is plausible that the actual production of IgA secreted into the intestinal lumen was derived mainly from CD11b$^+$ IgA$^+$ PCs in the early phase of the IgA response against orally immunized antigen.

Discussion

PCs could secrete antibodies to provide antigen-specific humoral immune responses in both systemic and mucosal tissues. Here, we demonstrated that intestinal IgA$^+$ PCs in mice could be categorized into two populations on the basis of CD11b expression. CD11b is an integrin αM that non-covalently associates with CD18 to form αMβ2 integrin (Mac-1) and binds to ICAM-1 (ref. 24). We therefore expected that CD11b$^+$ IgA$^+$ PCs were newly migrating cells whose migration was mediated by endothelial cells expressing ICAM-1, but in fact they were not. We also found no uptake of opsonized bacteria in either CD11b$^+$ or CD11b$^-$ IgA$^+$ cells (Supplementary Table S4 and Supplementary Fig. S13a), although CD11b is a receptor for complement (iC3b)[24]. In addition, unlike in human CD11b$^+$ B cells, which stimulate T cells strongly[32], major histocompatibility complex (MHC) class II (I-A^d) and costimulatory molecules (for example, CD80) were identically expressed on both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells (Supplementary Table S4 and Supplementary Fig. S13b).

A similar subset of CD11b$^+$ IgA$^+$ cells was observed in the systemic murine compartments (for example, spleen), but the immunological characteristics of these cells differed from those of the cells in the intestine. Indeed, intestinal CD11b$^+$ IgA$^+$ cells consisted exclusively of PCs, but not memory B cells, whereas splenic CD11b$^+$ IgA$^+$ cells included both PCs and memory B cells. We further found that CD11b could not be used as a marker of B1 cells in the intestine. Our current findings show for the first time that CD11b could be a specific marker for discriminating IgA$^+$ PCs that require microbial stimulation and IL-10, and presumably contribute to the early phase of the intestinal IgA response in mice.

We have identified unique CD11b$^+$ IgA$^+$ PCs in mice; the next question is whether or not the same population of IgA$^+$ PCs exists in humans. Our preliminary experiments have shown that no human intestinal IgA$^+$ cells express CD11b, but that some IgA$^+$ cells express Ki67, a marker of proliferating cells (unpublished data). One possible explanation for this difference between human and mice is difference in the composition of commensal bacteria. In this regard, we examined the involvement of segmented filamentous bacteria (SFB), which are a known major IgA stimulus in mice, but has not yet been confirmed as part of the human microbiota[19]. As expected, SFB stimulated IgA production following colonization of SFB-deficient C57BL/6 mice from the Jackson laboratory (JAX mice) with bacterial suspensions from SFB-monoassociated mice (JAX + SFB mice)[33]; however, we found that CD11b is expressed on IgA$^+$ cells independently of SFB colonization (Supplementary Fig. S14). It is possible that other commensal bacteria such as *Lactobacillus* (abundant in mice) and *Bifidobacterium* (abundant in human) are responsible for the species-specific expression of CD11b on IgA$^+$ cells. It is important to recognize the differences between the mouse and human immune systems, but it is obvious that proliferating IgA$^+$ cells are present in the iLP of both mouse and human. The immunological function of human proliferating IgA$^+$ cells in the intestine will therefore be the subject of our next study.

In the initial step of the antibody response to T cell dependent antigens, B cells are activated by antigens and form GCs in the lymph nodes[7]. As depleting antigen-specific GC B cells by CPM treatment during oral immunization resulted in complete loss of the IgA response to orally immunized antigen, it is likely that both CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs against T cell dependent antigen are derived from GC B cells. We also found that depletion of proliferating CD11b$^+$ IgA$^+$ PCs by CPM treatment after final immunization led to a decrease in the early-phase IgA response, although it is possible that proliferation activity and/or CD11b expression on IgA$^+$ cells might be wobble during immunization. Our *in vivo* findings indicated that the reduction in CD11b$^+$ IgA$^+$ PC numbers in MyD88 KO, IL-10 KO and PP-null mice did not affect the numbers of CD11b$^-$ IgA$^+$ PCs (Figs 1c, 3d and 5b). These findings, together with our *in vitro* data (Supplementary Fig. S6), indicate that it is likely that CD11b$^+$ IgA$^+$ PCs act as a separate lineage once they differentiate in the iLP.

Proliferating CD11b$^+$ IgA$^+$ PCs required microbial stimulation in the intestine. As proliferation is one of the characteristics of plasmablasts, it was possible that CD11b$^+$ IgA$^+$ cells have been recently committed to the PC fate. Notably, intestinal IgA$^+$ cells expressed MHC class II molecules; this expression is one of the unique characteristics of plasmablasts. Therefore, it is likely that intestinal IgA$^+$ PCs partly retain their plasmablast features. However, our findings indicated that CD11b$^+$ and CD11b$^-$ IgA$^+$ cells expressed identical levels of Blimp-1 and MHC class II. In addition, similar reduction was noted in CD11b$^+$ and CD11b$^-$ IgA$^+$ cells when cell trafficking from IgA inductive tissues (for example, PPs and the PerC) into the iLP was inhibited by treatment with FTY720. Thus, our findings suggest that CD11b$^+$ IgA$^+$ cells uniquely exhibit high proliferating and IgA-producing activity, although their other immunological features as PCs are similar to those of CD11b$^-$ IgA$^+$ PCs.

Proliferating CD138$^+$ PCs have been detected in the spleens of NZB/W mice with signs of systemic lupus erythematosus, but not in naive mice[34]. In contrast, the number of non-proliferating CD138$^+$ PCs is unchanged in the intestines of GF mice, as it is in the spleens of NZB/W mice[34]. These findings suggest that MyD88-dependent homeostatic stimulation of commensal bacteria determines the fate of proliferating CD11b$^+$ IgA$^+$ CD138$^+$ PCs in the intestine. Several lines of evidence have revealed the cellular and molecular mechanisms of microbe-dependent initiation of IgA responses. B cells express several toll-like receptors, and B cell-intrinsic MyD88-mediated signalling has been implicated in enhanced antibody production in some studies[35,36]. However, our current findings indicated that MyD88-mediated signalling in hematopoietic cells, including B cells, was not essential for intestinal CD11b$^+$ IgA$^+$ PC production. Additionally, we found IL-10 as a key molecule inducing CD11b$^+$ IgA$^+$ PC production. Previous studies have demonstrated that IL-10 promotes the proliferation of activated B cells and subsequent IgA production *in vitro*[37,38], which are consistent with our current findings of high-level proliferation of, and IgA production by, CD11b$^+$ IgA$^+$ PCs. Thus, our current findings proved that IL-10 functions in IgA production *in vivo* and that CD11b$^+$ IgA$^+$ PCs are the main targets in this