学 位 論 文 の 要 旨

氏名　　岡　明彦

学 位 論 文 名　Role of Regulatory B Cells in Chronic Intestinal Inflammation: Association With Pathogenesis of Crohn's Disease

発 表 雑 誌 名　Inflammatory Bowel Diseases
（巻，初頁～終頁，年）　(Volume 20, pages 315-328, 2014)

著 者 名　Akihiko Oka, Shunji Ishihara, Yoshiyuki Mishima, Yasumasa Tada, Ryusaku Kusunoki, Nobuhiko Fukuba, Takafumi Yuki, Kousaku Kawashima, Satoshi Matsumoto, Yoshikazu Kinoshita

論 文 内 容 の 要 旨

INTRODUCTION

Crohn's disease (CD) is a chronic intestinal immune-mediated disorder characterized by a relapsing-remitting course, but its etiology is not fully understood. A regulatory B cell (Breg) subset producing interleukin (IL)-10 plays essential roles to maintain innate immunity and autoimmunity in various organs. Lack or loss of this subset exacerbates symptoms in experimental mice models with immune-mediated disorders. We recently demonstrated that IL-10-producing Bregs were decreased in the murine model of CD (SAMP1/Yit mice). In this study we examined Bregs in patients with CD and ulcerative colitis (UC), another type of inflammatory bowel disease (IBD), and investigated the influence of Breg-depletion on experimental colitis in the adoptive transfer model of CD.

MATERIALS AND METHODS

Human experiments: Eighteen patients with CD, 23 patients with UC, and 26 healthy subjects. B cells were separated from isolated peripheral blood mononuclear cells (PBMCs) magnetically by positive selection with CD19 microbeads, and purified $CD19^+$ cells (B cells) were cultured with CpG DNA for 72 hours, and then IL-10 contents in culture media were measured using enzyme immunoassays (EIA). Multiple regression analysis was used to determine significant factors affecting the production of IL-10 in CpG DNA-stimulated B cells in CD and UC patients in association with clinical parameters. For intracellular staining of IL-10, PBMCs

from IBD patients and healthy subjects were cultured for 24 hours with CpG DNA, and then re-stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, as well as GolgiStop for the final 5 hours. The study protocol was approved by the Ethics Committee of Shimane University and written informed consent was obtained from all subjects. **Murine experiments:** AKR/N mice (Healthy control) share a genetic background with SAMP1/Yit mice and their entire MHC region is identical. Bregs were detected as $CD19^{hi}CD1d^{hi}$ B cells and were depleted from mesenteric lymph node (MLN)-derived $CD19^{+}$ cells of AKR mice by FACS sorting system. Breg-depleted B cells or whole B cells were transferred to experimental colitis model, induced by transfer of $CD4^{+}$ or $CD4^{+}CD25^{-}$ cells derived from SAMP1 mice to severe combined immunodeficient (SCID) mice. Body weight (BW) changes were monitored weekly using a top loading balance. All mice were euthanized at 6 or 7 weeks after cell transfer and the severity of colitis was examined using the disease activity parameters, including BW, and histological score. The expression levels of macrophage inflammatory protein (MIP)-2, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and IL-1β in intestinal tissues and MLNs were determined using real-time PCR. $CD4^{+}CD25^{-}$ T cells were magnetically isolated from MLNs of SCID mice transferred with SAMP1 $CD4^{+}$ T cells. T cells were co-cultured with whole B cells or Breg-depleted B cells isolated from MLNs of AKR mice in anti-CD3 antibody-coated 96-well plates with CpG DNA. The effect of the anti-IL-10 antibody was also examined. The supernatants were analyzed by EIA for detection of TNF-α. All experiments with animals in this study were approved by the Ethics Committee for Animal Experimentation of Shimane University and they were handled according to our institutional guidelines.

RESULTS AND DISCUSSION

Human data: The mean levels of IL-10 contents in culture supernatants were significantly lower in those from the CD and UC patients as compared to the controls. In addition, a decreased frequency of IL-10-producing peripheral blood B cells was also observed among cells obtained from the IBD patients. Since the IBD patients enrolled in this study had various clinical backgrounds including therapeutic regimens, we used multiple regression analysis to determine significant factors affecting CpG DNA-induced IL-10 production in B cells from those patients. Among several clinical parameters examined, the presence of CD was significantly related to decreased production of IL-10 by peripheral blood B cells. IL-10-producing B cells (Bregs) were mainly located in a population characterized by the cell surface markers $CD19^{hi}CD1d^{hi}$. **Murine**

data: Similar to the results of the human cell experiment, flow cytometry revealed that mouse B cells producing IL-10 were mainly located in the $CD19^{hi}$ and $CD1d^{hi}$ cell population. To investigate the regulatory role of B cells producing IL-10 in intestinal inflammation, we initially established 2 types of chronic colitis models of SCID mice by adoptive transfer of whole $CD4^+$ T cells or regulatory T cell (Treg)-depleted T cells ($CD4^+CD25^-$). Co-transferred B cells were detected for at least 7 weeks in spleens, intestines, and MLNs of recipient SCID mice. The colitis severity score was dramatically increased in Breg-depleted group. In Breg-depleted group the level of inflammatory cytokines (MIP-2, IFN-γ, TNF-α, and IL-1β) were significantly higher, as compared with control group. These results were similarly found in $CD4^+CD25^-$ mice (Treg-depleted adoptive transfer mice). Since the transferred $CD19^{hi}CD1d^{hi}$ B cells freshly isolated from the MLNs of recipient mice produced significant amounts of IL-10, colitis exacerbation might be dependent on depletion of $CD19^{hi}CD1d^{hi}$ B cells. CD3-stimulated $CD4^+CD25^-$ T cells were co-cultured with whole B cells or Breg-depleted B cells under a CpG DNA-stimulated condition. The results of our co-culture experiments indicated that the absence of $CD19^{hi}CD1d^{hi}$ B cells significantly increased CD3-induced production of TNF-α by $CD4^+CD25^-$ T cells. In addition, treatment with the anti-IL-10 antibody increased cytokine production by T cells co-cultured with whole B cells. In the present study, we demonstrated the immunoregulatory role of $CD19^{hi}CD1d^{hi}$ B cells in experimental colitis mouse models. Although we also found decreased production of IL-10 in CpG DNA-stimulated peripheral blood B cells in CD patients, it remains unknown whether Bregs dysfunction is directly correlated with the development and pathogenesis of CD. Moreover, recent reports have shown that total depletion of mature B cells with rituximab in autoimmune disease patients induced development of UC, suggesting that Bregs may also be associated with the pathogenesis of UC. To confirm these points, it will be important to address the various mechanisms related to the maturation and differentiation, and CpG DNA-dependent cellular signaling in Bregs, as well as the roles of intestinal microbial flora in Bregs functions. Such findings may lead to a novel therapeutic strategy targeting Bregs for IBD.

CONCLUSION

We found decrease production of IL-10 in CpG DNA-stimulated B cells in IBD patients, especially CD, and identified that Breg-depletion exacerbated experimental colitis in the presence or absence of Treg, suggesting that the function of this regulatory subset of B cells may be associated with the pathogenesis of IBD.

論文審査及び最終試験又は学力の確認の結果の要旨

(甲)・乙	氏名	岡　明彦
学位論文名	Role of Regulatory B Cells in Chronic Intestinal Inflammation: Association With Pathogenesis of Crohn's Disease	
学位論文審査委員	主査	原田　守
	副査	田島　義証
	副査	川内　秀之

論文審査の結果の要旨

Crohn's disease (CD) は，免疫応答が関与する代表的な炎症性腸疾患であるが，その病因は十分には解明されていない。一方，自己免疫疾患を抗CD20抗体で治療した場合，炎症性腸疾患を発症したという報告があり，免疫抑制性B細胞の存在が示唆されていた。さらに，最近の研究により免疫抑制性サイトカインである interleukin (IL)-10を産生する regulatory B (Breg) 細胞の存在が明らかになった。申請者らのグループも，CDを発症するSAMP-1マウスでBreg細胞が減少していることを報告している。そこで今回申請者は，CD患者においてBreg細胞の機能が低下しているか，また，免疫不全マウスにSAMP-1マウスのCD4陽性T細胞を移入して誘導するCDマウスモデルにおいて，Breg細胞が腸炎発症に抑制的に関与しているかを検討し，以下のことを明らかにした。①CD患者の末梢血中B細胞のIL-10産生能は，健常人のB細胞に比べて低下している。②SAMP-1マウスのB細胞の中でIL-10を産生するのは，CD19陽性CD1d陽性の細胞である。③免疫不全マウスにSAMP-1マウスのCD4陽性T細胞を移入して誘導するCDモデルで，CD19陽性CD1d陽性を除いたB細胞を同時に移入した場合には腸炎が悪化し，腸や腸間膜リンパ節での炎症性サイトカインのmRNAの発現が増加する。④regulatory T (Treg) 細胞を除去したSAMP-1マウス由来のCD4陽性T細胞を免疫不全マウスに移入した場合でも，Breg細胞を除いたB細胞の移入により腸炎の悪化や腸と腸間膜リンパ節での炎症性サイトカインのmRNAの発現が増加する。以上のように本研究は，CD発症におけるBreg細胞の抑制的役割をヒト検体と動物モデルで明らかにしたものであり，CDの発症機序の解明や治療法の開発に貢献する研究と考えられる。

最終試験又は学力の確認の結果の要旨

申請者は，CDにおけるBreg細胞の役割をヒト検体とCDマウスモデルを用いて検討し，Breg細胞がCD発症に抑制的に働いていることを明らかにした。研究成果は，CDの病態の理解と治療に貴重な情報を与えるものである。質疑応答も的確で，関連分野の知識も豊富であり，学位授与に値すると判断した。

（主査：原田　守）

申請者は，CD患者ならびにCDマウスモデルを用いて免疫学的解析を行い，Breg細胞がIL-10依存性に腸炎を抑制すること，また，CDではBreg細胞機能が低下していることを明らかにした。臨床応用につながる貴重な研究成果であり，質疑応答も適切で知識も豊富であり，学位授与に値すると判断した。

（副査：田島　義証）

本研究は，CD患者の末梢血リンパ球の免疫学的検討と動物モデルでのBreg細胞の移入実験により，CDの病勢の制御機構として，IL-10を産生するBreg細胞が重要であることを証明した優れた研究である。申請者の下部消化管炎症性疾患に関する知識や免疫学的方法論の理解も十分であり，本学の学位に値する。

（副査：川内　秀之）

（備考）要旨は、それぞれ４００字程度とする。