Involvement of IL-10 in the suppression of antibody production by in vitro immunized peripheral blood mononuclear cells

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Abstract Previously, we have established an in vitro immunization method to induce antigen-specific antibody-producing B cells. In the present study, we have attempted to clarify the mechanisms that regulate antibody production by in vitro immunized peripheral blood mononuclear cells (PBMC). Freshly isolated PBMC did not induce antibody production following in vitro immunization, but expressed the interleukin (IL)-10 gene. On the other hand, PBMC pretreated with L-leucyl-L-leucine methyl ester (LLME) induced antibody production, but did not express the IL-10 gene. IL-10 induced functional impairment of CD4⁺ Th cells and CD11c⁺ DC, resulting in the suppression of antibody production by in vitro immunized PBMC.

Keywords Antibody production · Interleukin-10 · CD11c⁺ dendritic cells · In vitro immunization · Peripheral blood mononuclear cells

Introduction

Recently, antibody therapy has been recognized to possess great potential for the treatment of cancer, autoimmune disorders, and viral/bacterial infections. However, thus far, methods for generating human monoclonal antibodies have not been completely established (Eren et al. 1998; Traggiai et al. 2004). We have established an in vitro immunization protocol to induce antigen-specific antibody production by peripheral blood mononuclear cells (PBMC). Using this protocol, we have successfully generated human B cell clones that produced monoclonal antibodies specific for cholera toxin B subunit, keyhole limpet hemocyanin, or rice allergen (Ichikawa et al. 1999; Xu et al. 2004). The aim of this study is to clarify the mechanisms that regulate antibody production by in vitro immunized PBMC.

During the course of our study, we observed that pretreatment of PBMC with L-leucyl-L-leucine methyl ester (LLME) is essential for inducing antibody production following in vitro immunization. Further, in the absence of pretreatment with LLME, PBMC expresses the IL-10 gene in the early phase of culture. Thus, we presumed that IL-10 plays an

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important role in suppressing antibody production by in vitro immunized PBMC.

IL-10 has originally been identified as being a Th2-type cytokine, which inhibits the secretion of cytokines by Th1-type cells (Fiorentino et al. 1989), and it is produced by a wide variety of cells of both hematopoietic and non-hematopoietic origin. Recently, increasing evidences have shown that IL-10 acts as a general inhibitor of proliferation and cytokine responses (de Waal Malefyt et al. 1991; Fiorentino et al. 1991). Furthermore, IL-10 has been identified as a major factor that prevents the differentiation of dendritic cells (DC) (Corinti et al. 2001). In this study, we focused on the effects of IL-10 on T cells and DC, and investigated the reason why IL-10 suppresses antibody production by in vitro immunized PBMC.

Materials and methods

Antigen and reagents

Mite extract was purchased from LSL (Tokyo, Japan). Recombinant human interleukin-2 (IL-2) and recombinant human IL-4 were purchased from Genzyme (Cambridge, MA) and PeproTech (London, UK), respectively. Muramyl dipeptide (MDP) was purchased from Chemicon (Temecula, CA). LLME was obtained from Bachem (Torrance, CA).

Isolation of human lymphocytes

Human PBMC were collected by density gradient centrifugation using lymphocyte separation medium (LSM; Organon Teknika, Durham, NC). In brief, 25 mL of peripheral blood was layered onto 15 mL of LSM and centrifuged at $400 \times g$ for 30 min at room temperature. PBMC were washed three times with ERDF medium (Kyokuto, Tokyo, Japan) and then treated with 0.25 mM LLME for 20 min at room temperature. After washing with the culture medium, these cells were used for in vitro immunization.

In vitro immunization

In vitro immunization of human PBMC was performed in 24-well culture plates (Becton Dickinson

Labware, Franklin Lake, NJ). The LLME-treated PBMC (5×10^6 cells) were sensitized with mite extract ($10 \mu\text{g mL}^{-1}$) in the presence of IL-2 (10 units mL^{-1}), IL-4 (10 ng mL^{-1}), and MDP ($10 \mu\text{g mL}^{-1}$) and then cultured in ERDF medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 2-mercaptoethanol ($50 \mu\text{M}$).

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Nunc, Naperville, IL) were coated with anti-human IgM antibody (TAGO, Burlingame, CA) or anti-human IgG antibody (TAGO) diluted with 0.1 M sodium carbonate buffer (pH 9.6) and incubated for 2 h at 37°C . The plates were washed three times with 2.24×10^{-2} M phosphate buffer containing 1.37×10^{-1} M NaCl and 0.05% Tween 20 (TPBS). Aliquots of serially diluted supernatants of in vitro immunized PBMC were added, and the plates were then incubated at 4°C overnight. After washing three times with TPBS, diluted horseradish peroxidase-conjugated anti-human IgM (TAGO) or anti-human IgG (TAGO) goat antibodies were added, and the plates were subsequently incubated for 2 h at 37°C . The plates were again washed three times with TPBS, and substrate solution (0.1 M citrate buffer (pH 4.0) containing 0.003% H_2O_2 and 0.3 mg mL^{-1} 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Wako, Osaka, Japan) was added followed by incubation for 20 min. The absorbance at 405 nm was measured using an ELISA plate reader.

Magnetic-activated cell sorting (MACS)

CD11c⁺ dendritic cells was sorted by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, PBMC suspension were stained with Phycoerythrin (PE)-labeled anti-CD11c mAb (Beckman Coulter, Miami, FL), incubated for additional 15 min with anti-PE microbeads and then applied to MACS system.

Flow cytometric analysis

Fluorescein isothiocyanate (FITC)-labeled anti-CD4 (Beckman Coulter, Miami, FL), PE-labeled anti-CD8

(Beckman Coulter), and PE-labeled anti-CD25 monoclonal antibodies (mAb) (Beckman Coulter) were used for flow cytometric analyses. A single-cell suspension in PBS supplemented with 5% FBS was stained with FITC-labeled mAb in combination with PE-labeled mAb at 4 °C for 30 min. After washing, the cells were analyzed using the EPICS XL (Beckman Coulter) and FlowJo software (Tree Star, San Carlos, CA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Cytokine mRNA was detected by RT-PCR. Total RNA was prepared from Th cells by using GenElute Mammalian Total RNA Miniprep Kit (Sigma, St. Louis, MO) according to manufacturer's protocol. The cDNA synthesis reaction was carried out in a total volume of 25 µL using M-MLV reverse transcriptase (Promega, Madison, WI) and total RNA (0.5 µg) as the template. Subsequent PCR cycles were performed by using 1 µL of the cDNA synthesis reaction mixture. The oligonucleotide primers used for amplifications were as follows: IL-1 β cDNA, 5'-GATAAGCCCACTCTACAGCTGG-3' and 5'-ATGTACCAGTTGGGGAAGTGGG-3'; IL-2 cDNA, 5'-CAACTCCTGTCTTGCAATTGC-3' and 5'-ATGGTTGCTGTCTCATCAGC-3'; IL-4 cDNA, 5'-GTTCTTCCTGCTAGCATGTGC-3' and 5'-GAGTCAACACAAGAACCTCCG-3'; IL-5 cDNA, 5'-TTGCTAGCTCTTGAGCTGC-3' and 5'-CCACTCGGTGTTTCATACACC-3'; IL-6 cDNA, 5'-TGAACCTCTTCTCACAAAGC-3' and 5'-ATCCAGATTGGAAGCATCA-3'; IL-10 cDNA, 5'-AACCTGCCTAACATGCTTCG-3' and 5'-CCAGAACCAAGAGTCGAACC-3'; IL-13 cDNA, 5'-ATTGCTCTCACTTGCCTTGG-3' and 5'-TCGACAGTCCAACCTACGAGG-3'; interferon (IFN)- γ cDNA, 5'-TCTGCATCGTTTGGGTTCT-3' and 5'-GTCGAAAAGCTTCAGTAGAG-3'; tumor necrosis factor (TNF)- α cDNA, 5'-GTGACAAGCCTGTAGCCCATGTTG-3' and 5'-GAGTAGATGAGGTACAGGCCCTC-3'; β -actin cDNA, 5'-CTACAATGAGCTGCGTGTGG-3' and 5'-TATCGTTGCATGTACCGACC-3'. Cytokine cDNAs were amplified by using these primers for 31–35 cycles (94 °C, 30 s; 60 or 63 °C, 30 s; and 72 °C, 1 min). The reaction products were resolved by electrophoresis on a 4% polyacrylamide gel and stained with

SYBR Gold (Molecular Probes, Eugene, OR). Band intensity was measured using NIH Image software. Relative expression levels were calculated by dividing the band intensity of each cytokine gene with that of β -actin.

Results and discussion

IL-10 existing prior to antigen sensitization suppressed antibody production by in vitro immunized PBMC

In the in vitro immunization system, freshly isolated PBMC were treated with LLME and subsequently sensitized with soluble antigen in the presence of IL-2 and IL-4. However, in the absence of LLME treatment, PBMC cannot induce antibody production upon antigen sensitization (Fig. 1a). To clarify the mechanisms that regulate antibody production in in vitro immunization, we first investigated the expression patterns of cytokine genes in LLME-treated and non-treated PBMC. The cytokine gene expression, including IL-2, IL-4, IL-13, TNF- α , GM-CSF, and IFN- γ , detected in LLME-treated PBMC, but not in the non-treated PBMC, while IL-6 and IL-10 showed characteristic expression patterns (Fig. 1b). IL-6 and IL-10 gene expressions were both detected in LLME-treated PBMC similarly to other cytokine genes. However, IL-6 gene expression was constantly detected during the culture period in non-treated PBMC. On the other hand, IL-10 gene expression was detected only in the early phase of culture during in vitro immunization. These results suggest that IL-10 existing prior to antigen sensitization suppresses antibody production by in vitro immunized PBMC.

To clarify the involvement of IL-10 in the suppression of antibody production, we treated PBMC with IL-10 or anti-IL-10 antibody prior to antigen sensitization. IL-10 markedly suppressed antibody production by LLME-treated in vitro immunized PBMC, whereas anti-IL-10 mAb augmented the antibody production (Fig. 2a). Further, IL-10 suppressed cluster formation of LLME-treated in vitro immunized PBMC, which is known to be correlated with antibody production (Fig. 2b). These results demonstrated that IL-10 existing prior to antigen

Fig. 1 Effect of LLME on in vitro immunization of PBMC. Non-treated PBMC (□) and LLME-treated PBMC (■) were sensitized with mite extract in the presence of IL-2 (10 U mL⁻¹), IL-4 (10 ng mL⁻¹), and MDP (10 μg mL⁻¹). **(a)** The amount of antibody secreted into the supernatants was determined by sandwich ELISA. **(b)** After 2, 4, 7, and 10 days

of culture, total RNA isolated from PBMC was subjected to RT-PCR analyses to detect gene expression of cytokines, including those of IL-2, IL-4, IL-6, IL-10, IL-13, TNF-α, GM-CSF, and IFN-γ. The relative expression level of cytokine genes are shown. The representative value from three independent experiments is shown

sensitization suppressed the antibody production by in vitro immunized PBMC.

IL-10 attenuated the cytokine expression of Th cells in in vitro immunized PBMC

Next, we investigated the effect of IL-10 on Th cells that were present in in vitro immunized PBMC. We could not detect any changes in the ratio of CD4⁺ and

CD8⁺ T cells in in vitro immunized PBMC upon pretreatment with IL-10 (Fig. 3a). However, we observed a notable decrease in the expression of cytokine genes including IL-2, IL-4, IL-5, IL-10, IL-13, IFN-γ, and TNF-α in CD4⁺ T cells upon pretreatment with IL-10 (Fig. 3b). The amounts of IL-2 and IFN-γ productions were important indicator of T cell activation. Moreover, IL-4, IL-5, and IL-13 were known to be involved in antibody production from B cells. These results demonstrate that the

Fig. 2 IL-10 suppresses antibody production and cluster formation in vitro immunization. **(a)** LLME-treated PBMC were pretreated with IL-10 or anti-IL-10 mAb for 2 days and then sensitized with antigen, as shown in the legend to Fig. 1. After 8 days of culture, the amount of antibody in the supernatant was assessed by ELISA. **(b)** After 7 days of culture, the cluster forming ability of individual PBMC was investigated under a phase-contrast microscope

cytokine-producing ability of Th cells was attenuated upon pretreatment with IL-10, resulting in the decreased antibody production.

IL-10-induced suppression of antibody production is not due to an increase in the number of CD4⁺/CD25⁺ Tr cells

IL-10 is known to act as a direct and/or indirect suppressor. IL-10 directly acts on T cells resulting in the suppression of proliferation and cytokine production (Groux et al. 1996; Joss et al. 2000). On the other hand, IL-10 was reported to block the maturation of DC (Corinti et al. 2001) and transform immature DC into tolerogenic antigen-presenting cells (APC) (Steinbrink et al. 1997), both resulting in the indirect inhibition of T cell function.

A growing body of evidences suggests that DC play a central role in the induction of peripheral tolerance via the selective development of adaptive regulatory T cells (Tr cells) (Steinman et al. 2003). At present, Tr cells are known to exhibit regulatory functions in vitro and in vivo, and these cells can be subdivided into a number of subsets based on the expression of cell surface markers, production of

cytokines, and mechanisms of action (Roncarolo and Levings 2000). One of the best-characterized subsets is CD4⁺ Tr cells that are defined by constitutive expression of the α chain of IL-2R (CD25). The CD4⁺/CD25⁺ Tr cells suppress the immune responses in some model systems via CTLA-4 (Baecher-Allan et al. 2001; Read et al. 2000; Takahashi et al. 2000). In order to clarify the involvement of Tr cells in the suppression of antibody production by in vitro immunized PBMC pretreated with IL-10, LLME-treated PBMC were pretreated with IL-10 for 2 days prior to antigen sensitization, and analyzed the ratio of CD4⁺/CD25⁺ Tr cells by using flowcytometer. However, we could not detect an increase in the number of CD4⁺/CD25⁺ Tr cells upon the treatment with IL-10 (Fig. 4); this indicates that IL-10-induced suppression of antibody production is not due to an increase in the number of CD4⁺/CD25⁺ Tr cells, and further suggests that IL-10 suppresses antibody production through provoking functional alteration of CD4⁺/CD25⁺ Tr cells and/or inducing other Tr subsets. For example, Tr1 cells have been reported to be involved in the downregulation of immune responses by producing IL-10 and TGF- β . Further studies are required to investigate the involvement of Tr1 cells in this repression.

IL-10 suppresses antibody production by in vitro immunized PBMC through functional alteration of DC

IL-10 exhibits a variety of functions against DC, such as inhibition of full maturation of DC (de Waal Malefyt et al. 1991) and development of regulatory DC (Sato et al. 2003), both of which interfere with DC function. Thus, we investigated the possibility that IL-10 affects DC function, thereby resulting in the suppression of antibody production by in vitro immunized PBMC. First, we collected CD11c⁺ DC from PBMC that were pretreated with IL-10 and subsequently immunized in vitro. As shown in Fig. 5a, the expression of TGF- β in CD11c⁺ DC, one of the most important cytokines for the negative function of CD11c⁺ DC, was augmented upon pretreatment with IL-10, particularly in the early phase of culture. These results suggest that enhanced secretion of TGF- β and IL-10 from CD11c⁺ DC activates Tr cells. This suppresses the immune

Fig. 3 Effect of IL-10 on CD4⁺ T cells in vitro immunized PBMC. LLME-treated PBMC were cultured in the presence of IL-10 for 2 days and then sensitized with antigen, as shown in the legend to Fig. 1. **(a)** After 8 days of culture, the cells were stained with FITC-conjugated anti-CD4 mAb and PE-conjugated anti-CD8 mAb. Following staining, the cells were analyzed using a flowcytometer. **(b)** After 0, 3, and 6 days of culture, total RNA prepared from CD4⁺ cells or CD8⁺ cells was subjected to RT-PCR analyses to detect cytokine gene expression

Fig. 4 Effect of IL-10 on CD4⁺CD25⁺ Tr cells in vitro immunized PBMC. LLME-treated PBMC were cultured in the presence of IL-10 for 2 days. After 2 days of preincubation, the cells were stained with FITC-conjugated anti-CD4 mAb and PE-conjugated anti-CD25 mAb. Following staining, the cells were analyzed using a flowcytometer

response of in vitro immunized PBMC upon pre-treatment with IL-10. We further investigated the involvement of CD11c⁺ DC in IL-10-induced suppression of antibody production by in vitro immunized PBMC. As shown in Fig. 5b, antibody production by PBMC pretreated with IL-10 and subsequently immunized in vitro was recovered by the removal of CD11c⁺ DC from PBMC.

Taken together, these results demonstrate that IL-10 negatively regulates antibody production from in vitro immunized PBMC through inducing functional impairment of Th cells and functional alteration of CD11c⁺ DC. Cytokine network between Th and CD11c⁺ DC might be important for induction and maintenance of suppressive state of immune response induced by IL-10. IL-10-producer cells and its target cells in PBMC, and their suppressive mechanisms should be clarified in the future study.

Fig. 5 Effect of IL-10 on CD11c⁺ DC in vitro immunized PBMC. **(a)** LLME-treated PBMC were cultured in the presence of IL-10 for 2 days and then sensitized with antigen, as shown in the legend to Fig. 1. After 0, 3, and 6 days of culture, total RNA prepared from isolated CD11c⁺ was subjected to RT-PCR analyses. **(b)** LLME-treated PBMC from which CD11c⁺ DC were removed by MACS were cultured in the presence of IL-10 for 2 days and then sensitized with antigen, as previously described. After 8 days of culture, the amount of antibody secreted into the supernatant was determined by ELISA

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