IL-10 Augments Antibody Production in in Vitro Immunized Lymphocytes by Inducing a Th2-Type Response and B Cell Maturation

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Received May 21, 2004; Accepted August 18, 2004

An in vitro immunization (IVI) protocol enables antigen specific antibody production from L-Leucyl-L-Leucine methyl ester (LLME)-treated human peripheral blood lymphocytes (PBL) upon antigen stimulation in the presence of IL-2, IL-4, and muramyl dipeptide. In the course of our studies, we have found that IL-10 added at the antigen sensitization significantly augmented antibody production level from the LLME-treated PBL. In the present study, we tried to demonstrate the role of IL-10 in the augmentation of antibody production in an IVI protocol by clarifying the cytokine expression profiles in CD4+ and CD8+ T cells. The results showed that IL-10 skewed the Th1/Th2 balance to Th2-type responses by suppressing Th1-type cytokine production and augmenting Th2-type cytokine production in CD4+ and CD8+ T cells, as well as in CD19+ B cells. Furthermore, IL-10 augmented the expression of CD38, an antigen marker of plasma cells, on B cells, which clearly indicates that IL-10 promoted differentiation and maturation of B cells in an IVI protocol. These results indicate that IL-10 plays an important role in setting the cellular milieu to produce antibodies in an IVI protocol.

Key words: in vitro immunization; antibody production; IL-10; CD38

Human monoclonal antibodies (mAb) have great potential for use in the treatment of cancer, autoimmune disease and bacterial infection. Human mAb are thought to be best suited for clinical use, but techniques to produce human mAb are not yet well established. One method to produce human mAb is the in vitro immunization (IVI) technique, in which human peripheral blood lymphocytes (PBL) are treated with L-Leucyl-L-Leucine methyl ester (LLME) and sensitized with specific antigen. Activated PBL are induced to produce antigen specific mAb.1,2 We previously improved this technique and found that muramyl dipeptide (MDP), IL-2, and IL-4 were effective as additives for inducing production of antigen-specific antibody from human PBL in vitro.1,3

In the course of our studies, we found that IL-10 gene expression was concordantly enhanced with augmented antibody production in LLME-treated PBL 1 week after antigen sensitization (Fig. 1A). These results suggest that IL-10 plays an important role in inducing antibody production in an IVI protocol. Here we tried to evaluate the functionalities of IL-10 in enhanced production of antibodies in an IVI protocol.

Materials and Methods

Antigen and reagents. Mite extract was purchased from LSL (Tokyo, Japan). Recombinant human IL-10 was purchased from R&D (Minneapolis, MN, USA). Recombinant human IL-2 was obtained from Genzyme (Cambridge, MA, USA). Recombinant human IL-4 was supplied by Pepro Tech (London, UK). MDP was purchased from Chemicon (Temecula, CA, USA). LLME was obtained from Boehringer GmbH (Mannheim, Germany). ERDF medium was purchased from Invitrogen (Carlsbad, CA).

Isolation of human lymphocytes. PBL were separated by density-gradient centrifugation from several healthy...
donors using lymphocyte separation medium (LSM; Organon Teknika, Durham, NC, USA). In brief, 25 ml of peripheral blood was layered onto 15 ml of LSM and centrifuged at 400 × g for 30 min at room temperature. PBL were harvested and washed three times with ERDF medium, and treated with 0.25 mM LLME for 20 min at room temperature. Cells were then washed three times in culture medium and utilized in *in vitro* immunization.

Experiments throughout this study were carried out in accordance with the principles of the Declaration of Helsinki.

*In vitro* immunization. *In vitro* immunization of human PBL was performed in 24-well flat-bottomed culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The LLME-treated PBL were cultured for 7 d in ERDF medium supplemented with 10% heat inactivated fetal bovine serum (FBS; Invitrogen), MDP (10 μg·ml⁻¹), IL-2 (10 units·ml⁻¹), IL-4 (10 ng·ml⁻¹), 2-mercaptoethanol (2-ME; 20 μM), and mite extract (10 μg·ml⁻¹). IL-10 (50 ng·ml⁻¹) was simultaneously added with antigen where indicated.

**Enzyme-linked immunosorbent assay (ELISA).** Culture supernatants were collected from *in vitro* immunized PBL and applied to ELISA to determine the amount of immunoglobulins and evaluate the specificities of antibodies produced in *in vitro* immunization.

![Graph A](image1.png)

**Fig. 1.** IL-10 Augments Antibody Production from *In Vitro* Immunized PBL.  
A. IL-10 gene expression in *in vitro* immunized PBL. LLME-treated or non-treated PBL were immunized *in vitro*. After 7 d of culture, IL-10 gene expression was evaluated by RT-PCR. B. Antibody production from PBL immunized *in vitro* in the absence or presence of IL-10 for 7 d. Amounts of IgM and IgG in the supernatants were assessed by ELISA. Values represent the mean ± s.e.m.; n = 3. Asterisks indicate P < 0.05 relative to no addition of IL-10. C. Titer of antigen-specific IgM and IgG produced in the supernatants of PBL immunized *in vitro* in the absence or presence of IL-10. Antigen specificities were assessed by direct ELISA. Values represent the mean ± s.e.m.; n = 3. A double asterisk indicates P < 0.01 relative to no addition of IL-10. An asterisk indicates P < 0.05 relative to no addition of IL-10.
Briefly, 96-well microtiter plates (Nunc, Naperville, IL, USA) were coated with 100 μl of 1000-fold diluted anti-human IgM (TAGO, Burlingame, CA, USA) or IgG (TAGO) antibodies in 0.1 M sodium carbonate buffer (pH 9.6) and incubated at 4 °C overnight. After the plates were washed three times with 2.24 × 10⁻³ M phosphate buffer containing 1.37 × 10⁻¹ M NaCl and 0.05% Tween 20 (TPBS), 50 μl aliquots of serially diluted supernatants of in vitro immunized PBL were added and incubated at 4 °C overnight. The wells were washed three times with TPBS and 100 μl well⁻¹ of 2000-fold diluted horseradish peroxidase-conjugated goat antibodies against human IgM (TAGO) or IgG (TAGO) were added, and subsequently incubated for 2 h at 37 °C. After washing three times with TPBS, 100 μl of substrate solution (0.1 M citrate buffer (pH 4.0) containing 0.003% H₂O₂ and 0.3 mg ml⁻¹ 2,2'-azino-di(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) (ABTS; WAKO, Osaka, Japan) were added and incubated for 20 min. Absorbance at 405 nm was measured using an ELISA reader. Concentrations of IgM and IgG were determined from a dose-response curve drawn by plotting the ELISA value vs. the logarithm of the concentration of control IgM and IgG. When examining mite extract-specific antibody production, microtiter plates coated with 1 mg ml⁻¹ mite extract were incubated with serially diluted supernatants of in vitro immunized PBL, and subsequently incubated with appropriately diluted horseradish peroxidase-conjugated goat antibodies against human IgM or IgG. Color development reaction was then carried out as above. All experiments were repeated at least 3 times, and reproducibilities were confirmed.

Magnetic-activated cell sorting (MACS). After the indicated days of culture, in vitro immunized PBL were collected and applied to MACS to separate cells to be analyzed. When positively isolating CD4⁺ and CD8⁺ T cell subsets, and CD19⁺ B cells, we used CD4- magnetic beads and CD8- magnetic beads respectively. B cells were separated by using a B cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer’s protocol, which enables magnetic depletion of T cells, NK cells, monocytes, granulocytes, platelets, and erythroid precursor cells from PBL. The purity of the isolated cells was found to be more than 95% as evaluated by flow cytometry.

Flow cytometric analysis. Isolated B cells were stained with PE-conjugated anti-CD38 mAb (Beckman Coulter, Miami, FL, USA). Stained B cells were analyzed with a flow cytometer (EPICS XL; Beckman Coulter) and FlowJo software (Tree Star, San Carlos, CA, USA).

Cytokine expression profiles. Total RNA was prepared from CD4⁺ and CD8⁺ T cells and CD19⁺ B cells using a Total RNA Extraction Kit (Sigma, St. Louis, MO, USA). Total RNA (200 ng) was used as a template for cDNA synthesis reactions using M-MLV reverse transcriptase (Promega, Madison, WI, USA). PCR reaction was undertaken using 1 × 10⁻¹ vol of cDNA reaction mixture, Taq DNA polymerase (Roche, Indianapolis, IN, USA), and specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPD) and several cytokines. The PCR primers were as follows: IL-2 sense, 5'-GAAGATGGATATATTACAAGATC-CC-3'; IL-2-antisense, 5'-TTTTCTAGATCTCCCTTTA-GTTCCAG-3'; INF-γ-sense, 5'-TCTGCTAGTTTT-GGGTTCT-3'; INF-γ-antisense, 5'-CACTTTTTCGAA-GTCATCTC-3'; IL-18-sense, 5'-GATAGCCAGCCTAGACTATGGG-3'; IL-18-antisense, 5'-CATGCGTCTAC-TACACTAGCC-3'; IL-4-sense, 5'-GTTTCCTCATGTG-AGCATGTCGC-3'; IL-4-antisense, 5'-CTCAGTGTTGTGT-TCCTGAGGCG-3'; IL-10-sense, 5'-AACCCTGGCTACATGCTTCG-3'; IL-10-antisense, 5'-GGTCTCGGTTTGCTG-3'; IL-6-sense, 5'-CAATAACCGCTCCCTAGACC-3'; IL-6-antisense, 5'-TACATTTGGCCCAGAGGCC-3'; TGF-β-sense, 5'-CGATAAAGCAGCCCTTCATGGTGGC-3'; TGF-β-antisense, 5'-CAGCTCATGCTTCCTGCAGGCTGT-3'; GAPDH-sense, 5'- AACAGCCCTAGATCATCGAC-3'; GAPDH-antisense, 5'-CATGAGTCCTTCCACGATACC-3'. Amplification was done through 25–30 PCR cycles (94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 1 min). Reaction products were fractionated by polyacrylamide gel electrophoresis on a 10% gel and stained with SYBR Gold (Molecular Probes, Eugene, OR, USA).

Statistical analysis. Data are expressed as the mean ± s.e.m. Statistical significance was tested by Student’s t-test. The differences were considered to be significance if P < 0.05.

Results

IL-10 enhanced antibody production from in vitro immunized PBL

In our normal in vitro immunization protocol, PBL are pretreated with LLME, and subsequently sensitized with antigen in the presence of IL-2 (10 U ml⁻¹), IL-4 (10 ng ml⁻¹), MDP (10 μg ml⁻¹), and 2-ME (20 μM). In the present study, we tried to evaluate the effect of IL-10 on the antibody production from in vitro immunized PBL, and then we performed in vitro immunization in the presence of IL-10 plus the additives described above. Supernatants were collected on day 7 after the start of in vitro immunization and applied to ELISA to determine the amount of immunoglobulins and to assess the specificities of antibodies against sensitized antigen produced from in vitro immunized PBL. The results showed that IL-10 markedly enhanced the production of IgM and IgG from in vitro immunized PBL increased on the addition of IL-10 (Fig. 1C). These results suggest that IL-10...
induces the culture milieu of in vitro immunized lymphocytes toward conditions to enhance antibody production and increase the titer of antigen-specific antibody production through activation of T cells, B cells, and other mononuclear cells.

**IL-10 induces Th2-type response in in vitro immunized PBL**

We tried to evaluate the effect of IL-10 on T cells included in in vitro immunized PBL. We performed in vitro immunization in the presence or absence of IL-10 and cultured the in vitro immunized lymphocytes for 7 d. First of all, we observed that the number of T cells remained unchanged, although the total number of mononuclear cells slightly decreased in in vitro immunized PBL upon the addition of IL-10 (data not shown). Next, CD4⁺ and CD8⁺ T cell subsets were separated from the in vitro immunized PBL at the indicated days of culture. Total RNA was prepared from these cells and cytokine expression profiles were investigated by RT-PCR. As shown in Fig. 2, we observe similar expression profiles in CD4⁺ and CD8⁺ T cells. IL-10 downregulated the expression of Th1-type cytokines including IL-2, IL-18, and IFN-γ in CD4⁺ and CD8⁺ T cells. Moreover, IL-10 upregulated the expression of Th2-type cytokines including IL-4, IL-6, IL-10, and TGF-β in CD4⁺ and CD8⁺ T cells. These results indicate that IL-10 shifts the Th1/Th2 balance to Th2-type responses by suppressing Th1-type cytokine production and enhancing Th2-type cytokine production in CD4⁺ and CD8⁺ T cells. Next we investigated the effect of IL-10 on the cytokine expression profiles of B cells after separating CD19⁺ B cells with MACS from the in vitro immunized PBL. In accordance with the results shown above, IL-10 downregulated the expression of the Th1-type cytokine of IL-18, and upregulated the expressions of the Th2-type cytokines of IL-10 and TGF-β in B cells in in vitro immunized PBL, although we could not detect significant changes in the expressions of IL-4 and IL-6 (Fig. 2C). Other cytokines could not be detected in B cells (data not shown). These results indicate that IL-10 induces B cells to secrete Th2-type cytokines, and further suggests that IL-10 functions to form an appropriate culture milieu to induce antibody production.

**IL-10 induces differentiation and maturation of B cells in in vitro immunization**

Since the differentiation of B cells into plasma B cells is crucial to specific antibody production, we next investigated the effect of IL-10 on B cell maturation, then analyzed cell size and the expression of specific surface marker antigen on B cells by flow cytometry. First we attempted to detect any changes in the cell size of B cells immunized in vitro in the absence or presence of IL-10. The results were that IL-10 increased the number of large B cells (encompassed in fields in Fig. 3A) from 2.68% (1,970 cells of a total of 73,528 cells) to 8.27% (5,108 cells of a total of 70,062 cells). Next we analyzed the expression of the cell surface marker antigen of plasma B cells, CD38, on large B cells. Although large B cells from PBL immunized in vitro in the absence of IL-10 weakly expressed CD38 antigen, large B cells from PBL immunized in vitro in
the presence of IL-10 augmented the expression of CD38 (Fig. 3B). We calculated the number of CD38\textsuperscript{high} cells showing log fluorescence intensity to be more than 100. CD38\textsuperscript{high} cells increased from 281 cells to 2,475 cells on the addition of IL-10, but expression levels of other surface molecules including CD86, CD80, CD40, and CD27 did not change on the addition of IL-10 (data not shown). All these results indicate that IL-10 induces the differentiation of B cells in in vitro immunized PBL into plasma B cells, resulting in augmentation of antibody production.

Effect of IL-10 on cell-cell interaction of in vitro immunized PBL

In in vitro immunization, one can observe many clusters of cells during culture, the sizes of which are correlated with the level of antibody production (Fig. 4). These results suggest that lymphocytes are activated in in vitro immunization, resulting in augmentation of cell-cell interactions and the efficient formation of clusters, which might lead to increased production of antibodies. Here we tested the effects of IL-10 on cluster formation in in vitro immunization. IL-10, however, did not significantly increase the number or size of cell clusters of in vitro immunized PBL (data not shown). These results suggest that IL-10 does not affect the cell-cell interactions of in vitro immunized PBL, and further that the size and number of clusters and the level of antibody production are separable factors in in vitro immunization.

Fig. 3. IL-10 Increased the Ratio of Large B Cells, and Augmented the Expression of CD38 on B Cells.

After 7 d of culture of PBL immunized in vitro in the absence or presence of IL-10, B cells were separated by a B cell isolation kit. Isolated B cells were stained with anti-CD38-PE conjugate antibody and analyzed by flow cytometry. A, Cell size was analyzed by front and side-scattering data. B, Gated cells were analyzed for their expression of CD38. Dotted line, PBL immunized in the absence of IL-10 stained with control antibody. Dashed line, PBL immunized in the absence of IL-10 stained with anti-CD38-PE. Straight line, PBL immunized in the presence of IL-10 stained with anti-CD38-PE.

Fig. 4. The Number and Size of Cell Clusters Generated in In Vitro Immunized PBL.

LLME-treated or non-treated PBL were in vitro immunized and cultured for 7 d. Photographs were taken at the same magnification (×200).
Discussion

We have established an in vitro immunization protocol to produce antigen-specific human antibody. In this protocol, human PBL are pretreated with LLME to remove NK and cytotoxic T cells, and then stimulated with antigen in the presence of IL-2, IL-4, and MDP. Stimulated PBL produce antigen-specific immunoglobulins during culture. During our study, we found that IL-10 gene expression was concordantly enhanced with augmented antibody production in LLME-treated PBL 1 week after antigen sensitization (Fig. 1A). These results suggest that IL-10 plays an important role in inducing antibody production in an IVI protocol. Here we investigated the effects of IL-10 on antibody production. Figure 1 shows that IL-10 markedly enhances the titer of antigen-specific antibody production from in vitro immunized PBL. These results suggest that IL-10 alters the culture milieu of in vitro immunized PBL into conditions to enhance the production of antigen-specific antibodies. We then tried to clarify any changes in the conditions to enhance the production of antigen-specific antibody production in an IVI protocol. Here we suggest that IL-10 plays an important role in inducing antibody production in an IVI protocol. Human PBL are pretreated with LLME to remove NK and cytotoxic T cells, and then stimulated with antigen in the presence of IL-2, IL-4, and MDP. Stimulated PBL produce antigen-specific immunoglobulins during culture. During our study, we found that IL-10 gene expression was concordantly enhanced with augmented antibody production in LLME-treated PBL 1 week after antigen sensitization (Fig. 1A). These results suggest that IL-10 plays an important role in inducing antibody production in an IVI protocol. Here we investigated the effects of IL-10 on antibody production. Figure 1 shows that IL-10 markedly enhances the titer of antigen-specific antibody production from in vitro immunized PBL. These results suggest that IL-10 alters the culture milieu of in vitro immunized PBL into conditions to enhance the production of antigen-specific antibodies. We then tried to clarify any changes in the culture milieu of in vitro immunized PBL on the addition of IL-10.

IL-10 is known to be a multifunctional cytokine with diverse effects on most hematopoietic cell types. A representative function of IL-10 is to suppress the production of Th1-type cytokines from T cells, B cells, and cells of macrophage/monocytes lineage. Focusing on the effects on B cells, IL-10 is known to improve the survival rate of B cells and promote their maturation. Furthermore, several researchers have reported that IL-10 induces memory B cells to differentiate into plasma cells.3–5) We then examined the effect of exogenously added IL-10 on T and B cells in in vitro immunized PBL. Figure 2A and B show that IL-10 downregulated expression of the Th1-type cytokines of IL-2, IL-18, and IFN-γ, but upregulated expression of the Th2-type cytokines of IL-4, IL-6, IL-10, and TGF-β in CD4+ and CD8+ T cells. These results indicated that IL-10 induced a Th2-type response in T cells included in in vitro immunized PBL. Next, we investigated the cytokine gene expression profiles of B cells separated from in vitro immunized PBL. Figure 2C shows that the Th1-type cytokine of IL-18 greatly decreased, while the Th2-type cytokines of IL-10 and TGF-β increased in B cells on the addition of IL-10. Basal IL-18 expression in B cells has been reported to be augmented on LPS stimulation, thus suggesting that inflammation is the major source of IL-18 expression.6) These results clearly indicate that IL-10 induced the cytokine balance in in vitro immunized PBL to incline toward the Th2-type, which leads to the formation of a milieu for efficient antibody production. Furthermore, phenotypic analysis of B cells after in vitro immunization (Fig. 3) showed that IL-10 promoted the differentiation of B cells in PBL immunized in vitro into plasma cells (CD38high). All these results demonstrate that IL-10 has several functions that facilitate efficient antibody production in an in vitro immunization protocol, and suggest that antigen-specific human antibody can be efficiently produced by in vitro immunization on the addition of IL-10. In fact, IL-10 is known to augment antibody production in activated B cells in some in vivo systems as well as some in vitro systems.3,4) Thus, the in vitro immunization protocol to generate antigen-specific human mAb for clinical use can be improved by using IL-10.

IL-10 can also indirectly elicit a Th2-type response by affecting the function of antigen presenting cells (APC) such as dendritic cells, macrophages, and monocytes.7–9) If the target APC of IL-10 in an in vitro immunization protocol can be identified, and its functionality leading to eliciting the Th2-type response understood, then we can refine the in vitro immunization protocol.

References

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