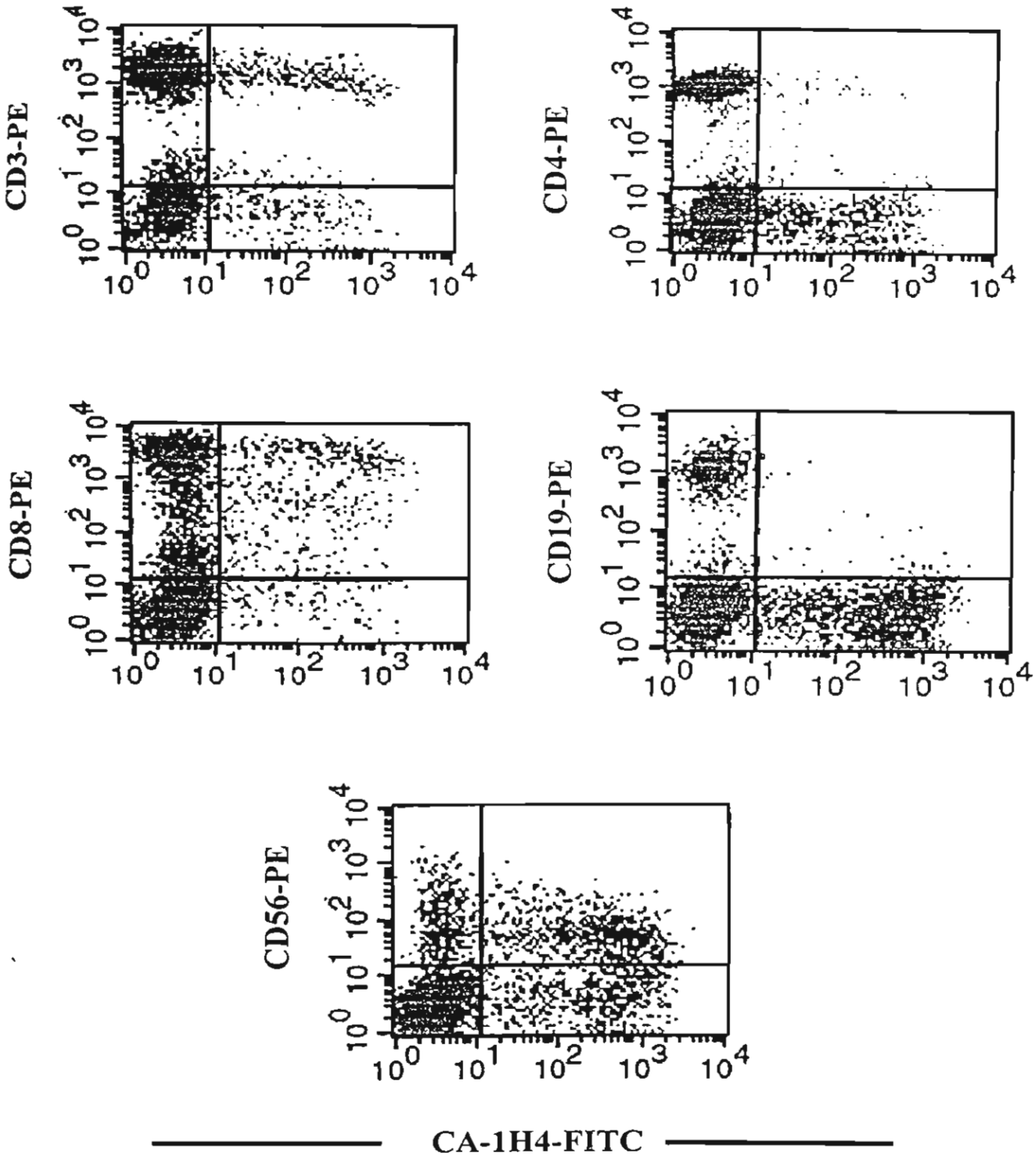
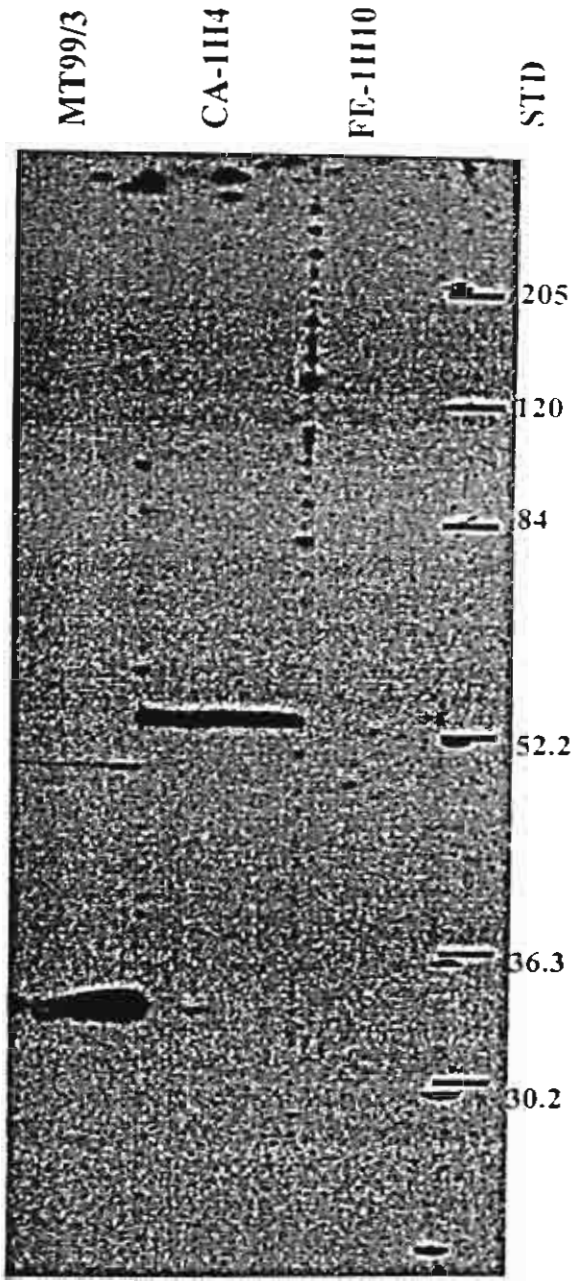
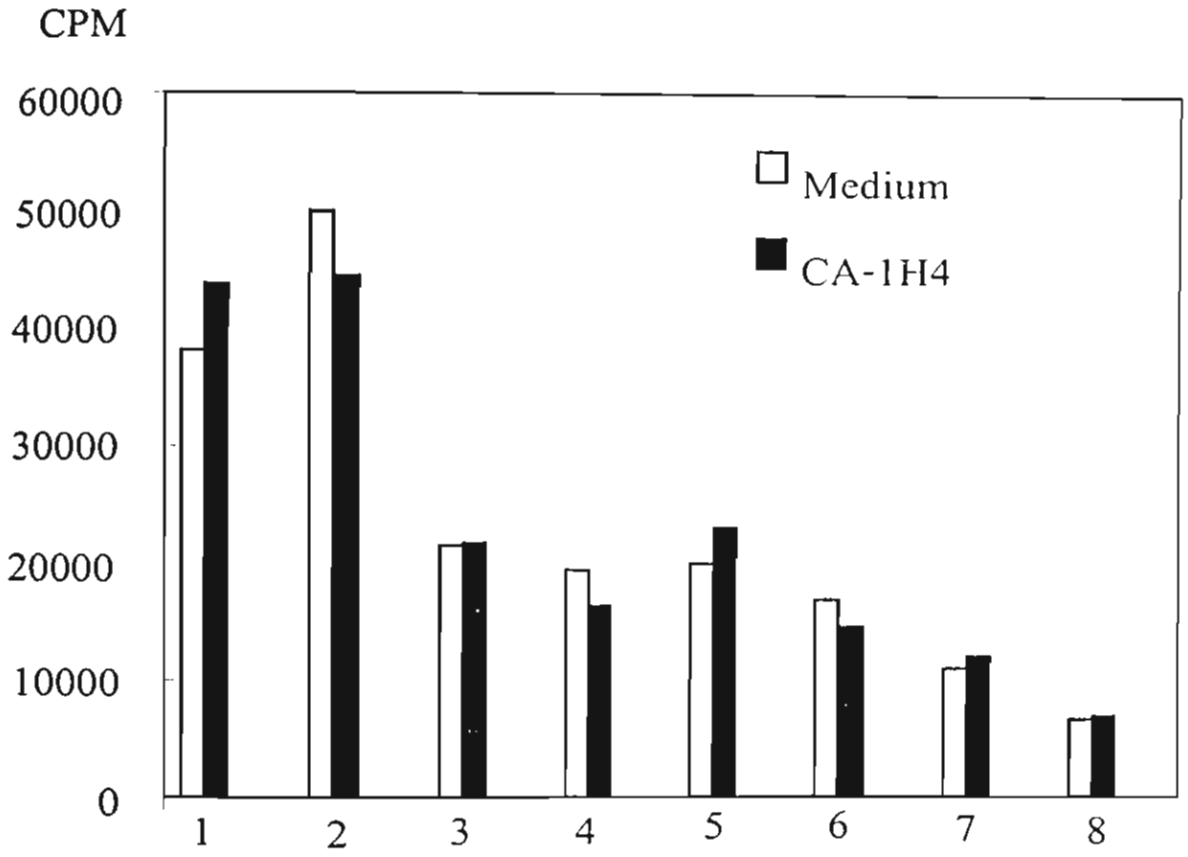


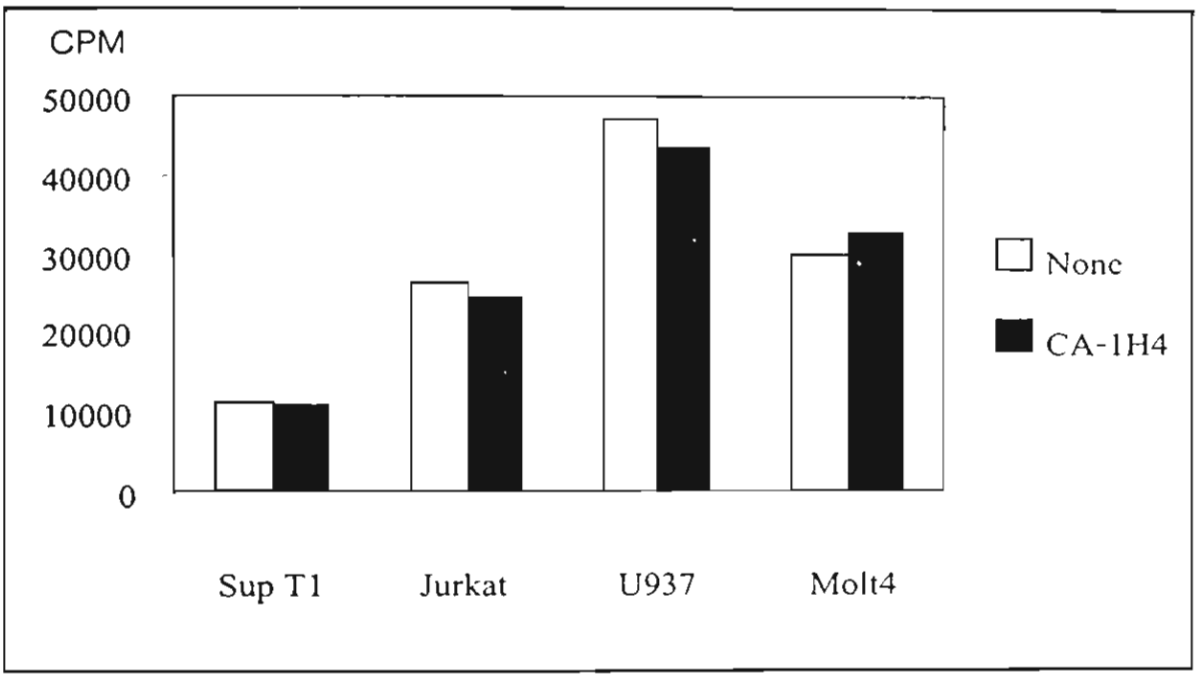

**รูปที่ 17.** Expression of CA-1H4 on lymphocyte sub-populations. Lymphocytes were stained with mAb CA-1H4-FITC and CD3-PE, CD4-PE, CD8-PE, CD19-PE or CD56-PE by direct immunofluorescence and analyzed by flow cytometry.



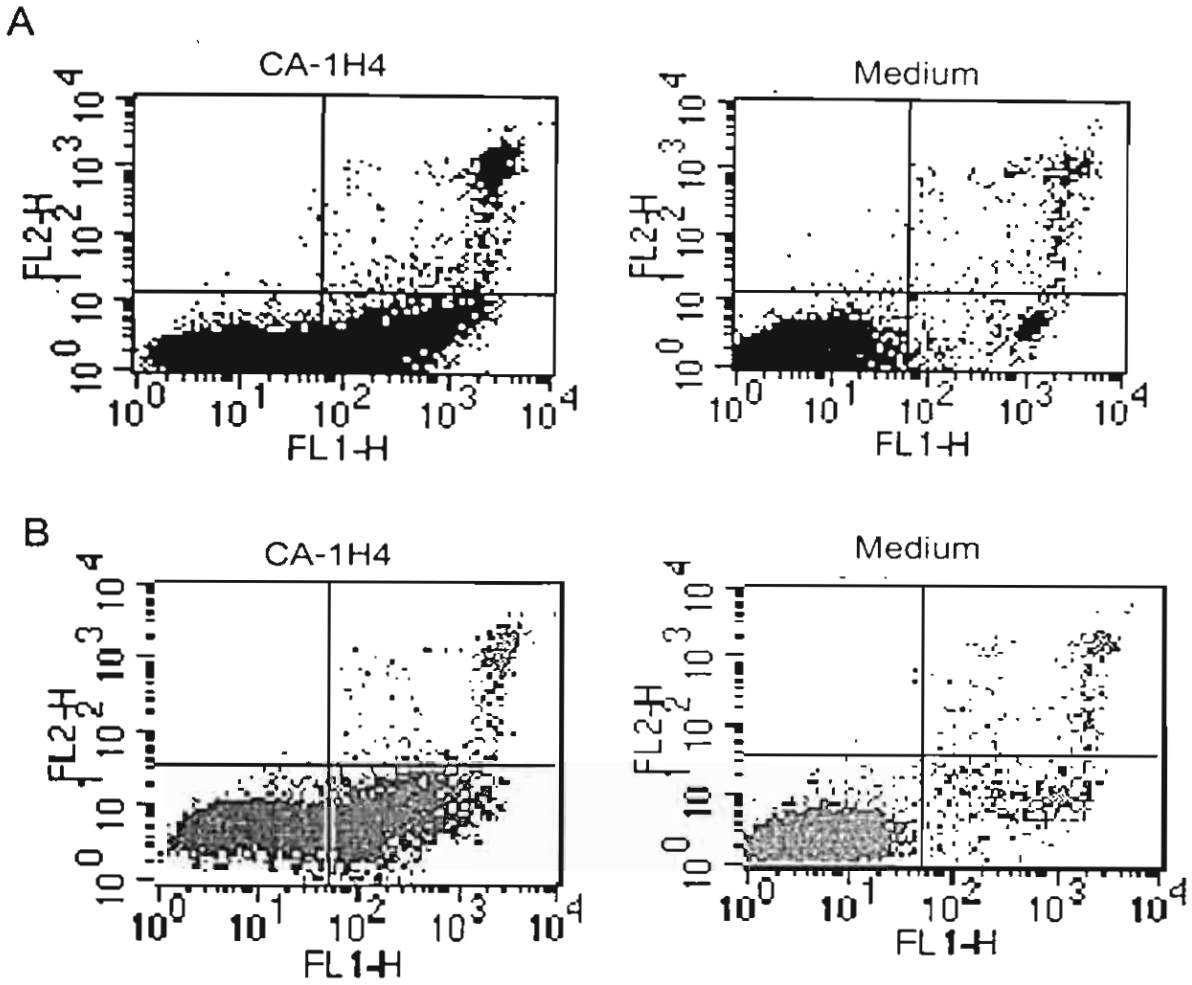

**รูปที่ 18** Western blot analysis of the cell surface molecule recognized by CA-1H4 mAb. Membrane was probed with MT99/3 (CD99 mAb), CA-1H4 mAb or negative control FE-1H10 mAb. Electrophoresis was performed under reducing. Molecular markers are shown on the right in kDa.




**รูปที่** 19 Effect of CA-1H4 mAb on CD3-induced T cell proliferation. PBMCs were activated with immobilized OKT3 mAb 1 □g/ml in the presence of CA-1H4 mAb or medium. The results of 8 healthy donors were shown.




**รูปที่** 20 Effect of CA-1H4 mAb on spontaneous proliferation of hemopoietic cell lines. Various cell lines including SupT1, Jurkat, U937 or Molt4 were cultured with CA-1H4 mAb (20 μg/ml) or medium for 5 hours. Cell proliferation was measured by [$^{3}$H]-thymidine incorporation assay.




**รูปที่** 21 Effect of CA-1H4 mAb on induction of apoptosis. SupT1 (A), or Molt4 T (B) cell lines were incubated with 20 μg/ml of CA-1H4 mAb or medium alone for 1 hours. Treated cells were analyzed for apoptotic cells by staining with Annexin V-FITC and PI.

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Output จากโครงการวิจัย

1. **ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ**


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2. **การนำผลงานวิจัยไปใช้ประโยชน์**

1. โมโนโคลนอล แอนติบอดีที่ผลิตได้ ได้นำมาใช้ในการเรียนการสอนระดับปริญญาตรี และระดับบัณฑิตศึกษาในคณะเทคนิคการแพทย์ มหาวิทยาลัยเชียงใหม่
2. ผลิตนักศึกษาปริญญาเอก 1 คน คือ น.ส. สาวิตรี เจียมพานิชยกุล (นักศึกษาในโครงการปริญญาเอกกาญจนาภิเษก)

3. **อื่นๆ**

1. **การจดสิทธิบัตร**

ได้ยื่นขอจดสิทธิบัตร จำนวน 1 เรื่อง คือ

ชื่อสิ่งประดิษฐ์ โมโนโคลนอล แอนติบอดีต่อ $Na^+$,$K^+$ ATPase $\beta$3 subunit กดการตอบสนองของทีและบี ลิมโฟซัยท์

ชื่อผู้ประดิษฐ์ นายวัชระ กสิณฤกษ์ และ น.ส. สาวิตรี เจียมพานิชยกุล

เลขที่คำขอ 075651

วันที่ยื่น 5 สิงหาคม 2545

ประเทศที่ยื่นขอ ประเทศไทย

## 2. การตีพิมพ์ในวารสารวิชาการในประเทศ

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## 4. รางวัลผลงานวิจัยที่ได้รับ จำนวน 4 เรื่อง ได้แก่

(1) รางวัลสภาวิจัยแห่งชาติ ประจำปี 2546ประเภทรางวัลผลงานวิจัย ระดับชมเชย

(2) อาจารย์ที่ปรึกษาวิทยานิพนธ์ ที่ได้รับรางวัลโปสเตอร์ดีเด่น ในงานมหิดล ปี 2546 จาก คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

(3) อาจารย์ที่ปรึกษาวิทยานิพนธ์ ที่ได้รับรางวัลโปสเตอร์ชมเชย ในงานมหิดล ปี 2546 จาก คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

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# ภาคผนวก

**Reprint จำนวน 4 เรื่อง คือ**


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2. Tayapiwatana C, Kasinrerk W. Construction and characterization of phage-displayed leukocyte surface molecule, CD99. Appl Microbiol Biotechnol. 2002; 60:336-341.
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HYBRIDOMA AND HYBRIDOMICS
Volume 21, Number 4, 2002


# Production of Antibodies by Single DNA Immunization: Comparison of Various Immunization Routes

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## ABSTRACT

**DNA immunization is a recent vaccination method that induces humoral and cellular immune responses in a range of hosts. Different immunization routes induce a different degree of the immune response. In the present report, we demonstrate that multiple intramuscular immunizations of plasmid DNA encoding various leukocyte surface molecules induced a specific antibody response. In contrast, a single intramuscular immunization could not induce antibody production. To study the induction of antibody response after a single immunization of plasmid DNA, mice were single-dose intramuscularly, intraperitoneally, intravenously and intrasplenically immunized, simultaneously, with the same preparation of plasmid DNA encoding CD147 membrane protein. We observed that only the intrasplenic route induced specific antibody production. The induction of antibody by intrasplenic immunization was confirmed by using plasmid DNA encoding CD54 molecule. By this single-dose DNA intrasplenic immunization, the generated antibodies could be detected in mice up to 6 months. These results suggest that the injected DNA is expressing the relevant protein antigen in the spleen for several months after injection. Our results demonstrate that direct immunization of antigen-encoding DNA into the spleen is a more effective method for induction of antibody production. This finding may support future investigations of DNA vaccination strategies that specifically promote the uptake of plasmid by splenocytes. Intrasplenic immunization may also be helpful in the understanding of the early events of the immune response to DNA vaccine and be useful as an effective route for the induction of immune responses.**

## INTRODUCTION

DNA IMMUNIZATION refers to the induction of an immune response to a protein expressed *in vivo*, subsequent to the introduction of its encoding DNA. In contrast to classical protein immunization, where antigens are administered, DNA immunization involves the administering of genetic material encoding the antigen. The antigen is, therefore, produced within the cells of the immunized individual and induces the immune responses.[1,2] Several investigators have demonstrated the feasibility of using direct injection of plasmid DNA for the induction of protective immunity against various pathogens[2,3] and the production of specific antibodies.[4–12] Immunization with DNA-based plasmid has been successfully attempted in several tissues by various routes of administration.[13] However, the intramuscular injection has been demonstrated as the most efficient route to transfer an aqueous solution of plasmid DNA.[13] This method, however, still results in a low efficiency of gene transfer and considerable variability in gene expression.[14,15] Induction of immune responses by a single intramuscular immunization of plasmid DNA has been demonstrated,[16–18] however, booster(s) were required in several reports.[19–22]

To obtain the appropriate immunization route for a single plasmid DNA injection for the induction of humoral immune response, in the present report we compared various administration routes including intramuscular, intraperitoneal, intravenous, and intrasplenic. We demonstrate here that, for a single plasmid DNA administration, intrasplenic immunization of antigen-encoding DNA is a more effective method in induction of antibody production. The induced antibody could be detected in mice up to 6 months after DNA immunization.

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## MATERIALS AND METHODS

### *Cells and antibodies*

Human hemopoietic cell lines, Sup T1 and U937, were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 40 μg/mL gentamicin and 2.5 μg/mL amphotericin B in a humidified atmosphere of 5% $CO_2$ at 37°C. COS7 cells were cultured in MEM (Gibco) containing 10% FBS and antibiotics. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by a Ficoll Hypaque density gradient centrifugation (Sigma-Aldrich Chemical, St. Louis, MO).

Purified CD54 monoclonal antibody (MAb) MT54 and CD147 MAb M6-1D4 were generated in our department.[12,23] Fluorescein isothiocyanate (FITC)-conjugated rabbit F(ab')2 anti-mouse immunoglobulin antibodies was purchased from Dako (Glostrup, Denmark).

### *Preparation of plasmid DNA*

cDNA encoding CD4, CD14, CD45, and CD54 membrane proteins inserted into an eukaryotic expression vector pCDM8 (designated CD4-DNA, CD14-DNA, CD45-DNA and CD54-DNA, respectively) and cDNA encoding soluble CD147-human IgG fusion protein (named CD147-Rg-DNA) were kind gifts from Dr. Hannes Stockinger, University of Vienna, Vienna, Austria. cDNA encoding CD147 membrane protein, named CD147-DNA, was generated in our Department.[24] For large-scale preparation, the plasmid DNA were transformed into *E. coli* MC1061/p3. The plasmid DNA were then isolated from transformed *E. coli* by Qiagen chromatography columns (Qiagen, Hilden, Germany). The plasmid DNA obtained were subsequently resuspended in phosphate-buffered saline (PBS). The concentration and purity of DNA preparation were determined by $OD_{260/280}$ reading. DNA were stored at −20°C, until injected into the mice.

The isolated plasmid DNA were proved for expression of the corresponding proteins by using the COS cell expression system. Indirect immunofluorescent staining of the transfected COS cells with specific MAbs was used for proving the expression of membrane protein. Enzyme-linked immunosorbent assay (ELISA) was used for proving the expression of soluble CD147-IgG fusion protein.

### *DNA immunization*

For intramuscular immunization, BALB/c mice were injected one or five times at 2-week intervals by intramuscular route at the hind legs (100 μg of DNA/dose). For intraperi-

TABLE 1. ANTIBODY RESPONSES IN MICE AFTER 5-DOSES OF INTRAMUSCULAR IMMUNIZATIONS WITH PLASMID DNA ENCODING VARIOUS LEUKOCYTE SURFACE MOLECULES

| | | *Dose of immunization* | | | | |
|---|---|---|---|---|---|---|
| ***DNA*** | ***Pre-immune*** | *1* | *2* | *3* | *4* | *5* |
| **CD4-DNA** | | | | | | |
| Mouse 1 | − | − | − | + | + | + |
| Mouse 2 | − | − | + | + | + | + |
| Mouse 3 | − | − | + | + | + | + |
| Mouse 4 | − | − | + | + | + | + |
| **CD14-DNA** | | | | | | |
| Mouse 1 | − | − | + | + | + | + |
| Mouse 2 | − | − | − | − | − | + |
| **CD45-DNA** | | | | | | |
| Mouse 1 | − | − | + | + | + | + |
| Mouse 2 | − | − | + | + | + | + |
| **CD54-DNA** | | | | | | |
| Mouse 1 | − | − | + | + | + | + |
| Mouse 2 | − | − | + | + | + | + |
| Mouse 3 | − | − | + | + | + | + |
| Mouse 4 | − | − | + | + | + | + |
| **CD147-DNA** | | | | | | |
| Mouse 1 | − | − | + | + | + | + |
| Mouse 2 | − | − | − | + | + | + |
| Mouse 3 | − | − | − | − | + | + |
| Mouse 4 | − | − | − | − | + | + |
| Mouse 5 | − | − | + | + | + | + |
| **CD147-Rg-DNA** | | | | | | |
| Mouse 1 | − | − | − | + | + | + |
| Mouse 2 | − | − | − | + | + | + |
| Mouse 3 | − | − | + | + | + | + |

Mice were immunized with 5 doses of various plasmid DNA. Sera were collected at pre-immunization and at 2 weeks after the indicated doses of DNA immunization. For detection of CD4 antibody, Sup T1 cells were used as target cells. For detection of CD14 antibody, peripheral blood monocytes were used as target cells. For detection of CD45, CD54, CD147 antibody, U937 cells were used as target cells. The target cells were stained with the collected sera (dilution 1:10) by indirect immunofluorescence and analyzed by flow cytometer. Antibody reactivity: −, negative; +, positive.

toneal immunization, 500 μL (100 μg) of plasmid DNA solution was injected into the peritoneal cavity. For intravenous immunization, 100 μL (100 μg) of plasmid DNA solution was injected into the tail vein. For intrasplenic immunization, mice were anesthetized with diethylether. The skin and peritoneum on the left side of the body was open to expose the spleen. Fifty microliters (100 μg) of plasmid DNA were injected into the spleen. The peritoneum and skin were then closed by fine sutures. Blood samples were collected from the immunized mice by tail bleeding at 2-week intervals. Sera were separated and stored at −20°C.

### *Immunofluorescence analysis*

To determine antibodies in the sera, indirect immunofluorescence was carried out. To block nonspecific Fc-receptor-mediated binding of antibody, cells were pre-incubated for 30 min at 4°C with 10% human AB serum before staining. Blocked cells were then incubated for 30 min at 4°C with tested sera. After washing, cells were incubated with FITC-conjugate for 30 min. Membrane fluorescence was analyzed on a FACSCalibur (Becton Dickinson, Sunnyvale, CA) flow cytometer.

### *DEAE-dextran transfection of COS7 cells*

Plasmid DNA were transfected into COS7 cells by the DEAE-dextran transfection method.[24] Briefly, $1 \times 10^6$ COS7 cells were transfered to 6-cm tissue culture dishes (NUNC, Roskilde, Denmark) on the day before transfection. Cells were incubated with 2 mL of MEM containing 250 μg/mL DEAE-dextran (Sigma), 400 μM chloroquine diphosphate (Sigma), and 2 μg DNA for 3 h at 37°C. Supernatant was removed and cells were treated with 10% dimethyl sulfoxide (DMSO) in PBS for 2 min at room temperature. Cells were then cultured in MEM containing 10% fetal bovine serum (FBS) overnight, washed once, and recultured with the same medium for another 2 days to allow expression of the corresponding proteins.

## RESULTS

### *Production of antibodies by intramuscular immunization of plasmid DNA*

To produce antibodies by injection of antigen-encoding plasmid DNA, intramuscular immunization was used as inoculation routes. In this study, plasmid DNA encoding CD4, CD14, CD45, CD54, CD147 membrane protein or secreted CD147-human IgG fusion protein was prepared by using Qiagen chromatography column. All plasmid DNA obtained were tested for its expression by COS cells transfection. The plasmid DNA encoding membrane proteins were to be able to express the corresponding proteins on COS cell membrane as determined by immunofluorescence technique (data not shown). The CD147-Rg DNA was able to produce soluble CD147-IgG fusion protein in culture supernatant of transfected COS cells as determined by ELISA (data not shown).

Mice were intramuscularly immunized with the plasmid DNA for 5 times at 2-week intervals or with a single injection. Sera collected from each mouse were first screened for the presence of specific antibodies by staining of cells expressing the

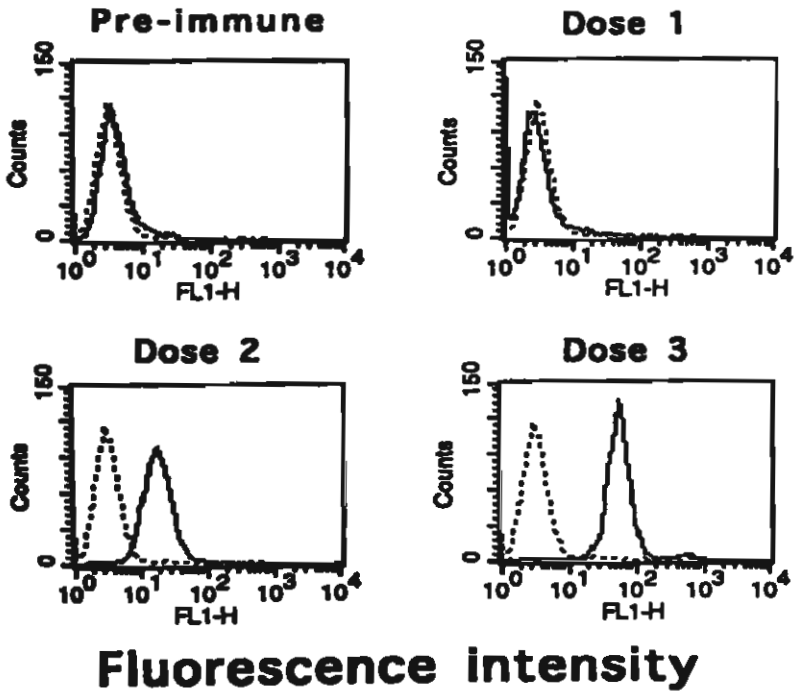


**FIG. 1.** FACS analysis of anti-CD147 antibodies generated by DNA immunization. Mouse was immunized with plasmid DNA encoding CD147 membrane protein. Sera were collected at pre-immunization and at 2 weeks after the indicated doses of DNA immunization. U937 cells were stained with indicated sera at 1:10 (solid lines) or without serum (dashed lines) by indirect immunofluorescence assay.

TABLE 2. ANTIBODY RESPONSES IN MICE AFTER SINGLE-DOSE IMMUNIZATION OF PLASMID DNA ENCODING CD147 MOLECULE BY VARIOUS IMMUNIZATION ROUTES

| | | *Weeks after DNA immunization* | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Route* | *Pre-immune* | *2* | *4* | *6* | *8* | *10* | *12* | *14* | *16* | *18* | *20* | *22* | *24* | *26* | *28* | *30* | *32* |
| Intraperitoneal | | | | | | | | | | | | | | | | | |
| Mouse 1 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Mouse 2 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Intramuscular | | | | | | | | | | | | | | | | | |
| Mouse 1 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Mouse 2 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Intravenous | | | | | | | | | | | | | | | | | |
| Mouse 1 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| Intrasplenic | | | | | | | | | | | | | | | | | |
| Mouse 1 | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

Mice were immunized with single-dose plasmid DNA encoding CD147 molecule by the indicated route. Sera were collected at pre-immunization and every 2 weeks after DNA immunization. U937 cells were stained with sera (dilution 1 : 10) by indirect immunofluorescence and analyzed by flow cytometer.

Antibody reactivity: −, negative; +; positive.

corresponding proteins. As shown in Table 1, sera from 5-doses intramuscularly either plasmid DNA encoding CD4, CD14, CD45, CD54, or CD147 immunized mice showed positive reactivity. Plasmid DNA encoding membrane CD147 and secreted CD147 protein induced antibody production in a similar pattern. For an example of flow cytometric analysis, antibody responses of a mouse immunized with plasmid DNA encoding CD147 membrane protein is shown in Fig. 1. In contrast, none of the sera, collected every 2 weeks up to 5 months, from single-dose intramuscularly immunized mice showed positive reactivity (data not shown).

To confirm that the generated antibodies after plasmid DNA immunization were specific for the corresponding proteins, the CD4-DNA, CD14-DNA, CD45-DNA, CD54-DNA, and CD147-DNA transfected COS cells were stained with the positive sera. As predicted, sera obtained from CD4, CD14, CD45, CD54, and CD147 and CD147-Rg-DNA immunized mice reacted to CD4, CD14, CD45, CD54, and CD147 transfectants, respectively, but they did not react to mock transfectants (data not shown). Pre-immune sera of each mouse did not react to any transfectants.

### *Production of antibodies by a single immunization of plasmid DNA*

In an attempt to produce specific antibody by only a single immunization of the encoding plasmid DNA, CD147-DNA were injected into mice by using intraperitoneal, intramuscular, intravenous and intrasplenic routes, simultaneously. As shown in Table 2, by a single intramuscular, intraperitoneal and intravenous route, no specific antibodies were detected. However, sera obtained from a single-dose intrasplenic immunized mouse showed positive reactivity. All positive sera were stained with COS cell transfectants and showed positive reactivity with CD147-DNA transfectants, and negative with mock transfectants. The results indicated that intrasplenic immunization is an effective route for single plasmid DNA injection.

To confirm that single intrasplenic immunization can induce antibody production, CD54-DNA were immunized into six mice. We found that all mice generated specific antibody (Table 3). Interestingly, the generated antibodies could be detected in the immunized mice sera up to several months (Table 3 and Fig. 2).

## DISCUSSION

The principle of DNA immunization has been demonstrated in several different animal models. Currently, it is clear that the

TABLE 3. ANTIBODY RESPONSES IN MICE AFTER SINGLE-DOSE INTRASPLENIC IMMUNIZATION OF PLASMID DNA ENCODING CD54 MOLECULE

| | *Mouse number* | | | | | |
|---|---|---|---|---|---|---|
| *Weeks after immunization* | *1* | *2* | *3* | *4* | *5* | *6* |
| Pre-immune | − | − | − | − | − | − |
| 2 | + | − | − | − | − | − |
| 4 | + | − | − | − | − | − |
| 6 | + | − | − | − | − | + |
| 8 | + | − | − | − | − | + |
| 10 | + | − | + | − | − | + |
| 12 | + | + | + | + | − | nd |
| 14 | + | + | + | + | + | nd |
| 16 | + | + | nd | + | + | nd |
| 18 | + | + | nd | + | + | nd |
| 20 | + | + | nd | + | + | nd |
| 21 | + | + | nd | + | + | nd |
| 22 | + | + | nd | + | + | nd |
| 24 | + | + | nd | + | + | nd |
| 26 | nd | + | nd | + | + | nd |
| 28 | nd | + | nd | + | + | nd |

Mice were immunized with single-dose CD54-DNA by intrasplenic route. Sera were collected at pre-immunization and every two weeks after DNA immunization. U937 cells were stained with sera (dilution 1 : 10) by indirect immunofluorescence and analyzed by flow cytometer.

Antibody reactivity: −, negative; +, positive; nd, not determine.

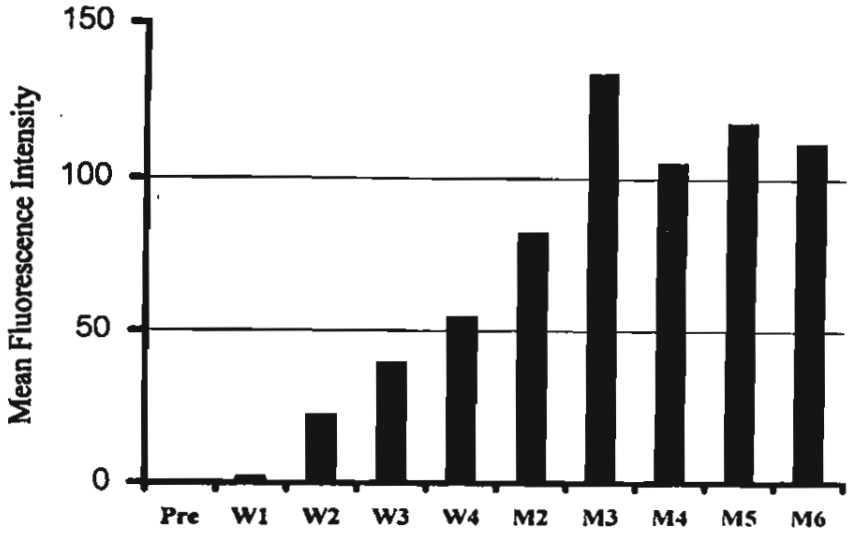


**FIG. 2.** Antibody response of a mouse intrasplenic immunized with CD54 encoding DNA. Mouse was single immunized on Days 0 and sera from blood drawn periodically were analyzed by indirect immunofluorescence and flow cytometer using U937 cells as antigen. Pre, pre-immune serum; W1-W4, sera obtained from 1–4 weeks after DNA immunization; M2–M6, sera obtained from 2–6 months after DNA immunization.

induction of humoral and cellular immunity is possible with the DNA immunization strategy. DNA immunization, therefore, promises to be an attractive alternative to the classical vaccines.[2,3] DNA immunization was also proven to be a good method for the production of polyclonal antibody as well as for the generation of MAbs.[4–12] This method may provide the best way to produce specific antibodies to proteins that are difficult to purify.

Several DNA delivery methods have been used to introduce plasmid DNA for the induction of immune responses.[13] The most common route to transfer pure plasmid DNA is intramuscularly.[13] In the present report, we found that by using the intramuscular route, antibody production was achieved after multiple immunizations of plasmid DNA expressing various types of leukocyte surface molecules. The specific antibodies were detected after two to five DNA inoculations. In contrast, no mice that obtained a single dose intramuscular immunization generated antibody. Intramuscular injection of plasmid DNA has been widely used with DNA vaccines. In most cases of DNA intramuscular immunization, high titers of antibodies have been found against the expressed protein. Some investigators demonstrated that a single intramuscular immunization of plasmid DNA induced antibody responses,[16–18] however, booster(s) was required in several reports including this report.[19–22] These differences appear to be due to the nature of the particular antigen, the expression vector used and may be due to the skill of the individual administering the DNA.[21,25] When DNA is administered by intramuscular injection, the plasmid DNA is taken up by myocytes. Due to the limitation of diffusion by physical factors such as the organization of the connective tissue, and extent of the extracellular matrix,[14,15] the intramuscular immunization of DNA results in a low efficiency of gene transfer and a considerable variability of gene expression. Therefore, induction of antibody production by intramuscular immunization of plasmid DNA requires booster dose(s).

Injection of protein antigen directly into lymphoid organs such as lymph node or spleen offers some strong theoretical advantages over other injection routes.[26,27] In this type of immunization, the immunogen is concentrated in one region that is specialized in dealing with it. This immunization route has been further used for polyclonal and MAb production purposes.[28–31] In an attempt to induce antibody production by a single-short plasmid DNA inoculation, in the present study, intrasplenic immunization was selected and compared with intramuscular, intraperitoneal and intravenous routes, simultaneously, by using the same preparation plasmid DNA. We found that only the intrasplenic route induced antibody production. Our results confirmed the previous reported possibility of using intrasplenic immunization for the induction of antibody responses by DNA immunization.[11,12] Enhancement of antibody responses by intrasplenic immunization is likely to be related to the fact that the injected plasmid DNA are directly transfected splenocytes, including antigen-presenting cells. The antigens are then expressed and concentrated in the spleen, where the immune responses are initiated. Antigen-presenting cells are thought to play at least three distinct roles in DNA immunization: (1) major histocompatibility complex (MHC) class II-restricted presentation of antigens secreted by neighboring, transfected cells, (2) MHC class I-restricted "cross" presentation of antigens released by neighboring, transfected cells, and (3) direct presentation of antigens by transfected antigen presenting cells themselves.[32] Therefore, injection of DNA into the spleen allows for the direct delivery of antigens to the spleen and the induction of antibody responses occur only with a single-short DNA immunization. In the present report, the induced antibody could be detected in mice up to 6 months after DNA intrasplenic immunization. These results suggest that the injected DNA is still expressing the relevant protein antigen, in the spleen, for several months after injection.

In protein immunization, it was clearly easier to obtain an

antibody response using minute amounts of antigen by intrasplenic route than by the intraperitoneal or intravenous route.[33] In agreement with protein immunization, this study demonstrated that a single injection of antigen-encoding DNA into the spleen induced antibody response better than via an intramuscular, intraperitoneal, or intravenous route. Recently, the use of intrasplenic immunization of plasmid encoding carcinoembryonic antigen (pCEA) for induction of immune responses was reported.[34] Intrasplenic administration of pCEA could induce specific antibody responses and partial immunoprotection against tumor challenge.[34] Our findings, therefore, support future investigations of DNA vaccination strategies that specifically promote the uptake of plasmid by splenocytes. This intrasplenic immunization may also be helpful in the further understanding of early events of the immune response to DNA vaccine as well as for the production of specific antibodies.

## ACKNOWLEDGMENTS

The authors thank Dr. Hannes Stockinger for providing plasmid DNA encoding various proteins. This work was supported by The Thailand Research Fund and The National Center for Genetic Engineering and Biotechnology (BIOTEC) of the National Science and Technology Development Agency.

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Received for publication February 21, 2002; Accepted for publication February 27, 2002

Appl Microbiol Biotechnol (2002) 60:336–341
DOI 10.1007/s00253-002-1146-x

ORIGINAL PAPER

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# Construction and characterization of phage-displayed leukocyte surface molecule CD99

Received: 11 July 2002 / Revised: 5 September 2002 / Accepted: 13 September 2002 / Published online: 5 October 2002


**Abstract** The phage display technique has been described for the production of various recombinant molecules. In the present report, we used this technique to display a leukocyte surface molecule, CD99. PCR subcloning of CD99 cDNA from the mammalian expression vector pCDM8 to the phagemid expression vector pComb3HSS was performed. The resulting phagemid, pComb3H-CD99, was transformed into *Escherichia coli* XL-1 Blue. CD99 was displayed on the phage particles following infection of the transformed *E. coli* with the filamentous phage VCSM13. Using sandwich ELISA, the filamentous phage-displayed CD99 was captured by a CD99 monoclonal antibody (mAb) then detected with anti-M13 conjugated to horseradish peroxidase, confirming that the CD99 molecule was displayed on the phage particles. The CD99-phages inhibited induction of Jurkat cell aggregation by CD99 mAb MT99/1. Proper folding of the displayed CD99 bioactive domain was inferred from this finding. Our results demonstrate that the phage display technique can be applied to the generation of full-length CD99 molecules. The phage carrying this cell surface protein will be useful for identification of its counter receptor or ligand.

## Introduction

The filamentous phage display technique, first described by Smith (1985), has been widely used for expression of polypeptides and proteins. Currently, several phagemid vectors are available for different purposes (McCafferty et al. 1994; Crameri and Blaser 1996; Persic et al. 1997). Basically, the recombinant molecules are fused with phage coat proteins, gp3 or gp8, and displayed on the surface of phage particles. The phage display technique delivers the recombinant molecule to the periplasm of *Escherichia coli* with the assistance of signal peptides. Due to the higher oxidizing conditions in comparison to the cytoplasm, the periplasmic environment effectively promotes disulfide bond formation (Becker and Hsiung 1986; Dracheva et al. 1995). Therefore, using phage display technique, the correct conformation of a recombinant protein is obtained. In 1995, Barbas and Wagner constructed the phagemid vector pComb3HSS for delivering a Fab fragment to the periplasmic space of *E. coli* (Barbas and Wagner 1995). Phage-displayed Fab libraries were produced and used to select Fabs specifically interacting with the epitope of interest. Recently, this vector was applied to the production of a tissue plasminogen activator deletion mutant (K2S) that contains nine disulfide bridges (Manosroi et al. 2001). Other complex molecules have also been displayed using this vector (Lasters et al. 1997; Appenzeller et al. 2001; Kurokawa et al. 2002). Production of recombinant protein by phage display technique has the major advantage that the displayed recombinant molecules can be directly and easily harvested from the *E. coli* culture supernatant by PEG precipitation.

Leukocytes express a large number of molecules on their surfaces. These leukocyte surface molecules are important for cell function. Antibodies raised against these molecules have become a major tool in characterizing the structure and function of these surface molecules. In addition to specific antibodies, the isolated cell surface molecules themselves have been broadly used for identification and functional characterization of their counter receptors or specific ligands (Bowen et al. 1996; Vilardell et al. 1998; Martinez-Pomares et al. 1999). However, to prepare these molecules from cell membranes, cumbersome steps of specific cell isolation are

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quired. In addition, contamination with undesired oteins is difficult to avoid. To overcome this problem, olecular techniques in mammalian cell expression stems have been employed. The Fc of immunoglobulin as used as a fusion partner of certain CD molecules, e.g., D31 (Prager et al. 1996) and CD147 (Koch et al. 1999). e fusion protein was then secreted into the culture edium and purified using a protein A column. Although e recombinant proteins obtained have an almost native nformation, mammalian cell expression systems are ore expensive and time-consuming in comparison to okaryotic expression systems. Moreover, the hydropho- c nature of the transmembrane region of the CD olecule makes it impossible to produce the entire length ersion as a secreted protein. An influence of the ansmembrane domain on the conformational structure f the external domain has already been shown (Gaudin et .. 1999). In contrast, lipocalin-1 interacting membrane ceptor, a molecule with nine putative transmembrane omains, was successfully expressed using phage display Wojnar et al. 2001).

In an attempt to produce recombinant leukocyte urface molecules using a prokaryotic expression system, ne potential of the phage display technique was evalu- ted. We demonstrated that the phage display technique ould be used to generate phage displayed CD99 nolecules. The constructed phages were able to inhibit urkat cell aggregation induced by monoclonal antibodies mAb) against CD99, indicating the presence of a ioactive domain. Our findings suggest that the phage lisplay technique is useful for displaying cell surface nolecules when the corresponding cDNA is available.

## Materials and methods

### Primer design

A pair of primers, CD99MatF 5'-GAGGAGGAGGTGGCCCAGG-CGGCCGATGGTGGTTTCGATTTA-3' and CD99MatR 5'-GAG-GAGGAGCTGGCCGGCCTGGCCTTTCTCTAAAAGAGTACG-3' (synthesized at the Bioservice Unit, National Center for Genetic Engineering and Biotechnology, Thailand) were designed to amplify the mature CD99-encoding gene carried by the mammalian expression vector pCDM 8 (Kasinrerk et al. 2000). The primers were designed with *Sfi*I end cloning sites (underlined) to maintain the correct reading frame of the inserted sequence from the ATG to the gpIII gene in the phagemid vector pComb3HSS.

### PCR amplification of the CD99 gene

Primers CD99MatF and CD99MatR (1 µg each) together with 50 ng CD99-encoding cDNA (pCDM 8-CD99) template were suspended in a 100 µl PCR mixture. *Taq* polymerase (2.5 U; Roche, Indianapolis, Ind.) was added last to the solution. The titrated amplification condition was initiated with a jump start at 85°C for 4 min, then denaturation at 95°C for 50 s, annealing at 42°C for 50 s, then extension at 72°C for 1.5 min for 35 cycles. The mixture was further incubated at 72°C for 10 min. The amplified product of 537 bp was subsequently purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The correct identity of the purified product was confirmed by restriction enzyme fragment analysis.

### Construction of a phagemid expressing CD99

The purified CD99 PCR product and the phagemid pComb3HSS (kindly provided by C.F. Barbas, Scripps Institute, La Jolla, Calif.) (Barbas and Wagner 1995) were digested with *Sfi*I (Roche) to prepare specific cohesive cloning sites. Purified PCR product (4 µg) was digested with 60 U *Sfi*I at 50°C for 18 h; for pComb3HSS, 20 µg phagemid was treated with 100 U *Sfi*I. Digested fragments of the purified PCR products and pComb3HSS (~3,300 bp) were subsequently gel-purified using a QIAquick Gel Extraction Kit (Qiagen). A ligation reaction was performed by introducing 5 U T4 DNA ligase (Roche) to a mixture of 0.7 µg purified *Sfi*I-digested pComb3HSS and 0.9 µg purified *Sfi*I-digested PCR product. Ligation was performed at 30°C for 18 h. The newly constructed phagemid was named pComb3H-CD99.

### Transformation of XL-1 Blue

$CaCl_2$ competent *E. coli* XL-1 Blue (200 µl) (Stratagene, La Jolla, Calif.) were transformed with 70 ng ligated product. The transformed cells were propagated by spreading on LB agar containing 100 µg/ml ampicillin and 10 µg/ml tetracycline (Sigma, St. Louis, Mo.). After cultivation at 37°C for 18 h, several antibiotic-resistant colonies were selected for plasmid minipreps using the alkaline lysis method. Each purified plasmid was subjected to *Sfi*I restriction site analysis. A transformant harboring a plasmid with the correct *Sfi*I restriction pattern was subsequently propagated for 18 h at 37°C in 100 ml LB broth with antibiotics as above. A plasmid maxiprep was performed using the Qiagen Plasmid Maxi Kit. The purified plasmid was re-examined by *Sfi*I digestion.

### Preparation of CD99-$\phi$

After transforming XL-1 Blue with pComb3H-CD99, the phage display technique was performed. A clone of pComb3H-CD99-transformed XL-1 Blue was propagated in 10 ml super broth containing 100 µg/ml ampicillin and 10 µg/ml tetracycline at 37°C until an OD at 600 nm of 1.5 was reached. The bacterial culture was subsequently propagated in 100 ml of the same medium and cultured for another 2 h. The transformed XL-1 Blue were infected with $10^{12}$ pfu VCSM13 helper phage (Stratagene). After 3 h incubation, kanamycin (final concentration 70 µg/ml) was added to the culture, which was then left shaking (200 rpm) for a further 18 h at 37°C. Bacteriophages harboring CD99 on gpIII (CD99-$\phi$) were then precipitated using 4% (w/v) PEG MW 8000 (Sigma) and 3% (w/v) NaCl. Finally, the harvested phage was resuspended in 2 ml phosphate-buffered saline (PBS), pH 7.4.

### Immunoassay for CD99-$\phi$

CD99-specific mAbs, MT99/1 (IgM isotype) and MT99/3 (Ig2a isotype), were generated in our laboratory (unpublished data, and Kasinrerk et al. 2000). Solid phase was separately coated with 1 µg MT99/1 and MT99/3. The same amount of a CD54 mAb, MT54 (Moonsom et al. 2001), was used as a control. Standard ELISA washing and blocking processes were performed. CD99-$\phi$ or VCSM13 phages (50 µl; $10^{11}$ pfu/ml) were added to each mAb-coated well. A suitable dilution of horseradish peroxidase (HRP)-conjugated sheep anti-M13 (Pharmacia, Uppsala, Sweden) was added to each reaction well after the washing step. The 3,3',5,5'-tetramethylbenzidine plus $H_2O_2$ substrate was added to every well and the reaction was finally stopped with $H_2SO_4$ solution after a 30-min incubation.

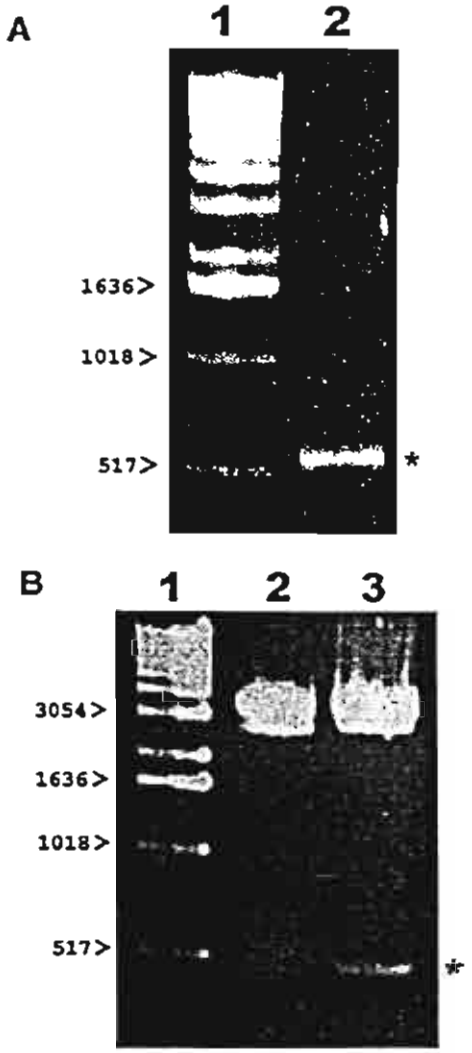


Fig. 1 a CD99 gene PCR amplification product from the pCDM 8-CD99 vector using primers CD99MatF and CD99MatR. Lanes: *1* DNA molecular weight marker (Roche), *2* 1 µl amplified product. A single band at 537 bp is depicted (*). b Restriction fragment analysis of pComb3-CD99. The constructed pComb3-CD99 was digested with *Sfi*I and electrophoresis was performed on a 1% agarose gel. Lanes: *1* DNA molecular weight marker, *2* uncut pComb3H-CD99, *3* *Sfi*I-digested pComb3H-CD99; inserted CD99 gene at 489 bp (*)

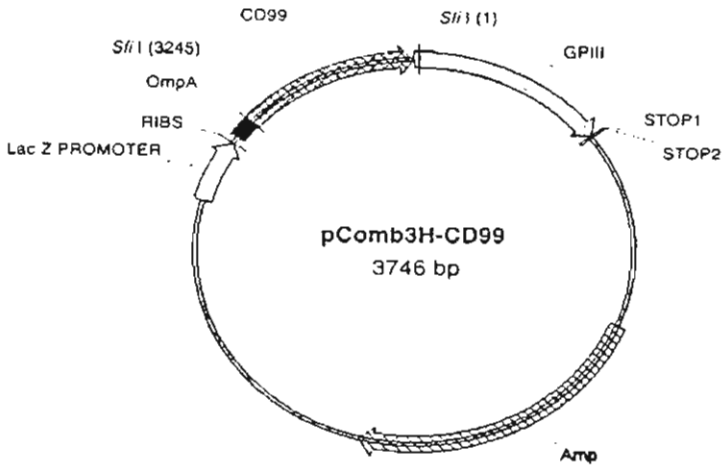


Fig. 2 Map of pComb3H-CD99. The two *Sfi*I cloning sites into which the CD99 gene was inserted are indicated. Signal sequence (*OmpA*), ribosome binding site (*RIBS*), *lac* promoter, and *gpIII* gene are also depicted

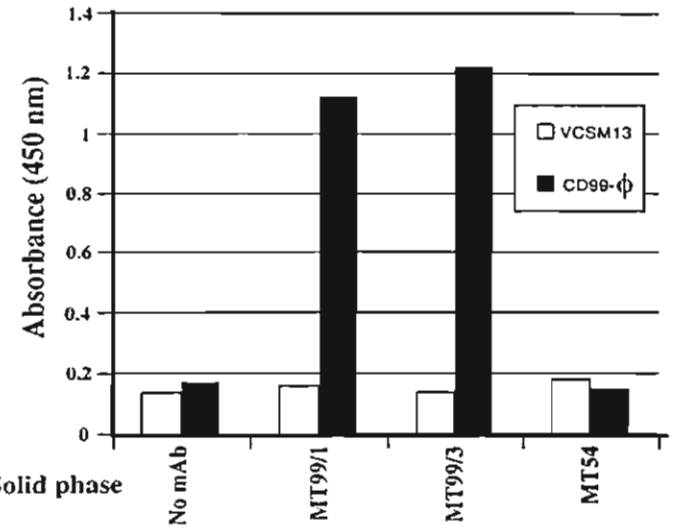


Fig. 3 Sandwich ELISA for the detection of phage bearing CD99. Solid phase was coated with either MT99/1, MT99/3, MT54 or no monoclonal antibody (mAb). VCSM13 was used as a negative control. The bound phage was traced with horseradish peroxidase (HRP)-conjugated sheep anti-M13

Validation of bioactive domain on CD99-$\phi$ by aggregation inhibition assay

The Jurkat human T-cell line was used as a target for homotypic cell aggregation. After washing three times, 75 µl Jurkat cells ($2.5\times10^5$ cells/ml) were transferred to a 96-well flat-bottomed tissue culture plate (Costar, Cambridge, Mass.). The aggregation base line was obtained by adding 50 µl of 0.15 µg/ml MT99/1 to the well. For the aggregation inhibition assay, 50 µl of $10^{12}$ pfu/ml CD99-$\phi$ were preincubated with 50 µl of 0.15 µg/ml MT99/1 for 1 h at 37°C before adding to the Jurkat cells. VCSM13 was used in place of CD99-$\phi$ as a negative inhibition control system. The final volume of each well was adjusted to 175 µl with culture medium. The culture was then maintained at 37°C in a humidified atmosphere with 5% $CO_2$ in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics. Cell aggregation was monitored every hour for 4 h under an inverted microscope (Olympus, Tokyo, Japan).

## Results

### Construction of a phagemid expressing CD99

In order to generate phage expressing CD99 molecules, a cDNA encoding CD99 protein cloned in the eukaryotic expression vector pCDM 8 (pCDM 8-CD99) (Kasinrerk et al. 2000) was used. From pCDM 8-CD99, we amplified the mature CD99 gene using primers CD99MatF and

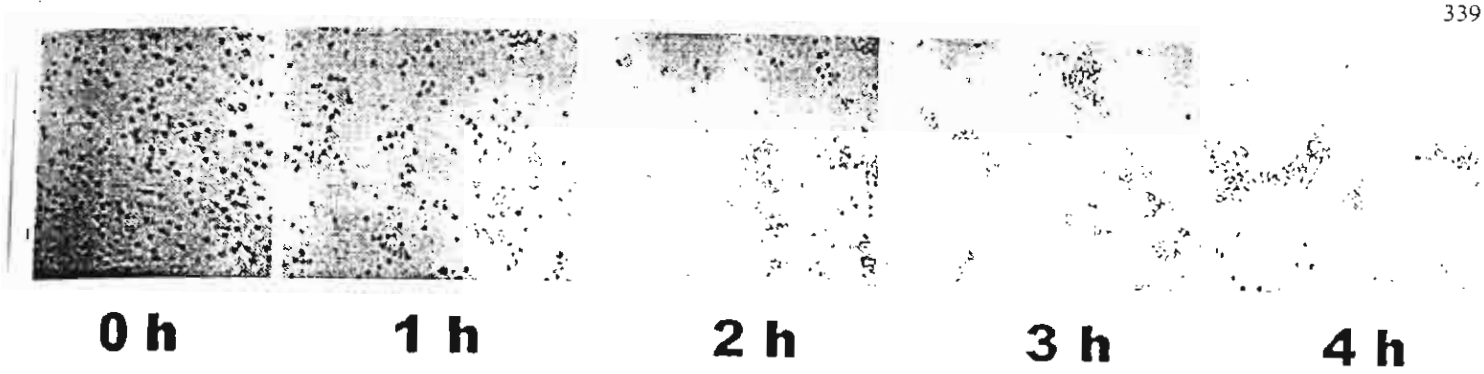


Fig. 4 Induction of Jurkat cell aggregation by MT99/1. Jurkat cells were incubated with MT99/1. Homotypic cell aggregation was monitored under an inverted microscope for 4 h

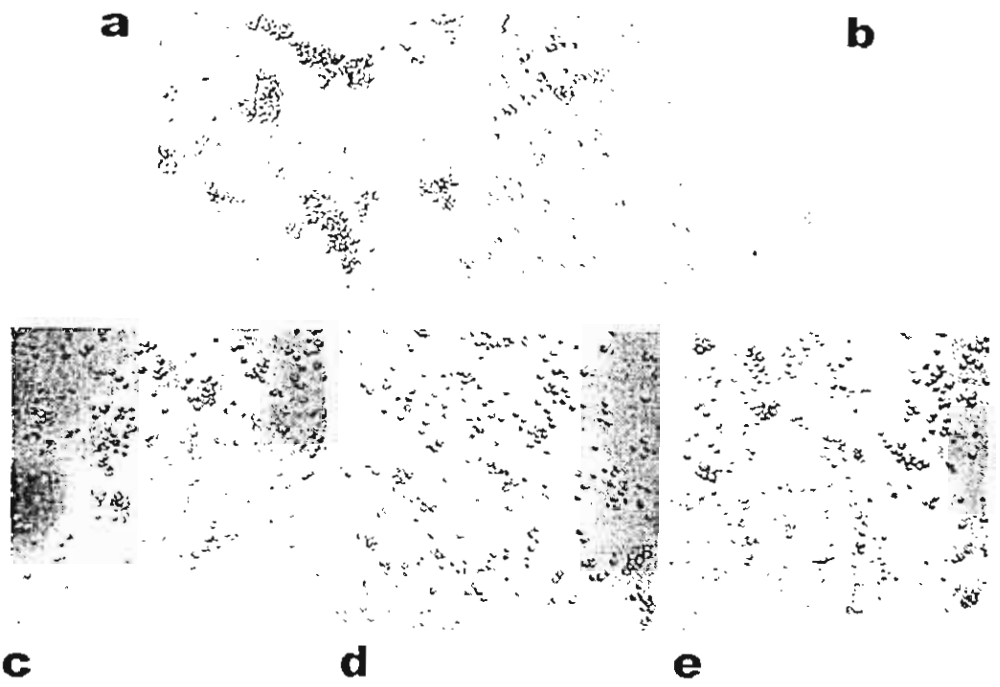


Fig. 5a–e Inhibition of MT99/1-induced Jurkat cell aggregation by CD99-$\phi$. Jurkat cells were incubated with MT99/1 preincubated with VCSM13 (a) or CD99-$\phi$ (b). As controls, Jurkat cells were cultured with VCSM13 (c) or CD99-$\phi$ (d) alone. Non-induced Jurkat cells were referred as auto-aggregation base-line control (e). The degree of aggregation was observed after 4 h of cultivation

CD99MatR. The amplified product of 537 bp obtained (Fig. 1A) was then inserted into pComb3HSS phagemid in the correct reading frame by means of the *Sfi*I cleavage sites on both the 5' and 3' ends. Thus, a new vector, pComb3H-CD99, harboring the CD99 gene was generated. In this vector, CD99-DNA is flanked upstream by the OmpA signal sequence and downstream by gpIII. The correct insertion of CD99 was verified by restriction analysis with *Sfi*I (Fig. 1B). PCR-analysis using primers CD99MatF and CD99MatR produced a single band of 537 bp. A map of pComb3H-CD99 is shown in Fig. 2.

## Generation of phage displaying the CD99 molecule

To produce phage displaying CD99 (CD99-$\phi$), VCSM13 filamentous phage was used to infect pComb3H-CD99-transformed *E. coli* XL-1 Blue. Propagation of VCSM13 results in incorporation of the CD99-gpIII fusion protein during the viral packaging process. The recombinant phage particles thus produced were screened for the expression of recombinant CD99 by sandwich ELISA. As shown in Fig. 3, the generated CD99-$\phi$ specifically bound to both CD99 mAbs (MT99/1 and MT99/3). In contrast, VCSM13 prepared from non-transformed XL-1 Blue was not captured by either CD99 mAb. A negative result was also obtained in wells coated with CD54 mAb MT54, irrespective of the phage type added (Fig. 3). These results suggested that CD99-expressing phage particles had been successfully produced.

## CD99-expressing phages carry a bioactive domain

It has been demonstrated that CD99 mAbs induce homotypic Jurkat cell aggregation (Kasinrerk et. al.

:000). In the presence of MT99/1, Jurkat cells started to how homotypic cell aggregation after 1 h incubation and eached maximum aggregation at 4 h incubation (Fig. 4). The induction of cell aggregation by MT99/1 was then ised to evaluate the CD99 bioactive domain on CD99-$\phi$. As shown in Fig. 5, preincubation of MT99/1 with CD99-$\phi$ inhibited Jurkat aggregation. In contrast, induction of cell aggregation was not altered after preincubation of MT99/1 with VCSM13. When Jurkat cells were cultured n the presence of VCSM13 or CD99-$\phi$ alone, very few auto-aggregation foci resulted after 4 h incubation (Fig. 5). The same degree of auto-aggregation degree also appeared in the non-induction Jurkat culture control (Fig. 5). These results indicated that the generated CD99-$\phi$ carry a properly folded bioactive epitope, which was recognized by MT99/1.

## Discussion

The phage display technique has been described for the production of recombinant molecules such as antibodies (Hoogenboom and Chames 2000), tissue plasminogen activator (Manosroi et al. 2001), or collagen-binding protein from *Necator americanus* (Viaene et al. 2001). The conformational structure of the heterologous molecules can be vastly improved as they are delivered to the periplasmic space of *E. coli*, which has higher oxidizing conditions compared to the cytoplasm. In the present report, we genetically engineered a cell surface molecule, CD99, using phage display. The PCR-amplified CD99 cDNA was inserted into *Sfi*I-cleaved pComb3HSS phagemid. The resulting phagemid (pComb3H-CD99) was then used to generate CD99-expressing phages using helper phage VCSM13. Expression of CD99 was demonstrated by sandwich ELISA; CD99-$\phi$ were recognized by CD99 mAbs MT99/1 and MT99/3. Since an HRP-labeled anti-M13 phage antibody was used as the tracing antibody, the CD99 molecules were manifestly linked to phage particles.

mAbs against CD99 protein produced in our department were previously shown to induce homotypic cell aggregation of Jurkat cells (Kasinrerk et al. 2000). In the present study, the inhibition of Jurkat cell aggregation induced by MT99/1 was used to evaluate the presence of CD99 bioactive domains on CD99-$\phi$. The degree of Jurkat cell aggregation was significantly reduced when MT99/1 was preincubated with CD99-$\phi$. The CD99 molecule was clearly implicated as the inhibitor since preincubation of MT99/1 with VCSM13 did not obstruct cell aggregation. The inhibition of MT99/1-induced Jurkat aggregation by CD99-$\phi$ suggested that the generated CD99-$\phi$ contained a properly folded bioactive domain. This successful preservation of the bioactive domain allows further use of CD99-$\phi$ in the screening of specific ligands on the leukocyte surface.

Taken together, our findings demonstrate the feasibility of using the phage display technique to display the CD99 molecule. This technique has a high potential to generate phage expressing other leukocyte surface molecules, providing the corresponding cDNA is available. In practical terms, the recombinant phages produced will be useful for identification and functional analysis of the receptors of the molecules of interest. In addition, the recombinant phagemid can be easily switched from a phage display version to a secretory version without subcloning to a new vector, as demonstrated in our recent study (Manosroi et al. 2001). As the defined fermentation conditions allowed protein levels of 100 mg/ml to be obtained, an adequate quantity of soluble molecule of interest can be produced (Manosroi et al. 2002). The soluble protein produced can be used for other immunological studies, e.g., epitope characterization and immunomodulation assays.

**Acknowledgements** This work was supported by The Thailand Research Fund and The National Center for Genetic Engineering and Biotechnology (BIOTEC) of the National Science and Technology Development Agency, Thailand. We thank S. Moonsom and W. Silaket for expert technical assistance.

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International Immunology, Vol. 14, No. 12, pp. 1407–1414


# Engagement of Na,K-ATPase β3 subunit by a specific mAb suppresses T and B lymphocyte activation

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*Keywords*: lymphocyte inhibition, mAb, Na,K-ATPase β3 subunit, retroviral cloning system

## Abstract

**In order to identify new molecules involved in regulation of T cell proliferation, we generated various mAb by immunization of mice with the T cell line Molt4. We found one mAb (termed P-3E10) that down-regulated the *in vitro* T cell proliferation induced by CD3-specific OKT3 mAb. The P-3E10 mAb was also able to inhibit IFN-γ, IL-2, IL-4 and IL-10 production of OKT3-activated T cells. The antigen recognized by P-3E10 mAb is broadly expressed on all hematopoietic as well as on all non-hematopoietic cell lines tested so far. Within peripheral blood leukocytes, the P-3E10 antigen was detected on lymphocytes, monocytes and granulocytes. Human umbilical vein endothelial cells (HUVEC) also scored positively. By evaluating the effect of P-3E10 mAb on these cell types we found that it also inhibited anti-IgM-induced B cell proliferation. However, it did not block growth factor-mediated proliferation of HUVEC, and spontaneous proliferation of SupT-1, Jurkat, Molt4 and U937 cell lines. Moreover, it did not influence phagocytosis of human blood monocytes and granulocytes. Biochemical analysis revealed that the P-3E10 antigen is a protein with a mol. wt of 45–50 kDa under non-reducing and 50–55 kDa under reducing conditions. By using a retroviral cloning system, the P-3E10 antigen was cloned. Sequence analysis revealed the P-3E10 antigen to be identical to the β3 subunit of the Na,K-ATPase.**

## Introduction

Highly orchestrated cooperation of stimulatory and suppressive immune pathways is required in order to defeat pathogens without causing harm to self tissues. Under certain circumstances, such as autoimmunity or hypersensitivity, the immune system fails to reach this harmony and becomes rather a threat than a favor. In such cases, therapy is directed to suppress autoreactive responses and thus to restore the natural balance of the immune system. Characterization of the molecular mechanisms underlying negative immune regulation is supposed to provide targets for clinical interventions (1–4). The interaction between T cells and antigen-presenting cells (APC) seems to be a key event leading to activation of the cellular branch of immunity. Examples of molecules and their ligands involved in this interaction include CD4–MHC class II (5), CD8–MHC class I (6) and CD28/CTLA-4–CD80/CD86 (7–9). Therefore, the most suppressive therapeutic agents being developed target those molecules on the surface of both T cells and APC which are essential for regulation of T cell activation (10,11). For instance, immunosuppressive agents specific to the TCR complex molecules CD3, CD4 or CD8 as well as to the co-stimulatory molecules and their ligands CD28–CD80, CD152–CD86 or CD40–CD154 have been designed and used with success (10–12). In addition, some molecules not directly involved in T cell activation also appear to be a reasonable target for immunosuppressive treatment, e.g. P-glycoprotein (13).

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*Transmitting editor*: I. Pecht

*Received* 5 March 2002, *accepted* 30 August 2002

In an attempt to identify new molecules involved in regulation of T cell proliferation, we generated hybridomas from BALB/c mice immunized with the T cell line Molt4 and screened them for the ability to inhibit T cell activation. From among others, we selected one mAb (termed P-3E10) that down-regulated the T cell proliferation induced by immobilized CD3-specific mAb OKT3 as well as production of IFN-γ, IL-2, IL-4 and IL-10 *in vitro*. To identify the molecule recognized by this mAb, we cloned the encoding cDNA using a retroviral expression cloning system (14–16) and found that it is identical to the Na,K-ATPase β3 subunit.

## Methods

### *Cells, reagents and antibodies*

All human hematopoietic and non-hematopoietic cell lines used in this study were maintained in RPMI 1640 medium supplemented with 10% FCS (Gibco, Grand Island, NY), 40 μg/ml gentamicin and 2.5 μg/ml amphotericin B in a humidified atmosphere of 5% $CO_2$ at 37°C. Phoenix packaging cells, an ecotropic retroviral packaging cell line developed by Nolan *et al.* (14), were maintained in DMEM supplemented with 10% FCS.

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by Ficoll-Hypaque density-gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden).

The CD99 mAb MT99/3 (IgG2a isotype), CD147 mAb M6-1E9 (IgG2a isotype) and the mAb KLH (IgG2a isotype) specific for the keyhole limpet hemocyanin molecule were generated by us [(17,18) and unpublished data]. The mAb MEM-188 (CD56; IgG2a isotype), MEM-M6/2 (CD147; IgG2a isotype) as well as the mAb against human α-fetoprotein AFP-01 (IgG1 isotype) were kindly provided by Dr V. Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic). Mouse IgG2a, UPC 10, was purchased from Sigma-Aldrich (St Louis, MO). The mAb 4G2 (IgG2a isotype) specific for E protein of dengue virus was kindly provided by Dr P. Malasit (Medical Biotechnology Unit, Mahidol University, Bangkok, Thailand). The IgG isotype mAb were purified by using a Protein A-coated Sepharose column (Zymed, San Francisco, CA) according to the methods described elsewhere (19).

### *Hybridoma production*

mAb P-3E10 was generated by immunization of a female BALB/c mouse 3 times i.p. at 1-week intervals using $1 \times 10^7$ Molt4 cells. Then, the mouse was boosted i.v. using $1 \times 10^6$ cells. Splenocytes were collected and fused with P3-X63Ag8.653 myeloma cells by standard hybridoma fusion techniques using 50% polyethylene glycol and HAT medium selection. The IgG2a isotype of the mAb was determined using an isotyping ELISA kit (Sigma-Aldrich).

### *Proliferation assay for lymphocytes*

Each culture was set up in a flat-bottom 96-well plate (Nunc, Roskilde, Denmark) in a final volume of 200 μl/well. Triplicate aliquots of $1 \times 10^5$ or $5 \times 10^5$ PBMC were activated using immobilized CD3 mAb OKT3 (20 ng/ml or 1 μg/ml; Ortho Pharmaceuticals, Raritan, NJ) or soluble goat anti-human IgM antibody (10 μg/ml; Hyland Diagnostics, Deerfield, MA) respectively in the presence or absence of various concentrations of tested mAb. The cultures were incubated for 3 days in a 5% $CO_2$ incubator at 37°C and then 1 μCi/well of [$^3$H]thymidine (Amersham Pharmacia Biotech, Freiburg, Germany) was added. The culture was incubated for an additional 18 h before harvesting. Incorporated radioactivity was counted in a liquid scintillation counter (MicroBeta; Wallac, Turku, Finland).

### *Proliferation assay for cell lines*

For the cell line proliferation assay, triplicate aliquots of $1.5 \times 10^4$ cells were cultured with 0.5 μCi/well [$^3$H]thymidine (Amersham Pharmacia) with or without 2.5 μg/ml P-3E10 mAb. The cultures were incubated for 3 and 5 h in a 5% $CO_2$ incubator at 37°C. Then the culture was harvested and the incorporated radioactivity was counted in a liquid scintillation counter (Wallac).

### *Proliferation assay of human umbilical vein endothelial cells (HUVEC)*

HUVEC were isolated by collagenase digestion. Briefly, human umbilical veins were flushed with Ringer's lactate and then incubated with 0.5 mg/ml collagenase type II (Sigma-Aldrich) at 37°C for 30 min. Detached HUVEC were collected, washed, and then cultured in fibronectin-coated flasks (Nunc, Naperville, IL) using M199 medium that contained 20% supplemented calf serum (SCS, Hyclone, Logan, UT), 25 μg/ml EC growth supplement (Technoclone, Vienna, Austria), 5 U/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. Confluent HUVEC were gently trypsinized, seeded onto 24-well plates ($1.5 \times 10^4$ cells/well) and cultured in the presence of P-3E10 mAb or isotype-matched control mAb at a final concentration of 20 or 1 μg/ml in M199 medium supplemented with 20% SCS (Hyclone). As negative control, the cells were cultured in M199 supplemented with 1% SCS. After 3 days of cultivation, cells were fixed by methanol and stained by crystal violet. After intense washings, cells were solubilized in 0.5% Triton X-100 and the number of cells was determined by measuring the absorbance at 595 nm using an ELISA reader and a standard curve.

### *Determination of cytokine production*

PBMC ($1 \times 10^5$) in the presence or absence of various concentrations of tested mAb (in a total volume of 200 μl) were culture in a flat-bottom 96-well plate (Nunc) precoated with mAb OKT3 (1 μg/ml). After incubation at 37°C in a $CO_2$ incubator for 24 or 72 h, the culture supernatants were harvested.

Cytokines were measured by sandwich ELISA using matched pairs of antibodies. Capture as well as detection antibodies to human IL-10 were obtained form R & D Systems (Minneapolis, MN). For the determination of IFN-γ a mAb (clone 25718.111) from R & D Systems was used as capture antibody and a mAb (clone GZ4) from Roche Diagnostics (Mannheim, Germany) as detection antibody. For IL-2 and IL-4, ELISA kits from Euroclone (Wetherby, UK) were used. Standards consisted of human recombinant material were obtained from R & D Systems. Assays were set up in

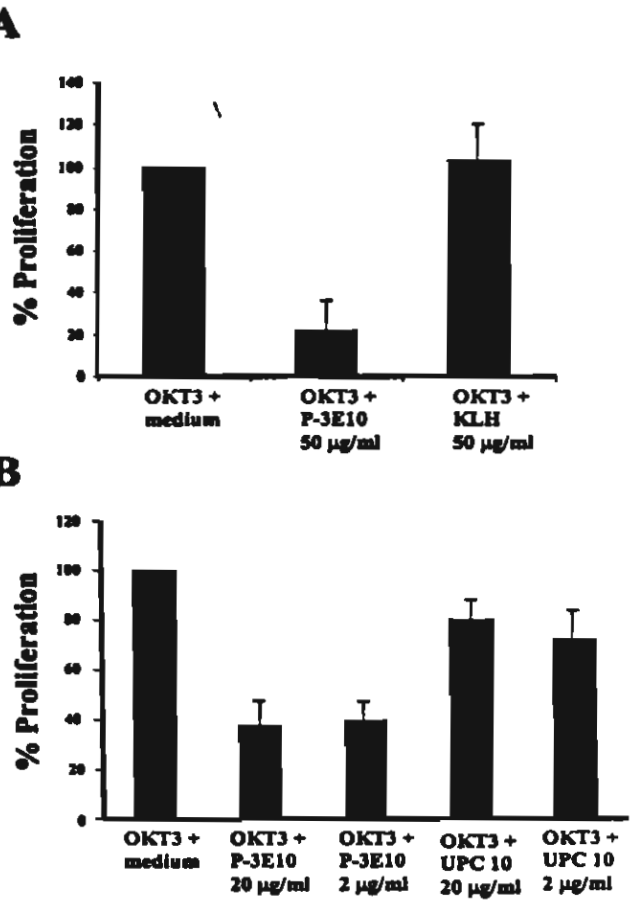


**Fig. 1.** P-3E10 mAb inhibits CD3-induced T cell proliferation. PBMC were activated with immobilized OKT3 mAb at 20 ng/ml (A) or 1 μg/ml (B) in the presence of the indicated concentrations of P-3E10 mAb, isotype-matched control mAb (KLH and UPC 10) or medium. The bars represent mean ± SD of 10 and six healthy donors for (A) and (B) respectively.

duplicates and performed according to the recommendations of the manufacturers.

### *Phagocytosis assay*

*Escherichia coli* was grown in LB broth (Gibco) overnight at 37°C. Cells were washed twice and resuspended in PBS. The optical density of the bacterial suspension was measured at 600 nm and adjusted to 2.5. For phagocytosis assay, 100 μl of EDTA–blood was incubated with 25 μl of the bacterial suspension in the presence or absence of 10 μg/ml of P-3E10 mAb or isotype-matched control mAb at 37°C for 30 min. The sample was then smeared on a grass slide and stained with Wright's stain. Phagocytic cells were counted by light microscopy.

### *Immunofluorescence analysis*

mAb binding to cells was analyzed by indirect immunofluorescence using FITC-conjugated sheep $F(ab')_2$ anti-mouse Ig antibodies (Immunotech/Coulter, Miami, FL). To block non-specific FcR-mediated binding of mAb, cells were pre-incubated for 30 min at 4°C with 10% human AB serum before staining. Membrane fluorescence was analyzed on a FACSCalibur (Becton Dickinson, Sunnyvale, CA) flow cytometer. Individual populations of blood cells were gated according to their forward and side scatter characteristics.

### *Labeling of cells and immunoprecipitation*

For surface labeling, PBS-washed cells were biotinylated with Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) (5 mM) for 1 h at 4°C. The reaction was quenched by washing once with 1 mM glycine in PBS and then twice with PBS. Cells ($1 \times 10^7$) were solubilized in 1 ml lysis buffer (1% NP-40, 50 mM Tris–HCl, pH 8.2, 100 mM NaCl, 2 mM EDTA, 5 mM iodoactamide, 1 mM PMSF and 10 μg/ml aprotinin). Cell lysates were precleared with Protein A–Sepharose beads coated with non-specific mAb. Precleared lysates were then mixed with specific mAb-coated Protein A–Sepharose beads at 4°C for 24 h. After immunoprecipitation and SDS–PAGE, biotinylated proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS for 1 h at room temperature. The blocked membrane was incubated for 1 h at room temperature with avidin–peroxidase (Dako, Glostrup, Denmark) and the biotinylated proteins were visualized by the chemiluminescence detection system (Pierce).

### *Retroviral cloning of the P-3E10 molecule*

The retroviral library construction was performed as described previously (15,16). In brief, a cDNA from human myeloid KG1a cells was cloned into the retroviral expression vector pBabeMN, kindly provided by G. Nolan (Stanford University).

For transfection of the library, Phoenix cells at 50% confluence were harvested by trypsinization, and $3 \times 10^7$ cells were added to a cocktail of 50 ml DMEM, 1% NuSerum (Genome Therapeutics, Waltham, MA), 200 μg/ml DEAE–dextran, 25 μM chloroquine diphosphate and 60 μg of the pBabeMN retroviral library. The cells were kept in suspension for 2 h at 37°C, washed once and cultivated in a 175 $cm^2$ flask (Nunc) in DMEM containing 10% FCS at 37°C. At 24 h post-transfection the medium was renewed. After an additional 48 h of cultivation at 32°C, the virus-containing supernatant was collected, supplemented with 10 μg/ml hexadimethrene bromide (Sigma) and added to $1 \times 10^6$/ml BW5147 mouse thymoma cells in 10 ml RPMI 1640 medium containing 10% FCS.

For the isolation of P-3E10-reactive cells, infected BW5147 cells ($4 \times 10^7$) were washed with PBS containing 1% BSA and incubated with P-3E10 mAb for 30 min on ice. After another washing step the cells were incubated with goat anti-mouse IgG microbeads (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions. After washing, cells were resuspended in 500 μl of MACS sorting buffer (0.5% BSA/2mM EDTA in PBS) and loaded onto MS$^+$ separation columns (Miltenyi Biotec) for positive selection of P-3E10 transduced cells. The isolated fraction was cultured in RPMI 1640 medium supplemented with 10% FCS. After three rounds of sorting, >95% of the isolated cells stained positively with P-3E10 mAb. Then, single-cell clones were obtained by limiting dilution.

For recovery of the P-3E10 cDNA, total RNA was extracted from the single-cell clone using Tri-Reagent (Sigma). RT-PCR was performed with Stratascript (Stratagene, La Jolla, CA) and

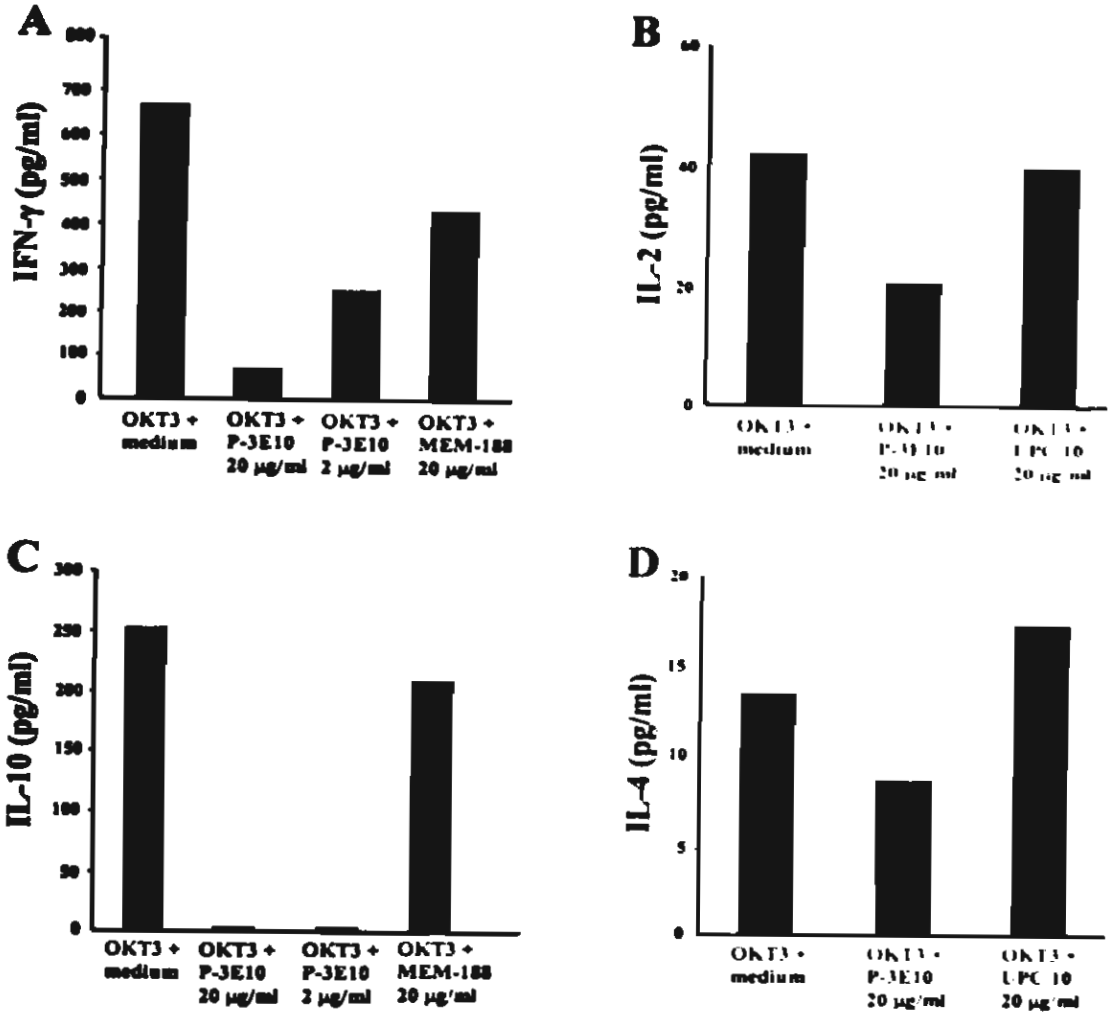


**Fig. 2.** P-3E10 mAb inhibits cytokine production. PBMC were activated with immobilized CD3 mAb OKT3 in the presence of indicated concentrations of soluble P-3E10 mAb, isotype-matched control mAb or medium alone. The culture supernatants were harvested and IFN-γ, IL-2, IL-10 and IL-4 were measured by ELISA.

the Advantage-GC polymerase system (Clontech, Palo Alto, CA) using primers flanking the multiple cloning site of the retroviral vector pBabeMN. The PCR was run for 30 cycles (30 s at 94°C, 30 s at 58°C and 4 min at 68°C). The purified PCR product was subcloned back into pBabeMN and transformed into *E. coli* DH5α. The plasmid DNA was isolated using a Qiagen Miniprep column according to the manufacturer's recommendation (Qiagen, Hilden, Germany). To confirm that the isolated plasmid encodes the P-3E10 antigen, we used it to infect BW5147 cells as described above, and analyzed the transductants for P-3E10 expression by indirect immunofluorescence and flow cytometry. The plasmid was sequenced at the VBC-Genomics sequencing facility (Bioscience Research, Vienna, Austria).

## Results

### *mAb P-3E10 inhibits the OKT3-induced T cell proliferation*

In an attempt to identify new molecules involved in the regulation of T cell proliferation, various mAb against leukocyte surface molecules were generated using the T cell line Molt4 as an immunizing agent. The mAb were examined for their ability to modulate T cell proliferation in mononuclear cell preparations isolated from peripheral blood. We found that one of the mAb, named P-3E10, inhibited the OKT3-induced T cell proliferation *in vitro*. As shown in Fig. 1, T cell proliferation was significantly inhibited by mAb P-3E10 ($n = 16$). In contrast, isotype-matched control mAb KLH and UPC 10, had no effect on the response of T cells to immobilized OKT3.

### *Inhibition of cytokine production of T cells by P-3E10 mAb*

PBMC were activated with immobilized OKT3 mAb in the presence or absence of soluble P-3E10 mAb, and IFN-γ, IL-2, IL-10 and IL-4 were measured in the culture supernatants by ELISA. In the presence of P-3E10 mAb, production of all cytokines tested was inhibited (Fig. 2). The isotype-matched control mAb, however, had no such effect.

### *Cellular distribution of the P-3E10 antigen*

To characterize the molecule recognized by P-3E10 mAb, various cell types were stained. All peripheral blood

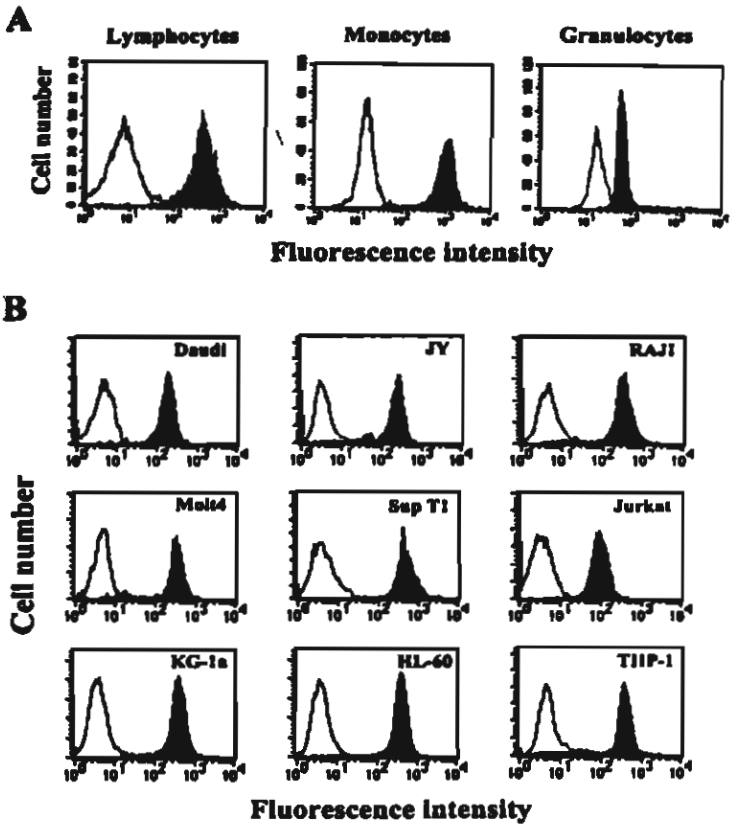


**Fig. 3.** Distribution of the antigen recognized by the mAb P-3E10 on peripheral blood leukocytes (A) and various hematopoietic cell lines (B). The indicated cells were stained with P-3E10 mAb (open) or AFP-01 control mAb (solid) by indirect immunofluorescence. Data are representative of 10 independent donors (A) and three independent experiments (B).

leukocytes ($n$ = 10) including lymphocytes, monocytes and granulocytes were positive with mAb P-3E10 (Fig. 3A). Then we examined expression on hematopoietic cell lines. As shown in Fig. 3(B), all cell lines tested, including B cell lines (Daudi, JY and RAJI), T cell lines (Molt4, Sup T1 and Jurkat) and myeloid cell lines (KG1a, HL-60 and THP-1), were strongly positive with P-3E10 mAb. We also analyzed several non-hematopoietic cells and cell lines, including HUVEC, 293 human embryonic renal epithelial, MCF-7 breast cancer, OVMZ ovarian cancer and TCL kidney cancer cell lines, and found that all were clearly stained by P-3E10 mAb (data not shown). Thus, our results indicate that the P-3E10 antigen is a broadly expressed plasma membrane molecule.

### *Effect of mAb P-3E10 on non-T cells*

Because of the broad expression of the P-3E10 molecule, we tested the effect of the P-3E10 mAb on cells other than T cells. When we treated anti-IgM-induced B cells with 10 μg/ml of mAb P-3E10, similar to the results obtained with T cells, proliferation was inhibited by 65 ± 14% (mean ± SD; $n$ = 3) (Fig. 4). However, in contrast to lymphocytes, the mAb did not block proliferation of HUVEC. Furthermore, it had also no effect on the growth of the hematopoietic cell lines Sup T-1, Jurkat, Molt4 and U937. Moreover, we analyzed the influence

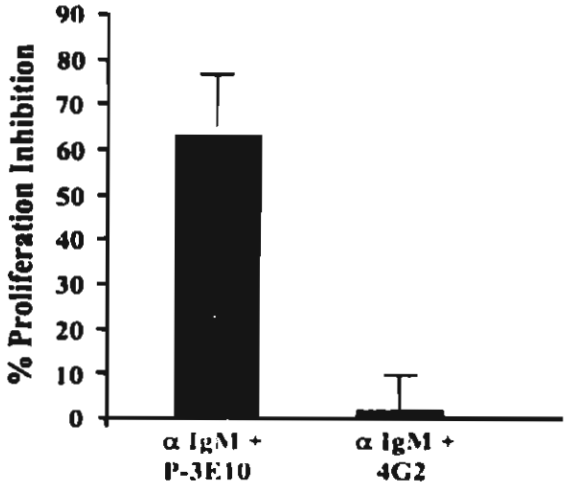


**Fig. 4.** P-3E10 mAb inhibits anti-IgM-induced B cell proliferation. PBMC were activated with 10 μg/ml of soluble anti-IgM antibody in the presence of 10 μg/ml P-3E10 mAb, isotype-matched control mAb (4G2) or medium alone. The bars represent mean ± SD of percent proliferation inhibition of three healthy donors.

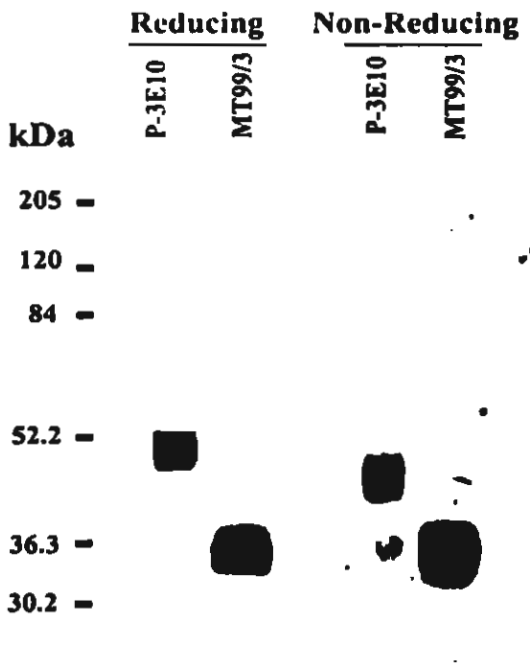


**Fig. 5.** Biochemical characterization of the cell surface molecule recognized by P-3E10 mAb. SDS-PAGE analysis of immunoprecipitates obtained with either P-3E10 mAb or CD99 mAb MT99/3 from lysates of surface biotin-labeled Sup T1 cells. Electrophoresis was performed under reducing and non-reducing conditions. Molecular markers are shown on the left in kDa.

of P-3E10 on phagocytosis of myeloid cells, monocytes and granulocytes, but no effect was observed. The percentages of monocytes that had phagocytosed *E. coli* in the presence or

```
   1                                                                  CCCGTAAC
   9 GAGGAGGTGTTCTCGGCCGTCCCACCCTTCACTGCCGTCTCCGGGCTGCGCCGCCGGAGCCGGGACG
  76 CGCCTCCGCAGCCCTCGCCGCCTCCATCCCCGCGGCCGCAGCTCCTCTCGCCGTCCGCGCGCACACC
     Met Thr Lys Asn Glu Lys Lys Ser Leu Asn Gln Ser Leu Ala Glu Trp Lys     17
 143 ATG ACG AAG AAC GAG AAG AAG TCC CTC AAC CAG AGC CTG GCC GAG TGG AAG
     Leu Phe Ile Tyr Asn Pro Thr Thr Gly Glu Phe Leu Gly Arg Thr Ala Lys     34
 194 CTC TTC ATC TAC AAC CCG ACC ACC GGA GAA TTC CTG GGG CGC ACC GCC AAG
     Ser Trp Gly Leu Ile Leu Leu Phe Tyr Leu Val Phe Tyr Gly Phe Leu Ala     51
 245 AGC TGG GGT TTG ATC TTG CTC TTC TAC CTA GTT TTT TAT GGG TTC CTG GCT
     Ala Leu Phe Ser Phe Thr Met Trp Val Met Leu Gln Thr Leu Asn Asp Glu     68
 296 GCA CTC TTC TCA TTC ACG ATG TGG GTT ATG CTT CAG ACT CTC AAC GAT GAG
     Val Pro Lys Tyr Arg Asp Gln Ile Pro Ser Pro Gly Leu Met Val Phe Pro     85
 347 GTT CCA AAA TAC CGT GAC CAG ATT CCT AGC CCA GGA CTC ATG GTT TTT CCA
     Lys Pro Val Thr Ala Leu Glu Tyr Thr Phe Ser Arg Ser Asp Pro Thr Ser    102
 398 AAA CCA GTG ACC GCA TTG GAA TAT ACA TTC AGT AGG TCT GAT CCA ACT TCG
     Tyr Ala Gly Tyr Ile Glu Asp Leu Lys Lys Phe Leu Lys Pro Tyr Thr Leu    119
 449 TAT GCA GGG TAC ATT GAA GAC CTT AAG AAG TTT CTA AAA CCA TAT ACT TTA
     Glu Glu Gln Lys Asn Leu Thr Val Cys Pro Asp Gly Ala Leu Phe Glu Gln    136
 500 GAA GAA CAG AAG AAC CTC ACA GTC TGT CCT GAT GGA GCA CTT TTT GAA CAG
     Lys Gly Pro Val Tyr Val Ala Cys Gln Phe Pro Ile Ser Leu Leu Gln Ala    153
 551 AAG GGT CCA GTT TAT GTT GCA TGT CAG TTT CCT ATT TCA TTA CTT CAA GCA
     Cys Ser Gly Met Asn Asp Pro Asp Phe Gly Tyr Ser Gln Gly Asn Pro Cys    170
 602 TGC AGT GGT ATG AAT GAT CCT GAT TTT GGC TAT TCT CAA GGA AAC CCT TGT
     Ile Leu Val Lys Met Asn Arg Ile Ile Gly Leu Lys Pro Glu Gly Val Pro    187
 653 ATT CTT GTG AAA ATG AAC AGA ATA ATT GGA TTA AAG CCT GAA GGA GTG CCA
     Arg Ile Asp Cys Val Ser Lys Asn Glu Asp Ile Pro Asn Val Ala Val Tyr    204
 704 AGG ATA GAT TGT GTT TCA AAG AAT GAA GAT ATA CCA AAT GTA GCA GTT TAT
     Pro His Asn Gly Met Ile Asp Leu Lys Tyr Phe Pro Tyr Tyr Gly Lys Lys    221
 755 CCT CAT AAT GGA ATG ATA GAC TTA AAA TAT TTC CCA TAT TAT GGG AAA AAA
     Leu His Val Gly Tyr Leu Gln Pro Leu Val Ala Val Gln Val Ser Phe Ala    238
 806 CTG CAT GTT GGG TAT CTA CAG CCA TTG GTT GCT GTT CAG GTC AGC TTT GCT
     Pro Asn Asn Thr Gly Lys Glu Val Thr Val Glu Cys Lys Ile Asp Gly Ser    255
 857 CCT AAC AAC ACT GGG AAA GAA GTA ACA GTT GAG TGC AAG ATT GAT GGA TCA
     Ala Asn Leu Lys Ser Gln Asp Asp Arg Asp Lys Phe Leu Gly Arg Val Met    272
 908 GCC AAC CTA AAA AGT CAG GAT GAT CGT GAC AAG TTT TTG GGA CGA GTT ATG
     Phe Lys Ile Thr Ala Arg Ala Stop                                      279
 959 TTC AAA ATC ACA GCA CGT GCA TAG TATGAGTAGGATATCTCCACAGAGTAAATGTTGTG
1018 TTGTCTGTCTTCATTTTGTAACAGCTGGACCTTCCATTCTAGAATTATGAGACCACCTTGGAGAAAG
1085 GTGTGTGGTACATGACATTGGGTTACATCATAACGTGCTTCCAGATCATAGTGTTCAGTGTCCTCTG
1152 AAGTAACTGCCTGTTGCCTCTGCTGCCCTTTGAACCAGTGTACAGTCGCCAGATAGGGACCGGTGAA
1219 CACCTGATTCCAAACATGTAGGATGGGGGTCTTGTCCTCTTTTTATGTGGTTTAATTGCCAAGTGTC
1286 TAAAGCTTAATATGCCGTGCTATGTAAATATTTTATGGATATAACAACTGTCATATTTTGATGTCAA
1353 CAGAGTTTTAGGGATAAAATGGTACCCGGCCAACATCAAGTGACTTTATAGCTGCAAGAAATGTGGT
1420 ATGTGGAGAAGTTCTGTATGTGAGGAAGGAAA
```

**Fig. 6.** Nucleotide and deduced amino acid sequence of the cDNA encoding the P-3E10 antigen. Nucleotide positions are indicated on the left and amino acid positions on the right. The putative transmembrane-spanning domain is underlined. The six cysteines are boxed.

absence of P-3E10 mAb were 72 ± 4.6 and 70 ± 6.2; those of granulocytes 56 ± 5.3 and 53 ± 3.8 (mean ± SD; $n$ = 3), respectively.

### *Biochemical characterization of the surface molecule recognized by mAb P-3E10*

To biochemically characterize the molecule bearing the P-3E10 antigen, we performed an immunoprecipitation experiment. As shown in Fig. 5, we were able to precipitate a 50- to 55-kDa protein under reducing conditions from lysates of surface-biotinylated Sup T1 cells using P-3E10 mAb. The protein shifted to 45–50 kDa under non-reducing conditions (Fig. 5) indicating that it contains intramolecular disulfide bonds. In comparison the isotype-matched control mAb MT99/3 (to the CD99 antigen) precipitated, as described previously (17), a protein band of 32 kDa both under reducing and non-reducing conditions.

### *Molecular cloning of the cDNA coding for the antigen recognized by P-3E10 mAb*

A KG1a cDNA library in the retroviral vector pBabeMN and the packaging cell line Phoenix were used to produce ecotropic viruses for transducing the target cell line BW5147. BW5147 transductants expressing the P-3E10 antigen were sorted by P-3E10 mAb using MACS and cloned to single-cell cultures by limiting dilution. One strongly positive cell clone was selected to isolate the P-3E10 cDNA. For this, RT-PCR was performed with the RNA extracted from the clone using primers flanking the multiple cloning site of the retroviral vector pBabeMN. The PCR product was digested with *Eco*RI to remove the plasmid sequences flanking the cDNA. Sequencing of the cDNA revealed a length of 1451 bp with an open reading frame of 840 bp coding for 279 amino acids (Fig. 6). Comparison of the sequence using the BLAST program at the NCBI (Bethesda, MD) resulted in 100% homology to the Na,K-ATPase β3 subunit (20). This molecule is a type II transmembrane protein

with a single transmembrane segment. The predicted extracellular domain contains six cysteine residues, which is in accord with our biochemical data in Fig. 5.

To confirm that the isolated Na,K-ATPase β3 subunit cDNA encodes the P-3E10 antigen, the cDNA was re-ligated via *Eco*RI into the pBabeMN vector and transduced into BW5147 cells, which were subsequently tested for binding of the P-3E10 mAb. The transduced BW cells were specifically stained by P-3E10 mAb, demonstrating that the P-3E10 antigen is indeed a determinant on the Na,K-ATPase β3 subunit.

## Discussion

In our study, we prepared a set of mAb reactive with the Molt4 T cell line. The generated mAb were screened for the ability to modulate CD3-induced T cell proliferation. P-3E10 mAb was found to be of interest, as it significantly inhibited T cell proliferation as well as IFN-γ, IL-2, IL-4 and IL-10 production *in vitro*. P-3E10 mAb also inhibited anti-IgM-induced B cell proliferation. However, it had no effect on HUVEC and hematopoietic cell line proliferation, as well as phagocytosis of monocytes and granulocytes. By using a retroviral cloning system we proved that the P-3E10 antigen is a determinant on the human Na,K-ATPase β3 subunit (20).

The Na,K-ATPase is a membrane-associated enzyme responsible for the active transport of $Na^+$ and $K^+$ in most animal cells (21–23). By using the energy from the hydrolysis of one molecule of ATP, it transports three $Na^+$ out in exchange for two $K^+$ that are taken in. By coupling the hydrolysis of ATP to the movement of $Na^+$ and $K^+$ ions across the plasma membrane, the enzyme produces the electrochemical gradient that is the primary energy source for the active transport of nutrients, the action potential of excitable tissues and the regulation of cell volume (21–23). In all tissues, Na,K-ATPase is characterized by a complex molecular heterogeneity that results from the expression and differential association of multiple isoforms of both its α and β subunits. At present, as many as four different α polypeptides (α1, α2, α3 and α4) and three distinct β isoforms (β1, β2 and β3) have been identified in mammalian cells (23). The α subunits are multispanning membrane proteins with a molecular mass of ~100 kDa that are responsible for the catalytic and transport properties of the enzyme. The β polypeptides cross the membrane once, depending on the degree of glycosylation in different tissue, and their molecular mass ranges from 40 to 60 kDa. The β subunit is essential for the normal activity of the enzyme, and it appears to be involved in the occlusion of $K^+$, and the modulation of $K^+$ and $Na^+$ affinity of the enzyme (22,23). In addition, in vertebrate cells, the β subunit may act as a chaperone, stabilizing the correct folding of the α subunit to facilitate its transport to the plasma membrane (22,23).

In immune cells, induction of Na,K-ATPase-mediated $K^+$ fluxes in mitogen-activated lymphocytes has been reported (24–28). Increase in mRNA encoding the α and β subunits of Na,K-ATPase in phytohemagglutinin-activated lymphocytes was also demonstrated (26,29). Several mechanisms have been proposed to explain the increase in the Na,K-ATPase activity in lymphocyte activation (26,28,30). Prasad *et al.* have argued that the increase in intracellular calcium may lead to a subsequent protein kinase-induced enhancement of Na,K-ATPase enzymatic activity (28). Na,K pump activation, accompanying human lymphocyte blast transformation, also plays a critical role in the expression of IL-2 receptor and initiates IL-2 expression (24,31,32). Inactivation of Na,K-ATPase by specific inhibitors causes inhibition of both mitogen- and antigen-induced lymphocyte activation (31–33).

In the present study we demonstrate that engagement of the Na,K-ATPase β3 subunit by a mAb down-regulates both T and B lymphocyte proliferation as well as production of IFN-γ, IL-2, IL-4 and IL10 of T cells. This is, to the best of our knowledge, the first study to demonstrate that a specific mAb to the Na,K-ATPase β chain is able to block lymphocyte activation. Although, the precise mechanism of this inhibition is unknown, engagement of Na,K-ATPase by P-3E10 mAb may block Na,K-ATPase activation and subsequently suppress downstream events, which in turn lead to the inhibition of lymphocyte activation. Further investigation of the mechanisms of the Na,K-ATPase β3 chain in inhibition of lymphocyte activation may lead to a better understanding of immune regulation, which may provide new avenues for clinical intervention.

## Acknowledgements

We would like to thank Dr Garry Nolan for providing the retroviral vector pBabeMN-lacZ and the Phoenix packaging cell line, and Dr Václav Horejsí for providing mAb. This work was supported by the Thailand Research Fund, the Royal Golden Jubilee PhD program of Thailand, the National Center for Genetic Engineering and Biotechnology (BIOTEC) of the National Sciences and Technology Development Agency, and the Competence Center for Biomolecular Therapeutics in Austria.

## Abbreviations

| | |
|---|---|
| APC | antigen-presenting cell |
| PBMC | peripheral blood mononuclear cell |
| HUVEC | human umbilical vein endothelial cell |
| SCS | supplemented calf serum |

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Journal of Immunological Methods 281 (2003) 177–185

JIM Journal of Immunological Methods

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Recombinant Technology

# Displaying and epitope mapping of CD147 on VCSM13 phages: influence of *Escherichia coli* strains

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Received 7 January 2003; received in revised form 16 July 2003; accepted 16 July 2003

**Abstract**

The external domain of a human leukocyte surface molecule, CD147 was displayed on the surface of phage. Two *Escherichia coli* laboratory strains, XL-1 Blue and TG-1, were chosen to separately propagate the recombinant phages. By sandwich enzyme linked immunosorbent assay (ELISA), CD147 on phage particles were individually captured by six CD147 mAbs and subsequently detected by anti-M13 conjugated HRP. All mAbs specifically bound the CD147 on phage particles derived from TG-1. On the contrary, only four of them could recognize the CD147 on phages produced by XL-1 Blue. The results indicate that the environment in the TG-1 periplasm is more appropriate than that of XL-1 Blue for promoting the suitable folding of CD147. This finding emphasizes the importance of selecting the appropriate *E. coli* host for display of a complex protein. The epitopes of CD147 displayed on the phage were further mapped by competitive inhibition ELISA, which is a reliable and economical method. Certain clusters of mAb recognition areas were identified and will provide valuable information for the discovery of the ligand for CD147.


*Keywords:* CD147; Monoclonal antibodies; Phage display; Epitope mapping; Periplasm

## 1. Introduction

Expression of heterologous proteins in *Escherichia coli* has long been an essential tool in the study of the structure and function of proteins, in part because of the ease and low cost of manipulation and production. However, not every heterologous protein can be successfully produced in this prokaryotic host, since most recombinant heterologous proteins tend to aggregate, hampering their activity and antigenicity. The periplasm can be a more suitable environment for expression of soluble complex proteins, due to its resemblance to the endoplasmic reticulum of eukaryotic cells (Glockshuber et al., 1992). Compared with the cytoplasm, the periplasm has an oxidizing environment, which promotes better disulfide bond for-

*Abbreviations:* T.U., transforming unit; ELISA, enzyme linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RBS, ribosome binding site; *Omp*A, signal sequence of the outer membranes protein A of *E. coli*.

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doi:10.1016/S0022-1759(03)00270-9

mation. The phage display technique, which relies on periplasmic expression of the displayed proteins, has been used successfully for producing various recombinant proteins. The binding activity of ScFv (Andris-Widhopf et al., 2000), antigenicity of tissue plasminogen activator (Manosroi et al., 2001) and bioactive domain of CD99 (Tayapiwatana and Kasinrerk, 2002) are retained.

CD147 is a broadly expressed leukocyte surface molecule. Since the first identification of CD147 (or M6) characteristics (Miyauchi et al., 1991; Kasinrerk et al., 1992), the only CD147-ligand reported was secreted cyclophilin A and B (Yurchenko et al., 2001, 2002). No cell surface ligand-partner of CD147 has been clearly shown. Recently, we reported the effects of CD147 mAbs in inducing homotypic cell aggregation of U937 cells. Interestingly, not all of the mAbs tested bound to the bioactive domains of CD147. We subsequently discovered that the mechanism was depended on the LFA-1/ICAM-1 pathway (Kasinrerk et al., 1999) and the signaling was accomplished through protein kinases (Khunkeawla et al., 2001). It is thus of interesting to determine the ligand for CD147.

Epitope mapping with mAb can provide useful information about the bioactive domains of molecule. Several techniques may be used for epitope characterization, and two have been reported for CD147: the epitope map of a soluble CD147-Fc fusion protein produced from transfected COS cells has been evaluated by BIAcore biosensor, which in principle is accurate but extremely expensive (Koch et al., 1999). More recently, the epitope mapping of CD147 mAbs was analyzed in our laboratory by a fluorescence inhibition technique (unpublished observations). The method is reliable but the eukaryotic expression system is time consuming and requires sophisticated processing. In addition, the competitive mAbs must be labeled with fluorescein dye, which is labour-intensive.

In the present study, we generated phage-displayed CD147 (CD147-ϕ) and mapped its epitopes with defined CD147 mAbs (Kasinrerk et al., 1999; Khunkeawla et al., 2001) by competitive inhibition ELISA. Practically, a number of *E. coli* $F^+$ strains, e.g. TG-1 (Schlebusch et al., 1997), XL-1 Blue (Lekkerkerker and Logtenberg, 1999), SS320 (Sidhu et al., 2000) and JM109 (Rondot et al., 2001) have been used by various groups for displaying recombinant molecules. The choice of an *E. coli* host strain has been reported as one of the important parameters for producing high level expression of functional heterologous proteins (Friehs and Reardon, 1993; Dueñas et al., 1994; Balbás, 2001). In this study, the efficiency of two *E. coli* laboratory strains, XL-1 Blue and TG-1, in synthesizing the properly folded CD147 on phage particles was evaluated.

## 2. Materials and methods

### 2.1. E. coli strains and primers

Two *E. coli* strains, TG-1 {*supE hsd*Δ5 *thi*Δ(*lac-proAB*) F′ [*traD36proAB*+, *lacI*$^q$ *lacZ*ΔM15]} (kindly provided by Dr. A.D. Griffiths, MRC, Cambridge, UK) and XL-1 Blue {*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*F′ [*proAB*$^+$, *lacI*$^q$ *lacZ*ΔM15 Tn10 (tet$^r$)]} (Stratagene, La Jolla, CA), were used as hosts for the production of phages displaying the CD147 molecule.

Each primer was synthesized with 5′-overhangs containing a *Sfi*I restriction site (small letters): CD147ExF [5′-GAG GAG GAG GTg gcc cag gcg gcc GCT GCC GGC ACA GTC TTC-3′] and CD147ExR [5′-GAG GAG GAG CTg gcc ggc ctg gcc GTG GCT GCG CAC GCG GAG-3′]. They were suitable for annealing the ectodomain of the human CD147 gene from the mammalian expression vector, pCDM8-CD147 (Kasinrerk et al., 1992, 2002), and gave the correct orientation of CD147 gene which was inserted into pComb3HSS phagemid vector, kindly provided by Dr. Carlos F. Barbas, (Scripps Institute, CA).

### 2.2. CD147 gene amplification by PCR

The external domain of the CD147 gene was amplified using pCDM8-CD147 as a template. Briefly, 50 ng of template was annealed with 1 μg of each primer in 100 μl of a PCR mixture containing 2.5 U of *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). The amplification condition included a jump start at 85 °C for 4 min and followed by the three cycles of PCR amplification: denaturation at 95 °C for 50 s, annealing at 42 °C for 50 s

and extension at 72 °C for 1.5 min. After 35 amplification cycles, the mixture was incubated at 72 °C for 10 min. Gel electrophoresis was performed to analyze the molecular weight of the PCR product. The amplified product was purified by QIAquick PCR purification Kit (QIAGEN, Hilden, Germany) and cleaved with *Hae*II.

### 2.3. Construction of phagemid expressing CD147

The phagemid expressing CD147 was constructed by inserting the *Sfi*I-digested ectodomain of CD147 gene into the *Sfi*I-digested pComb3HSS phagemid vector. Fifty nanograms of CD147 amplified product was treated with 1 U of *Sfi*I (Roche Molecular Biochemicals), while 100 ng of pComb3HSS was treated with 5 U of the same enzyme and incubated at 50 °C for 18 h. After purification, the ligation step was performed by adding 1 U of $T_4$ ligase enzyme (Roche Molecular Biochemicals) into a mixture containing 100 ng of vector and 50 ng of insert. The reaction mixture was subsequently incubated at 4 °C for 16 h. The ligated product was named pComb3H-CD147.

### 2.4. Bacterial cell transformation

The ligated product, pComb3H-CD147, was transformed into $CaCl_2$ competent *E. coli* XL-1 Blue or TG-1. After culture for 3 h in antibiotic-free LB, the transformed cell pellet was harvested by spinning down at 1100 g, 25 °C for 10 min. The pellet was resuspended in 500 μl of the same medium and plated on LB agar containing ampicillin (100 μg/ml) and cultured overnight at 37 °C. The ampicillin resistant colonies were selected for plasmid miniprep (QIAGEN). Restriction fragment analysis of the purified plasmid was performed using *Sfi*I. Finally, the PCR amplified product was checked for an insert in the purified plasmid as described above.

### 2.5. Preparation of phage-displayed CD147

For displaying CD147 on filamentous phages, 10 ml of XL-1 Blue bacteria transformed with pComb3H-CD147 was precultured at 37 °C in super broth (3%[wt/vol] tryptone, 2%[wt/vol] yeast extract, and 1%[wt/vol] morpholinepropanesulphonic acid [MOPS]) containing ampicillin (100 μg/ml) and tetracycline (10 μg/ml). When an $OD_{600}$ of 1.5 was reached, the bacteria were transferred to 100 ml of the same medium. Two hours later, the $10^{12}$ transforming unit (t.u.) of VCSM13 helper phage (Stratagene) was added and cultured for another 3 h. Subsequently, kanamycin (70 μg/ml) was added to the culture, which was continuously shaken at 180 rpm for 18 h at 37 °C. The bacteriophages were harvested by precipitation with PEG 8000 as described previously (Tayapiwatana and Kasinrerk, 2002). Finally, the phages were reconstituted with 0.15 M PBS pH 7.2 and stored at −70 °C. This protocol was regarded as standard growth condition of XL-1 Blue host.

The pComb3H-CD147 plasmid was transformed into TG-1 strain precultured in 2 × TY broth (1.6% [wt/vol] tryptone, 1%[wt/vol] yeast extract, and 0.5%[wt/vol] sodium chloride) containing ampicillin (100 μg/ml) until an $OD_{600}$ of 0.8 was reached. The precultured bacteria were subsequently propagated in 100 ml of the same medium containing 2 ml of 50% glucose. After 2 h, the 30 ml of culture was infected with 2.4 ml of $10^{12}$ t.u. of the VCSM13 helper phage and kept at 37 °C without shaking for 30 min. Phage-infected TG-1 was spun down at 1100 g, 4 °C for 10 min. The pellet was reconstituted with 30 ml of 2 × TY broth containing ampicillin (100 μg/ml) and kanamycin (70 μg/ml). Fifteen milliliters of culture was resuspended in 250 ml of the same medium and shaken at 180 rpm for 18 h at 37 °C. The procedures for harvesting and storing the recombinant phages were performed as above. This protocol was regarded as standard growth condition of TG-1 host.

To evaluate the influence of growth conditions, displaying of CD147 on phage particle in TG-1 host was performed under the standard growth condition used for XL-1 Blue host but tetracycline was omitted. CD147-ϕ was conversely assembled in XL-1 Blue host with TG-1 standard growth condition.

### 2.6. Immunoassay for phage-displayed CD147 by ELISA

Microtiter plates (NUNC, Roskilde, Denmark) was coated with 50 μl of 10 μg/ml CD147 mAbs (M6-1B9; $IgG_3$, M6-2B1; IgM, M6-1D4; IgM, M6-1E9; $IgG_{2a}$, M6-1F3; IgM, and M6-2F9; IgM) (Kasinrerk et al., 1999; Khunkeawla et al., 2001) in carbonate/

bicarbonate buffer pH 9.6 for 2 h at room temperature. The plate was then blocked with 2% skimmed milk in 0.15 M PBS pH 7.2 for 1 h at room temperature. The wells were washed four times with 0.05% Tween-20 in 0.15 M PBS pH 7.2 and $10^7$ t.u. of recombinant phages were added and the mixture incubated for 1 h at room temperature. The unbound phages were washed out and detection of bound phage was performed using peroxidase-labeled sheep anti-M13 antibodies (Amersham Biosciences, Buckinghamshire, UK). Subsequently, peroxidase activity was determined by treatment with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and measured the optical density (OD) measured at 450 nm after adding 1 M $H_2SO_4$ to stop the reaction. MT54 mAb specific for CD54 (Moonsom et al., 2001) was used as an antibody control in the ELISA system.

### 2.7. SDS-PAGE and Western immunoblotting

Phage-expressing CD147 protein were diluted in 5 × non-reducing buffer (3.7%[wt/vol] Tris–HCl, pH 6.8, 5%[wt/vol] sodium dodecyl sulfate, 50%[vol/vol] glycerol) and heat-denatured for 5 min before loading to a 12% separating gel for SDS-PAGE. The separated proteins were blotted to a nitrocellulose membrane. Blocking was performed for 2 h at room temperature with 5% skimmed milk in 0.15 M PBS pH 7.2 and further incubated with six CD147 mAbs (M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9) for 1 h. The membrane was washed three times with 0.05% Tween 20 in 0.15 M PBS pH 7.2 and then incubated with peroxidase-labeled sheep anti-mouse immunoglobulins (DAKO Diagnostica, Hamburg, Germany) diluted in 5% skimmed milk in 0.15 M PBS pH 7.2 for 1 h. Unbound conjugate was washed out three times with 0.05% Tween 20 in 0.15 M PBS pH 7.2 and once with 0.15 M PBS pH 7.2; the specific bands were visualized using a chemiluminescent substrate detection system (Pierce, Rockford, IL).

### 2.8. Epitope mapping

Epitope mapping was carried out by competitive inhibition ELISA. Fifty microliters of 10 μg/ml CD147 mAbs (M6-1B9, M6-1E9, M6-1F3 and M6-2F9) in carbonate/bicarbonate buffer pH 9.6 was individually absorbed on a solid phase of 96-well plates for 2 h at room temperature. These coated mAbs are referred as catcher. Any nonspecific binding sites were blocked with 2% skimmed milk in 0.15 M PBS pH 7.2. During the blocking period, $10^7$ t.u. of CD147-phage (CD147-ϕ) were separately pre-incubated with the same panel of 500 ng of CD147 mAbs which were termed competitors. After the washing step, the pre-incubated CD147-ϕ/CD147 mAbs were added into the CD147 mAbs-coated wells and incubated for 1 h at room temperature. The bound phages were detected by incubating for 1 h at room temperature with peroxidase-labeled sheep anti-M13 antibodies (Amersham Biosciences). After washing, the TMB substrate was added and the reaction was stopped with 1 M $H_2SO_4$. The reaction of competitive inhibition ELISA was detected at wavelength 450 nm and compared with the OD of non-competitor wells. The cut off value of the inhibition was taken 35% reduction of absorbance units in competitive wells in comparison with the non-competitor wells.

## 3. Results

### 3.1. Construction of CD147 phagemid

The ectodomain gene of CD147 in vector pCDM8-CD147 was amplified by PCR using primers CD147ExFw and CD147ExRev. The PCR product containing the double *Sfi*I restriction sites with molecular weight of 552 bp was demonstrated by agarose gel electrophoresis (data not shown). The amplified CD147 gene was sub-cloned into the phagemid-expressing vector, pComb3HSS, in the correct reading frame. The engineered phagemid bearing CD147 ectodomain gene, flanked upstream by *Omp*A signal sequence and downstream by gpIII (Fig. 1), was named pComb3H-CD147.

### 3.2. Detection of phage-displayed CD147 from different E. coli strains

Recombinant bacteriophages were produced by infecting the pComb3H-CD147-transformed *E. coli* with VCSM13 helper phage. During the assembly of progeny viruses, the CD147-gpIII fusion proteins were concomitantly incorporated into phage particles. To detect phage carrying CD147 molecules released

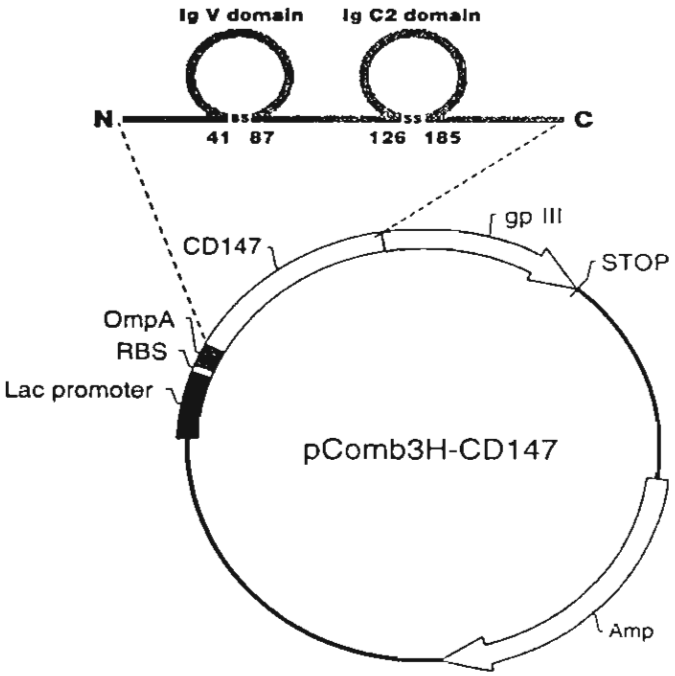


Fig. 1. Schematic representation of pComb3H-CD147: Double *Sfi*I-cloning sites where the CD147 ectodomain gene was inserted; the signal sequence (*Omp*A), ribosome binding site (RBS), *lac* promoter and gpIII gene are depicted. STOP represents the stop codon for CD147-gpIII translation. The derived primary structure of CD147 ectodomain containing two immunoglobulin-like domains is shown. The cysteine residuals which form disulfide bridges are labeled.

into culture supernatant, the polystyrene plate was coated with six CD147 mAbs (M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9) for sandwich ELISA. Only four of the six mAbs (M6-1B9, M6-1D4, M6-1E9, and M6-2F9) reacted against CD147-ϕ derived from *E. coli* XL-1 Blue host when culturing in its standard growth condition (Fig. 2). In contrast, the CD147-ϕ produced in *E. coli* TG-1 under its standard growth condition could be recognized by all CD147 mAbs used. No binding was seen to CD54 mAb (MT54)-coated well which was used as a control. None of CD147 mAbs-captured phages expressing the irrelevant protein, CD99 (Tayapiwatana and Kasinrerk, 2002) (data not shown). This indicated the specificity of CD147 mAbs used. Our results demonstrated that different *E. coli* strains produced different conformation of the expressed protein on phage particles. However, this effect may influence from the different growth conditions. To address this question, CD147-ϕ was produced in TG-1 using standard growth condition of XL-1 Blue and vice versa.

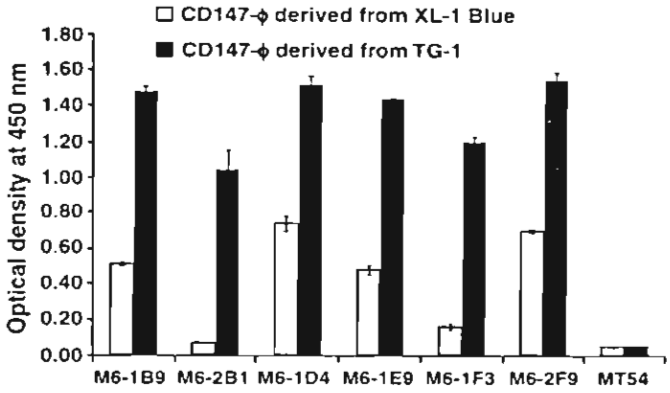


Fig. 2. Comparison of the binding efficiency of CD147-ϕ derived from *E. coli* XL-1 Blue or TG-1 host to the indicated CD147 mAbs by sandwich ELISA. Six CD147 mAbs (M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9) and one irrelevant CD54 mAb (MT54) were individually immobilized on polystyrene plates. The antibody-bound phages were detected with anti-M13 conjugated HRP. The experiment was performed twice with two preparations of CD147-ϕ from both bacterial strains. The histograms demonstrated the mean value and standard deviation.

The CD147-ϕ produced in TG-1 with the standard growth condition of XL-1 Blue could be recognized by all CD147 mAbs used (Fig. 3). However, the number of CD147-ϕ captured by mAb M6-1F3 was remarkably decreased in comparison to CD147-ϕ produced in

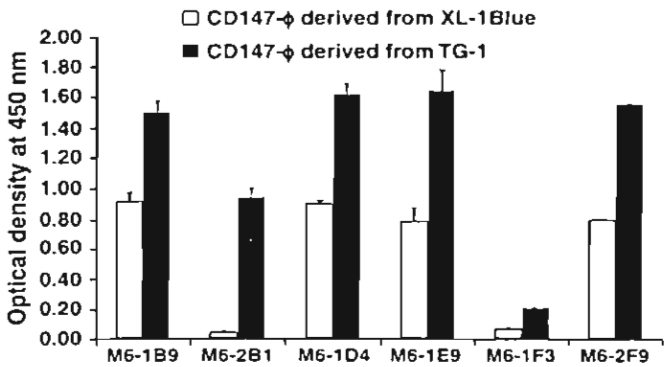


Fig. 3. Influence of growth condition on the folding of CD147 epitopes. CD147-ϕ derived from XL-1 Blue host using the standard growth condition of TG-1 and vice versa were detected with six CD147 mAbs (M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9). The antibody-bound phages were detected with anti-M13 conjugated HRP. The experiment was performed twice with two preparations of CD147-ϕ from both bacterial strains. The histograms demonstrated the mean value and standard deviation.

TG-1 with its standard growth condition (Fig. 2). CD147 mAbs, M6-2B1 and M6-1F3, could not capture CD147-ϕ derived from XL-1 Blue which was cultured in the standard growth condition of TG-1.

### 3.3. Western immunoblotting

Protein components of CD147-ϕ generated from *E. coli* TG-1 were separated by SDS-PAGE under non-reducing conditions. The polypeptides were transferred onto a nitrocellulose membrane and subsequently probed with the six CD147 mAbs. An immuno-reactive band located at approximately 38 kDa was obtained with four CD147 mAbs (M6-1B9, M6-1D4, M6-1E9, and M6-1F3) (Fig. 4). This suggests the fusion protein of CD147 ectodomain (20 kDa) and truncated gpIII (18 kDa). The separated polypeptides did not interact with CD54 mAb (MT54), which was used as a negative control. A specific band with molecular weight of 40 kDa was observed when probing with anti-gpIII mAb, demonstrating the presence of the VCSM13 component in the loaded sample.

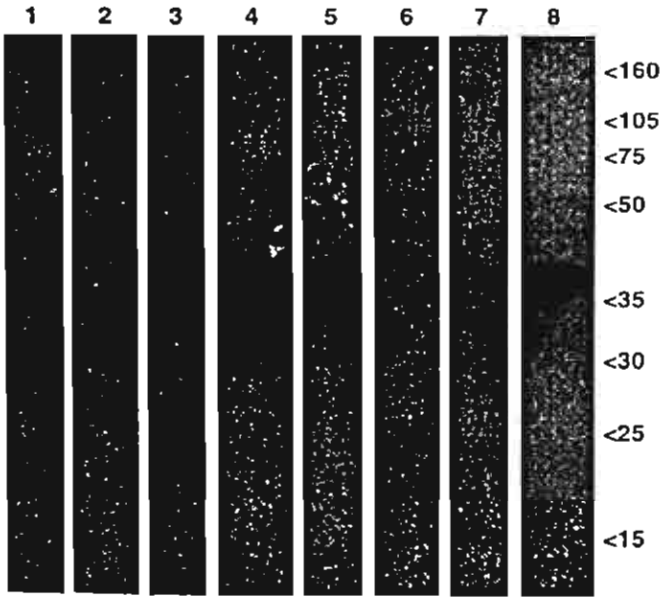


Fig. 4. Western immunoblotting of CD147-ϕ proteins separated by non-reducing SDS-PAGE. Immunological assay was performed by probing with CD147 mAbs; M6-1B9, M6-2B1, M6-1D4, M6-1E9, M6-1F3, and M6-2F9 (lanes 1–6, respectively), CD54 mAb (MT54) (lane 7) or anti-gpIII of VCSM13 mAb (lane 8). The immunoreactive bands were visualized by chemiluminescent substrate detection system. Molecular weight markers in kDa were indicated by arrows.

Table 1
The optical density of competitive inhibition ELISA for epitope mapping of CD147 mAbs

| Inhibitor | Catcher | | | |
|---|---|---|---|---|
| | M6-1B9 | M6-1E9 | M6-1F3 | M6-2F9 |
| M6-1B9 | **1.06** | 0.78 | 0.07 | 1.69 |
| M6-1E9 | 1.07 | **0.96** | 0.06 | 1.32 |
| M6-1F3 | 1.63 | 1.59 | **0.22** | 1.50 |
| M6-2F9 | 1.50 | 1.57 | 0.07 | **1.04** |
| No inhibitor | 1.69 | 1.64 | 1.56 | 1.61 |

The absorbance units of self-inhibition were designated in bold letter. The absorbance units which showed more than 35% reduction in comparison with no inhibitor were underlined.

### 3.4. Epitope mapping

Competitive inhibition ELISA was used for epitope mapping analysis of CD147 ectodomain presented on phage particles derived from *E. coli* TG-1. Four CD147 mAbs (M6-1B9, M6-1E9, M6-1F3, and M6-2F9) were used for the epitope mapping. In this experiment, each CD147 mAb, which was used as the inhibitor, was incubated with CD147-ϕ in the soluble phase. The same set of CD147 mAb was separately immobilized on ELISA wells and used as the catcher. The peroxidase-labeled sheep anti-M13 antibodies were used to determine whether CD147 mAb pre-incubated CD147-ϕ was captured on the solid phase by the catcher. If the competitor and catcher bound to the same region on CD147 molecule, CD147-ϕ would not be caught on the solid phase. Self-inhibition was used to indicate maximal inhibition control. The data of competitive inhibition ELISA is shown as absorbance units in Table 1. Each reaction pair of inhibitor and catcher, which gave more than 35% reduction of absorbance unit in comparison with the non-competitor well, was taken as indicating an overlapping epitope. In this experiment, mAbs M6-1B9 and M6-1E9 inhibited each other. MAb M6-2F9 did not hamper the binding of either mAb M6-1B9 or M6-1E9, and vice versa. Binding of mAb M6-1F3 was interfered with by all tested mAbs. In contrast, mAb M6-1F3 did not block the occupation of other mAbs. As a result, the epitopes of extracellular domain CD147 were proposed as falling into four groups (Fig. 4).

## 4. Discussion

Since phage display technology was invented in 1985 (Smith, 1985), certain investigations have demonstrated that phage display is a high potential technology for producing functional recombinant proteins (Appenzeller et al., 2001; An et al., 2002) Recently, we have applied this technique to generate phage expressing a leukocyte surface molecule, CD99 (Tayapiwatana and Kasinrerk, 2002). By this technique, the bioactive domain of the CD99 protein expressed on phage particles was preserved. The effects on cellular changes of haematopoietic cell lines by CD99-ϕ, i.e. homotypic cell aggregation, proliferation and apoptosis, suggested the presence of a counter-receptor (unpublished observations).

In an attempt to characterize the ligand of CD147, we decided to generate phage expressing a fragment of CD147. The aim is to use CD147-ϕ to search for its counter-receptor on various cell types. However, for this objective, the expressed CD147 fragment must contain bioactive determinants and has to retain the native-like conformation. As CD147 contains two consecutive disulfide bridges in its extracellular domain (Kasinrerk et al., 1992), we compared the efficiency of the two *E. coli* host strains, XL-1 Blue and TG-1, in expressing phage carrying the proper conformation of CD147. By sandwich ELISA, phage generated from XL-1 Blue and TG-1 host strains reacted with the CD147 mAb panel in different ways. All CD147 mAbs used could capture CD147-ϕ produced in TG-1. However, only four CD147 mAbs directed to the CD147 were able to bind the CD147-ϕ derived from XL-1 Blue. This result suggested that the conformation of the CD147 epitopes displayed on phage particles delivered from TG-1 was more accurate. The influence of some unknown properties of *E. coli* affecting the production of heterologous proteins is commonly found (Dueñas et al., 1994; Miksch et al., 2002). However, to the best of our knowledge, no report has been described for this phenomenon in phage display technique. Since the properly structural folding is tremendously significant in using the recombinant phages as probes for discovering a neo ligand-partner, our findings indicating that care must be taken in using different *E. coli* strains for this purpose.

Since the growth condition for XL-1 Blue and TG-1 were different, we raised a question whether the phenomenon described above is the effect of *E. coli* strains alone. Hence, the standard growth condition of XL-1 Blue was used to produce CD147-ϕ in TG-1 and vice versa. It was found that phage generated from XL-1 Blue and TG-1 host strains in the switched growth conditions reacted with the CD147 mAb panel with pattern almost the same as those obtained from its standard growth conditions. Surprisingly, mAb M6-1F3 reacted much better to CD147-ϕ produced from TG-1 in its standard growth condition than those generated in XL-1 Blue growth condition. This finding suggested that both *E. coli* strain and growth condition are important. A suitable culturing condition and a proper *E. coli* host must be cautiously selected for obtaining the correct conformation of CD147 displayed on phage particle.

The correct size of CD147-truncated gpIII fusion protein, 38 kDa, was demonstrated by Western immunoblotting. The antigenic determinants recognized by M6-1B9, M6-1D4, M6-1E9 and M6-1F3 mAbs are in non-tertiary structure. In contrast, M6-2F9 and M6-2B1 mAbs react with conformational epitopes.

In our previous CD147 functional study, mAbs M6-1F3 and M6-2F9 were found to induce U937 homotypic cell aggregation, whereas M6-1E9 was not (Kasinrerk et al., 1999; Khunkeawla et al., 2001). In addition, mAbs M6-1B9 and M6-1E9 inhibited CD3 inducing T cell proliferation (unpublished observations). From these findings together with the results of epitope mapping (Table 1), topographic information of CD147 bioactive epitopes on the CD147-phage was predicted (Fig. 5). Since mAbs M6-1B9 and M6-

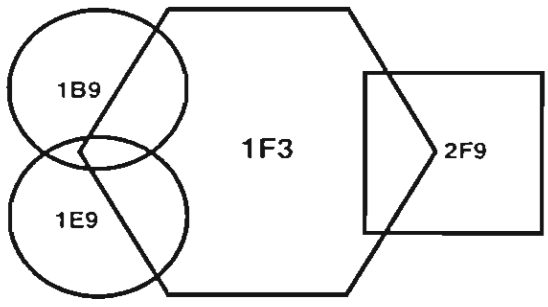


Fig. 5. Topographic illustration of the predicted CD147 bioactive epitopes. Each geometric form represented individual epitope recognized by mAb. The intersections of the polygons indicate the overlapping regions of the different epitopes.

1E9 showed similar result in inhibition of T cell proliferation as well as competition of each other in the epitope-mapping experiment, we proposed that the epitopes recognized by these mAbs are contiguous. In contrast, mAb M6-2F9 could not induce cell aggregation or inhibit T cell proliferation and did not block the binding of mAb M6-1B9 and M6-1E9. The epitope of mAb M6-2F9, therefore, does not overlap or associate with the epitopes recognized by mAbs M6-1B9 and M6-1E9.

The binding of mAb M6-1F3 was interfered with all tested mAbs together with that M6-1F3 could also induce homotypic cell aggregation; the epitope of M6-1F3 was therefore predicted to overlap with other mAbs. We observed that the occupation of mAbs M6-1B9, M6-1E9 and M6-2F9 obstructed the binding of mAb M6-1F3, however, mAb M6-1F3 did not influence the binding of any mAbs tested. This finding may be explained by conformational change after mAbs M6-1B9, M6-1E9 and M6-2F9 interacted with their epitopes. Consequently, other parts of CD147 hampered the epitope recognized by mAb M6-1F3. This phenomenon is regarded as allosteric effect which was reported in certain studies (Davies and Cohen, 1996; Towbin et al., 1996; Aguilar et al., 2000). In contrast, binding of mAb M6-1F3 to its epitope could not induce the conformational change of CD147 structure. Another possibility, which could not be excluded, was the affinity difference of the CD147 mAbs. The binding affinity of mAb M6-1F3 may be less effective than other CD147 mAbs, thus it could not block the binding of mAbs M6-1B9, M6-1E9 and M6-2F9 to their epitopes.

In summary, our study achieved the generation of CD147-ϕ and emphasized the necessity of selecting a suitable *E. coli* host strain for proper folding of the displayed molecule. However, there is no common rule applying for each displayed protein. A novel expression strategy for obtaining functional recombinant protein from *E. coli* by co-expression of DsbABCD in periplasm is supposed to overcome this hurdle (Kurokawa et al., 2000). In addition, the relationship between epitope location and bioactive domain was demonstrated by a conventional method. The CD147-ϕ will be considered as a screening tool for finding its binding partners on the target cells.

## Acknowledgements

This work was supported by The Thailand Research Fund and The National Center for Genetic Engineering and Biotechnology (BIOTEC) of the National Science and Technology Development Agency, Thailand. We are thankful to W. Silaket for expert technical assistance and P. Klangsinsirikul for proofreading of the manuscript.

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