# III. MATERIALS AND METHODS

## 1. Reagents

The following is a list of the reagents used and their sources:
-RPMI 1640 (Gibco, Grand Island, NY)
-Fetal Calf Serum (FCS; Gibco)
-Bovine serum albumin fraction V (Sigma, MO, USA)
-Ficoll 400 (Pharmacia, Uppsala, Sweden)
-Hypaque sodium 50% (Winthrop-Pharmaceuticals, NY, USA)
-6% Macrodex (Pharmacia)
-Gentamycin (Roussel, London, UK)
-AmphotericinB (Gibco)
-Purified-protein derivative (PPD; Staten Seruminstitut, Denmark)
-Phytohemagglutinin (PHA; Wellcome, Beckenham, UK)
-Concanavalin A (Con A type-IV; Sigma)
-recombinant Granulocyte-macrophage colony-stimulating factor (rGM-CSF; Sandoz Research Institute, Vienna, Austria)
-recombinant Tumor necrosis factor (rTNF α; Ernst Boehringer Institute, Vienna, Austria)
-[$^3$H]thymidine (Amersham, Buckinghamshire, UK.)
-ECL enhanced chemiluminescence reagent (Amersham)

## 2. Antibodies

The following is a list of the antibodies used in this study:
-1B2 mAb obtained from hybridomas derived from BALB/c mouse spleen cells after immunization with U-937 cell lines
-CD25-8D8 (anti-IL2Rα), VIT6 (anti-CD1a), 9C8 (undefined mAb) MEM18 (anti-CD14) and anti-CD31 were a kind gift from Dr. Hannes Stockinger (University of Vienna, Vienna, Austria)
-3E10 mAb (undefined mAb; W. Kasinrerk, unpublished observation)
-Leu-4 (anti-CD3; Becton Dickinson, Sunnyvale, CA, USA)
-The murine isotype-matched IgG2bκ (Sigma, St. Louis)
-Biotinylated rabbit anti mouse Igs and Peroxidase-conjugated Avidin (Egg white) (Dakopatts,Glostrup, Denmark)
-Fluorescein isothiocyanate (FITC) labelled sheep F(ab′)$_2$ anti mouse IgG+IgM antibodies (Grub, Scandic, Vienna, Austria)

## 3. Cell lines

The cell lines used in this study were divided into two groups, those being, haematopoietic and non-haematopoietic cells (Table 1.). Haematopoietic cell lines including U-937, K-562, KG-1, HL-60, HPB-ALL, SupT-1 and JY, and non-haematopoietic cell lines including HEp-2, PC9 and PC14. HEp-2, PC9, and PC14 were gifts from Dr. Porn-ngarm Limtrakul, Department of Biochemistry, Faculty of Medicine, Chiang Mai University.

**Table 1. Specifications of all the cell lines used in this study**

| Type | Designation | Source |
|---|---|---|
| **Haematopoietic cell lines** | | |
| T cell lines | HPB-ALL | acute lymphoblastic leukemia |
| | SupT-1 | acute lymphoblastic leukemia |
| B cell line | JY | Burkitt's lymphoma |
| Myeloid cell line | U-937 | histiocytic lymphoma |
| | KG-1 | acute myelogenous leukemia |
| | HL-60 | promyelocytic leukemia |
| Erythro/myeloid cell line | K-562 | chronic myelogenous leukemia |
| **Non-haematopoietic cell lines** | | |
| | PC9 | human CA lung |
| | PC14 | human CA lung |
| | HEp-2 | human CA larynx |

All cell lines were maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 40 mg/L Gentamycin and 2.5 mg/L Amphotericin B in a fully humidified atmosphere of 5% $CO_2$ at 37 °C.

**4. Isolation of peripheral blood mononuclear cells**

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood. The PBMC fraction of human peripheral blood cells was separated by density gradient centrifugation over Ficoll-Hypaque. Briefly, 20 ml of heparinized whole blood was diluted with 20 ml of sterile PBS pH 7.2 in a 50 ml tube and mixed thoroughly. Then, 10 ml of sterile Ficoll-Hypaque was underlayered and centrifuged at 400 g for 30 minutes at room temperature. The PBMC rich interphase layer was collected. Prior to any experiment, the cells were washed three times with RPMI 1640 medium and resuspended in 10% FCS-RPMI 1640.

**5. Isolation of peripheral blood granulocytes**

Granulocytes were isolated by centrifugation through the Ficoll-Hypaque density gradient, followed by dextran sedimentation. Briefly, the pellet from Ficoll-Hypaque density gradient isolation was washed twice with sterile PBS pH 7.2 and resuspended with 30 ml of PBS in a 50 ml tube and 20 ml of 6% Macrodex was added. The tube was left to stand in a 37 °C incubator for 45 minutes at a 45° angle. The leukocyte rich fraction was then collected and washed twice with PBS. The washed cells were mixed with 5 ml of lysing buffer and left to stand at room temperature for 5 minutes. After that, the cells were washed twice with PBS. The granulocytes rich population was evaluated for cell survival by using the dye-exclusion test.

**6. Isolation of leukocytes from leukemic whole blood**

With the advantage of ammonium chloride Tris ($NH_4Cl$-Tris pH 7.2), the mononuclear cells and granulocytes could be isolated from the small amount of blood samples. Briefly, 100 µl of leukemic whole blood was mixed with 1.5 ml of $NH_4Cl$-Tris and incubated for 10 minutes at room temperature. The treated blood was then centrifuged at 600 g for

10 minutes at room temperature and the supernatant was then discarded. The cell pellet was washed three times and resuspended with 1% BSA-PBS azide.

**7. Indirect Immunofluorescence analysis**

Cell were washed twice with cold 1% BSA-PBS azide and the cell concentration was adjusted to $1x10^6$ cells/ml with the same reagent. To block non-specific Fc receptor binding, cells were incubated for 30 minutes at 4 °C with 10% human AB serum before staining. Aliquot of 50 µl cell suspension was incubated with 50 µl mAb (20 µg/ml in 1%BSA-PBS azide) on ice for another 30 minutes. The cells were washed twice with cold 1% BSA-PBS azide and resuspended to 50 µl with the same reagent. Then, 50 µl of FITC labelled sheep $F(ab')_2$ anti mouse IgG+IgM antibodies, diluted to 1:30 with 1% BSA-PBS azide, was added and incubated on ice for 30 minutes. Finally, the stained cells were washed three times with 1% BSA-PBS azide and analyzed for fluorescent staining by a fluorescent microscope. Furthermore, the stained cells were fixed with paraformaldehyde (0.5% w/v in PBS pH 7.2) and membrane fluorescence was analyzed on a FACScan flow cytometer (Becton Dickinson). Individual of blood cell populations were gated according to their forward and side scatter characteristics.

**8. Detection of 1B2 molecule on the cell surface of stimulated PBMC**

Freshly isolated PBMC from PPD-positive healthy donors were cultured in RPMI 1640 medium, supplemented with 10% FCS at a density of $1x10^6$ cells/ml. This was performed in the presence or absence of 30 µg/ml of PPD, 2 µg/ml of PHA, 40 µg/ml of ConA, 40 ng/ml of rTNFα or 100 ng/ml of rGM-CSF at a final volume of 5.0 ml in 25-$cm^2$ tissue culture flasks (Nunc, Roskilde, Denmark) for 1, 3 and 5 days. The activated cells were collected and washed twice with 1% BSA-PBS azide followed by the indirect immunofluorescent technique. The membrane fluorescence was analyzed by a fluorescent microscope and on a FACScan.

## 9. Optimization of the antigen and mitogen concentrations for PBMC stimulation

Each culture was set up in a 96-well plates (Nunc) in a final volume of 200 µl/well. Triplicate aliquots of $1x10^5$ PBMC from PPD-positive donors were cultured with various concentrations of PPD (0-20 µg/ml), PHA (0-2 µg/ml) or ConA (0-40 µg/ml). The cultures were incubated in a $CO_2$ incubator at 37 °C (7 days for PPD, and 3 days for PHA and ConA stimulation). The cultures were pulsed with 0.4 µCi/well of [$^3$H]thymidine 18 hrs. before being harvested. Incorporated radioactivity was counted in a Beckman LS3000 liquid scintillation counter. Values were given by means of the c.p.m. of triplicate cultures.

## 10. Assay of the effect of 1B2 mAb on the proliferation of activated PBMC

PBMC were cultured at a cell density of $1x10^6$ cells/ml in a final volume of 200 µl, in the presence or absence of 2.5 µg/ml of PPD, 0.03125 µg/ml of PHA or 2.5 µg/ml of ConA and various concentrations of 1B2 mAb. The cultures were incubated in a $CO_2$ incubator at 37 °C (7 days for PPD, and 3 days for PHA and ConA stimulation). [$^3$H]thymidine (0.4 µCi/well) was pulsed 18 hours before being harvested. The incorporated radioactivity was counted. Values of triplicate cultures were given by means of c.p.m. and compared between each condition, with or without the presence of various concentration of 1B2 mAb.

## 11. Assay of the effect of 1B2 mAb on the proliferation of U-937 and K-562

U-937 and K-562 were collected and washed once with RPMI 1640 medium. The cells were individually cultured at a cell density of $1x10^5$ cells/ml and $1x10^6$ cells/ml in a final volume of 150 µl/well in 96-well plates. This was performed in the presence or absence of 50 µl of various concentrations of 1B2 mAb or control mAb, including MEM18 (anti-CD14), 9C8 (undefined mAb) and isotype matched mouse IgG2b. In each culture, 50 µl of 0.2 µCi/well of [$^3$H]thymidine was added and the cultures were then incubated in a $CO_2$ incubator at 37 °C for 3 and 5 hrs. Then the cultures were harvested, and incorporated radioactivity was

counted. A comparison between each condition was determined by means of the c.p.m. of triplicate cultures.

## 12. Assay for the cytotoxicity of 1B2 mAb

The cell lines (U-937 and K-562) were collected, washed once with RPMI 1640 medium and resuspended to $1x10^7$ cell/ml with 10% FCS-RPMI. 12 µl of cell suspension was added to the sterile 12x75 mm test tubes containing 40 µl of 1B2 mAb (60 µg/ml in 10% FCS-RPMI) or 40 µl of mouse IgG2bκ (60 µg/ml in 10% FCS-RPMI), and 68 µl of 10% FCS-RPMI. The cells were cultured in a $CO_2$ incubator at 37 °C for five hours. After that, the cell culture was spinned down and its supernatant was discarded. Ten microliters of cell suspension were mixed with 10 µl of 0.5% trypan blue solution, and the percentage of cell survival was calculated from the number of living cells in the total of 200 cells counted.

## 13. SDS-PAGE and Western blotting

Four parts of the cell lysate were mixed with one part of 5x sample buffer (2% SDS, 50 mM Tris-HCl pH 6.8, 10% glycerol and 0.1% bromphenol blue) and heated at 96 °C for 4 minutes. SDS-PAGE was performed according to Laemmli (1970) on 0.75 mm slab gels of 10% polyacrylamide at 120 V. The separated samples (in the gel) were blotted at a constant 8 $mA/cm^2$ for 90 minutes onto 0.45 µm nitrocellulose membranes (Nitrobind, MSI, WESTBORO, MA, USA) using a semi-dry electrophoretic transfer instrument (Semi-Phor, Hoefer, San Francisco, USA). After being transferred, the membrane was immersed in cold PBS pH 7.2 and stored at 4 °C overnight.

## 14. Optimization of conditions for a chemiluminescent technique

Normal mouse serum was diluted five-fold in PBS pH 7.2 to 1:500, 1:2500. and 1:12500. Four parts of diluted serum were mixed together with one part of 5x sample buffer (2% SDS, 50 mM Tris-HCl pH 6.8, 10% glycerol and 0.1% bromphenol blue) and heated at 96 °C for 4 minutes. SDS-PAGE was performed and the separated proteins were blotted onto nitrocellulose membranes as in, the methods described in 13. Each membrane was blocked with blocking solution (5% non-fat dried

milk, 0.05% tween 20 in PBS pH 7.2) for 1 hour at room temperature with agitation and then washed for five minutes four times with PBSTween. The blocked nitrocellulose membranes were individually incubated with biotinylated rabbit anti mouse Igs (Dakopatts; diluted to 1:2500 and 1:5000 in PBSTween) for 1 hour at room temperature on a rocking plate, and then washed with changes of PBSTween three times in five minute periods. The membranes were cut into strips and each one was individually incubated with various concentrations of peroxidase-conjugated avidin (Dakopatts; diluted 1:5000, 1:10000, 1:20000 in PBSTween) for another 1 hr. at room temperature. Finally, the membranes were washed four times: three times with PBSTween and finally with PBS until ready for chemiluminescent reaction. The enhanced chemi-luminescence (Amersham) solution-1 was mixed together with solution-2 in equal volume to make a working solution immediately prior to use. The membrane strips were then placed on a plastic sheet, flooded with enhanced chemiluminescent working solution and left to stand at room temperature for exactly two minutes. Excess solution was rinsed out completely and the membranes were covered with saran wrap. They were then, immediately exposed to the X-ray film (Hyperfilm, Amersham) for 2.5 minutes. The exposed films were developed for 90 seconds in the developer solution (Kodak), followed by 60 seconds fixing in the rapid fixing solution (Kodak). Finally, the films were rinsed with tap water and left to air dry.

## 15. Preparation of U-937 lysate

U-937 was collected and washed twice with cold PBS pH 7.2. The washed cells were resuspended to the final concentration of $50x10^6$ cells/ml with precooled lysis solution (20 mM Tris, 140 mM NaCl, 2 mM EDTA, 2 mM PMSF, 5 mM iodoacetamide, and 1% Nonidet p-40, pH 8.2) and incubated for 30 minutes on ice, mixing every ten minutes. The cells were spinned down in a microcentrifuge at 12000 rpm, at 4 °C for five minutes. The cell lysate was collected into a new precooled microtube (Treff AG, Degersheim, Switzerland) and stored at -20 °C.

## 16. Immunoblotting for the detection of 1B2 mAb-binding protein

Lysate of U-937 was prepared, applied on SDS-PAGE and blotted onto nitrocellulose membrane, as described in 13. The membrane was blocked by using blocking solution (5% non-fat dried milk in PBS pH 7.2) for 1 hour at room temperature on a rocking plate. Then the blocked membrane was washed for five minutes four times with PBSTween. The membrane was then incubated with 1B2 mAb (20 µg/ml in PBSTween) for one hour at room temperature with agitation. The membranes were washed for five minutes three times with PBSTween, and then incubated in biotinylated rabbit anti-mouse Igs (diluted 1:5000 in PBSTween) for a further one hour at room temperature with agitation. The membrane was washed again with changes of PBSTween three times in five minute periods and incubated in peroxidase-conjugated avidin (diluted 1:20000 in PBSTween) for one hour at room temperature with agitation. Finally, the membrane was washed four times: three times with PBSTween and finally with PBS.

The antigen-antibody reaction on the membrane was visualized by the enhanced chemiluminescence detection system of Amersham, as described in 14.