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(54) **UTILISATION D'AGENTS LIANTS A CD47 ET CES LIGANDS POUR LE TRAITEMENT OU PROPHYLAXIE DE MALADIES INFLAMMATOIRE, AUTOIMMUNITAIRE ET ALLERGIQUE ET POUR LE TRAITEMENT DE REJET DE GREFFONS**

(54) **USE OF BINDING AGENTS TO CD47 AND ITS LIGANDS IN THE TREATMENT OR THE PROPHYLAXIS OF VARIOUS INFLAMMATORY, AUTOIMMUNE AND ALLERGIC DISEASES AND IN THE TREATMENT OF GRAFT REJECTION**

TITLE

Use of binding agents to CD47 and its ligands in the treatment or the prophylaxis of various inflammatory, autoimmune and allergic diseases and in the treatment of graft rejection.

FIELD OF THE INVENTION

The present invention relates to new uses of binding agents to CD47 antigen or its ligands, and more particularly to monoclonal antibodies specific to the CD47 or thrombospondin, in the treatment or prophylaxis of various inflammatory, autoimmune and allergic diseases as well as in treatment of tumor metastasis, cachexia and graft rejection.

BACKGROUND OF THE INVENTION

Integrin superfamily of adhesive receptors are transmembrane heterodimeric molecules which function in cell-matrix and cell-cell adhesion (4, 5). The CD47 Ag, a surface glycoprotein of ~50 KD MW is physically and functionally associated with β3 integrin mainly αvβ3 (the vitronectin receptor) on a variety of cell types (6, 7). Integrin αvβ3 is a highly promiscuous receptor recognizing Arg-Gly-Asp (RGD) in a wide variety of proteins as well as being expressed by many cell types including endothelial cells, osteoclasts, monocytes, activated lymphoid cells, platelet, fibroblasts and malignant cells (8, 9). The αvβ3 integrin plays a role in diverse biologic processes such as cell migration and differentiation, tumor cell invasion, angiogenesis, bone resorption and immune response (9-12). Taken together, the

role of αvβ3 in cell directed mobility underlies the importance of this molecule in development, wound repair, cancer and inflammation.

The cDNA sequence of CD47 Ag predicts a multispanning membrane protein with 5 transmembrane domains; the large extracellular N-terminal domain is homologous to members of IgV superfamily and harbours several potential N-glycosylation sites (13, 14). It appears that CD47 Ag affects both ligand affinity and signal transduction of β3 integrins (6, 15). Indeed, mAb directed against CD47 Ag inhibited ligand binding to αvβ3 receptor and blocked activation of PMN phagocytes, respiratory burst, chemotaxis as well as stimulation of Ca^{+2} entry in endothelial cells in response to RGD containing proteins. Recent studies have shown that CD47 Ag is also involved in transendothelial and transepithelial migration of neutrophils (16, 17).

Although CD47 does not bind to vitronectin, its natural ligand was recently identified as being thrombospondin (TSI) (18), one of the several ligands of αvβ3 to which it binds via RGD-containing sequence. TSI interacts with CD47 through its cell binding domain (non RGD sequence). Both anti-TSI and anti-CD47 mAbs partially inhibited TSI-stimulated Ca^{+2} entrance in fibroblasts providing a possible mechanism for TSI directed cell mobility via CD47. It is also speculated that TSI-CD47 interactions would modulate the function of αvβ3 during angiogenesis (18). Interestingly, CD47 Ag is an ubiquitous molecule, present on a variety of cell types including lymphocytes and erythrocytes which express low levels or no αvβ3 integrins, respectively (19).

It is also known that the infiltrate at the extravascular site of inflammation during acute (ex: following invasion by microorganisms) or chronic (ex:

autoimmune) diseases consists of diverse accumulation of leukocytes (i.e., T, B, granulocytes and macrophages). Despite the specific immune reaction that triggers the disease, the majority of cells found in the inflammatory infiltrate are non-specifically activated leukocytes. Recovery from the disease is intimately related to the regression of this infiltrate and this can be achieved by the elimination of the few Ag specific T cells strongly suggesting that rare cells may regulate the recruitment and most likely the function of the vast majority of non-specific cells (1). Recently an "in vitro model" of non-specific T cell activation has been developed whereby cocultures of human resting T cells with autologous monocytes and IL-2 or IL-12 lead to large production of IFN-γ in the absence of Ag (2). Results obtained with this method indicates that CD40-CD40L interactions as well as IL-12 are key regulators of this bystander T cell activation and that sCD23 further amplifies it by triggering monokine release by monocytes (ex: TNF-α, IL-1) (3).

SUMMARY OF THE INVENTION

The present invention is directed to a plurality of new uses of binding agents to CD47 antigen (Ag), and more particularly to new uses of monoclonal antibodies (mAbs) specific to the CD47. The invention is based on the discovery that mAbs directed against CD47 Ag strongly abrogate both IFN-γ production and monokine release (i.e. IL-1, IL-12 and TNF-α), and downregulate cytokine production by anti-CD3 or allogenic cell-stimulated T cells. These results were obtained with both an antibody named 10G2 produced by the applicant, and with different commercial anti-CD47 antibodies, including an antibody named B6H12 (ATCC HB-9771). Thus, it is believed that anti-CD47 antibodies in general, and CD47 ligands such as thrombospondin could be useful in the treatment or prophylaxis of various

inflammatory, autoimmune and allergic diseases as well as in treatment of tumor metastasis, cachexia and graft rejection. These human diseases include rheumatoid arthritis, lupus erythematosus, multiple sclerosis, diabetes, uveitis, ulcerative colitis, Crohn's disease, inflammatory bowel disease, thyroiditis, glomerulonephritis, Sjögren disease, graft versus host disease (GVH), allergies, asthma, rhinitis and eczema.

Preferred binding agents include ligands, antibodies, fragments thereof or artificial constructs comprising antibodies or fragments thereof or artificial constructs designed to mimic the binding of antibodies or fragments thereof. Such binding agents are discussed in Dougall et al in Tibtech (1994) 12:372-379. They include complete antibodies, $F(ab')_2$ fragments, Fab fragments, Fv fragments, ScFv fragments, other fragments, CDR peptides and mimetics. These can be obtained/prepared by those skilled in the art. For example, enzyme digestion can be used to obtain $F(ab')_2$ and Fab fragments (by subjecting an IgG to molecule to pepsin or papain cleavage respectively). References to "antibodies" in the following description should be taken to include all of the possibilities mentioned above. Recombinant antibodies may be used. The antibodies may be humanized or chimerised. The CDRs may be derived from a rat or mouse monoclonal antibody. The framework of the variable domains, and the constant domains, of the altered antibody may be derived from a human antibody. Such a humanized antibody elicits a negligible immune response when administered to a human compared to the immune response mounted by a human against a rat or mouse antibody.

Alternatively, the antibody may be a chimeric antibody. A chimeric antibody comprises an antigen binding region and a non-immunoglobulin region. The antigen binding region is an antibody light chain variable domain or heavy chain variable

domain. Typically, the chimeric antibody comprises both light and heavy chain variable domains. The non-immunoglobulin region is fused as its C-terminus to the antigen binding region. The non-immunoglobulin region is typically a non-immunoglobulin protein and may be an enzyme region, a region derived from a protein having known binding specificity, from a protein toxin or indeed from any protein expressed by a gene. The two regions of the chimeric antibody may be connected via a cleavable linker sequence.

The antibody may be a human IgG such as IgG1, IgG2, IgG3, IgG4, IgM, IgA, IgE or IgD carrying rat or mouse variable regions (chimeric) or CDRs (humanized). Primatizing techniques may also be used.

The binding agents of the present invention may be used alone or in combination with immunosuppressive agents such as steroids, cyclosporin, or antibodies such as an anti-lymphocyte antibody or more preferably with a tolerance-inducing, anti-autoimmune or anti-inflammatory agent such as a $CD4^+$ T cell inhibiting agent e.g. an anti-CD4 antibody (preferably a blocking or non-depleting antibody), an anti-CD8 antibody, a TNF antagonist e.g. an anti-TNF antibody or TNF inhibitor e.g. soluble TNF receptor, or agents such as NSAIDs.

The binding agent will usually be supplied as part of a sterile, pharmaceutically acceptable composition. This pharmaceutical composition may be in any suitable form, depending upon the desired method of administering it to a patient. It may be provide in unit dosage form and may be provided as part of a kit. Such a kit would normally (although not necessarily) include instruction for use.

Binding agent administrations are generally given parenterally, for example intravenously, intramuscularly or sub-cutaneously. The binding agents are generally given by injection or by infusion. For this purpose a binding agent is formulated in a pharmaceutical composition containing a pharmaceutically acceptable carrier or diluent. Any appropriate carrier or diluent may be used, for example isotonic saline solution. Stabilizers may be added such as a metal chelator to avoid copper-induced cleavage. A suitable chelator would be EDTA or sodium citrate. They may be given orally or nasally by means of a spray, especially for treatment of respiratory disorders. They may be formulated as creams or ointments, especially for use in treating skin disorders. They may be formulated as drops, or the like, for administration to the eye, for use in treating disorders such as vernal conjunctivitis. For injectable solutions, excipients which may be used include, for example, water, alcohols, polyols, glycerine, and vegetable oils.

The pharmaceutical compositions may contain preserving agents, solubilizing agents, stabilizing agents, wetting agents, emulsifiers, sweeteners, colorants, odorants, salts (substances of the present invention may themselves be provided in the form of a pharmaceutically acceptable salt), buffers, coating agents or antioxidants. They may also contain other therapeutically active agents.

Suitable dosages of the substance of the present invention will vary, depending upon factors such as the disease or disorder to be treated, the route of administration and the age and weight of the individual to be treated. Without being bound by any particular dosages, it is believed that for instance for parenteral administration, a daily dosage of from 0.01 to 50 mg/kg of a binding agent of the present invention (usually present as part of a pharmaceutical composition as indicated above) may be suitable for treating a typical adult. More suitably the dose

might be 0.05 to 10 mg/kg, such as 0.1 to 2 mg/kg. This dosage may be repeated as often as appropriate. Typically administration may be 1 to 7 times a week. If side effects develop the amount and/or frequency of the dosage can be reduced. A typical unit dose for incorporation into a pharmaceutical composition would thus be at least 1 mg of binding agent, suitably 1 to 1000 mg.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be better understood from the following detailed description given with reference to the accompanying drawings.

Fig. 1 and Fig. 2 are graphical representations which show 10G2 and B6H12 mAbs recognizing CD47 antigen. Untransfected COS and COS cell lines transiently expressing CD47 Ag (Fig. 1) or stable CH0-transfected cell line with CD47 cDNA (Fig. 2) were stained with either 10G2 (Fig. 1 and 2) or B6H12 (Fig. 2) mAbs or isotype-matched cont mAbs as described in materials and methods. One representative experiment out of 3.

Figures 3 to 5 are graphical representations which show the measurements of IFN-γ production by autologous monocytes. T cells ($1X10^6$/ml) were cultured in the presence of autologous monocytes ($2.5x10^5$/ml) and stimulated by IL-2 (50 U/ml) (Fig. 3), IL-2 (2 U/ml) plus sCD23 (25 ng/ml) (Fig. 4) or IL-15 (100 ng/ml) plus sCD23 (25 ng/ml) (Fig. 5) in the presence or absence of anti-CD47 mAbs used at 5 μg/ml final concentration. IFN-γ production was measured in the CSN by RIA after 3 days culture. Data represent mean ± SEM of 5 experiments ($p<0.001$).

Figures 6 is a graphical representation which shows that anti-CD47 mAbs suppressed in a dose-dependent manner IL-2 plus sCD23 induced IFN-γ production. Cultures of T cells and monocytes were stimulated with IL-2 (10 U/ml) and sCD23 (25 ng/ml) in the presence of various concentration of anti-CD47 mAb (clone 10G2) (Panel A), F(ab')2 fragments of clone B6H12 (Panel B) or different anti-CD47 mAbs (Panel C). IFN-γ production was measured after 3 days cultures. Data is one representative experiment out of 3.

Figures 7 to 9 are graphical representations which show Anti-CD47 mAbs suppressed IL-12 production. T cells ($1X10^6$/ml) were cocultured with autologous monocytes ($2.5X10^5$/ml) as described in Figures 3-5. After 3 days cultures, IL-12 p40 release was measured in the culture supernatant by ELISA. Data represent mean ± SEM of 5 (Fig. 7), 4 (Fig. 8) and 1 out of 3 experiments (Fig. 9) ($p<0.01$).

Figures 10 is a graphical representation which shows that Anti-CD47 mAbs suppressed IL-12 plus sCD23-induced IFN-γ production. T cells ($1X10^6$/ml) were cultured with autologous monocytes ($2.5X10^5$/ml) and stimulated by IL-12 (40 pM) and sCD23 (25 ng/ml) in the presence or absence of anti-CD47 mAbs (2.5 μg/ml). IFN-γ production was measured in the CSN after 3 days culture. Data represent mean ± SD of 4 experiments ($p<0.01$).

Figures 11 is a graphical representation which shows that F(ab')2 and Fab fragments of anti-CD47 mAb suppressed IFN-γ production. T cells ($1X10^6$/ml) were cocultured with monocytes ($2.5X10^5$/ml) and stimulated by IL-2 (20 U/ml) with or without F(ab')2 or Fab fragments of anti-CD47 mAb (clone B6H12). IFN-γ was measured after 3 days of culture. Shown is one representative experiment out of 2.

Figures 12 is a graphical representation which shows that clones 10G2 and B6H12 recognize different CD47 epitopes. Jurkat T cell line (Panel A) and THP-1 monocyte cell line (Panel B) were stained with various concentrations of clone 10G2, B6H12 mAbs and isotype-control matched mAbs as described in materials and methods.

Figures 13 is a graphical representation which shows the cellular distribution of 10G2 and B6H12 antigens on dendritic cells and erythrocytes. Erythrocytes (Fig. 13a) and dendritic cells (Fig. 13b) were stained with either 10G2 or B6H12 mAbs as described in materials and methods. One representative experiment out of 3.

Figures 14 is a graphical representation which shows that anti-CD47 mAbs suppressed TNF-α production by purified monocytes. Enriched monocytes ($2X10^5$/ml) were stimulated by sCD23 (25 ng/ml) or LPS (10 μg/ml) in the presence or absence of 2.5 μg/ml anti-CD47 mAbs (clone 10G2 or B6H12). After overnight culture, TNF-α, IL-1β, IL-8 and PGE2 were measured in the CSN by ELISA. Data represent mean ± SD of 8 (Panel A) and 3 experiments (Panel B) ($p<0.001$).

Figures 15 is a graphical representation which shows that sCD23 costimulates IL-2 or IL-15-induced IL-12 p40 release. T cells (10^6/ml) were cultured with autologous monocytes ($2X10^5$/ml) and stimulated with IL-2 (50 U/ml) or IL-15 (100 ng/ml) in the presence or absence of sCD23 (25 ng/ml). Anti-CD47 or isotype-control matched mAb (5 μg/ml) were added to the cultures. After 3 days culture, IL-12 p40 release was measured in the CSN. Data represent mean ± SD of 4 experiments.

Figures 16 is a graphical representation which shows that anti-CD47 mAb suppresses IL-12 p75 production induced by T-cell dependent or independent costimulatory signals. Monocytes (10^6/ml) were stimulated with SAC alone, SAC plus IFNγ or sCD40L plus IFNγ and GM-CSF in the presence of anti-CD47 or control mAb. After overnight culture, IL-12 p75 release was measured in the CSN. Data represent mean ± SD of 7 experiments.

Figures 17 is a graphical representation which shows the effect of anti-CD47 mAb on SAC or SAC plus IFNγ-induced monokine release. Monocytes (10^6/ml) were stimulated with SAC plus IFNγ in the presence of anti-CD47 or isotype control-matched mAb. Monokine release (i.e. IL-1β, IL-6, TNFα and IL-10) were measured in the CSN after overnight culture. Data represent mean ± SD of 5 experiments.

Figures 18 is a graphical representation which shows that anti-CD47 mAb suppresses Ag-dependent T cell IFN-γ response. Purified T cells ($1X10^6$/ml) were stimulated by soluble anti-CD3 mAb (clone 64.1) plus IL-2 (25 U/ml) or IL-12 (60 PM) with or without anti-CD47 mAb (5 μg/ml). ^{3}H thymidine uptake was measured during the last 16 hrs of 5 days culture and CSN were collected for the measurement of IFNγ production. Data represent mean ± SD of 3 experiments ($p<0.05$).

Figures 19 is a graphical representation which shows that anti-CD47 mAbs suppresses allogeneic mixed lymphocyte reaction. T cells ($0.5X10^6$/ml) were cocultured with allogeneic mitomycin C-treated dendritic cells ($0.3X10^5$/ml) in 96-well U-bottom plate in the presence or absence of anti-CD47 mAb (5 μg/ml). ^{3}H-thymidine uptake was measured after 5 days culture. Data are one representative experiment out of 3.

Figures 20 is a graphical representation which shows that anti-CD47 mAbs specifically suppresses IgE synthesis with no effect on B cell proliferation. Tonsillar B cells ($1X10^6$/ml) were stimulated by soluble CD40-ligand (sCD40L) (1 µg/ml) and IL-4 (10 ng/ml) in the presence or absence of anti-CD47 mAbs (5 µg/ml) (clone 10G2 or B6H12). ^{3}H-thymidine uptake (B-cell proliferation) was measured after 5 days culture and IgE production after 14 days. Data represent mean ± SD of 8 experiments ($p<0.001$).

DETAILED DESCRIPTION OF THE INVENTION

Production of mAbs

Clone 10G2 secreting anti-CD47 antibodies (IgM class) has been produced according to conventional procedures such as described by Köhler and Milstein (Nature (1975) 256:495-497). Accordingly, a non-IgG secreting mouse myeloma cell line (NSI) rendered azaguanine resistant are fused to spleen cells from immunized mice with Jurkat T cell line to obtain hybrid cells that produce large amounts of monoclonal antibody. This method employed polyethylene-glycol (PEG) as the fusing agent followed by selection in HAT medium (hypoxanthine, aminopterin and thymidine). Screening of mAbs was performed according to their "anti-inflammatory biological activity: i.e. inhibition of IFN-γ response" in T cells/monocytes coculture system in the absence of TCR engagement.

Cell separation and culture conditions

Monocytes: PBMC were isolated by density gradient centrifugation of heparinized blood from normal healthy volunteers using Lymphoprep (Nycomed, Oslo, Norway). Monocytes were prepared as described (2). Briefly, PBMC were

resuspended at 50 X 10^6 cells/ml in RPMI 1640 containing 10% FCS (BioWittaker, Inc., Walkersville, MD) and incubated 40 min at 4°C under rotation (to allow aggregation of monocytes) followed by 10 min incubation on ice. Pellets of aggregated enriched monocytes were further separated from non-aggregated PBMC by a gradient of FCS and another 10 min incubation on ice. Enriched monocyte preparations were further depleted in T and/or NK cells by rosetting with S-(2-aminoethyl) isothiouronium bromide (Aldrich Chemical Co., Milwaukee, WI) treated sheep red blood cells (AET-SRBC). Monocyte purity was shown to be >95% by flow cytometry (FACScan, Becton Dickinson) using phycoerythrin-conjugated anti-CD14 mAb (Becton Dickinson). For some experiments, monocytes were positively selected according to CD14 expression by means of a FACSort (Becton Dickinson), and monocyte purity was >99% $CD14^+$ cells. Cellular viability was >90% using trypan blue exclusion.

T cells: Enriched T cell populations were obtained from the monocyte-depleted PBMC by rosetting with AET-SRBC and treatment with ammonium chloride. To obtain highly purified T cells, rosette forming cells were washed and incubated for 20 min at 37°C in Lympho-Kwik T (One Lambda, Los Angeles, CA). Cell purity was assessed by flow cytometry (FACScan, Becton Dickinson) using phycoerythrin-conjugated anti-CD3 mAb (Becton Dickinson) and shown to be >98% in all the cases.

All cultures were performed in complete serum-free HB101 medium (Irvine Scientific, Santa Ana, CA) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, 100 IU penicillin and 100 µg/ml streptomycin. When cultured alone, monocytes were incubated in 5ml sterile Falcon tubes (Becton Dickinson, Lincoln Park, NJ) at 2 X 10^5 cells/ml for cytokine measurement in the

presence of polymyxin B (10 µg/ml) (Sigma Chem., St. Louis, MO). For coculture experiments, T cells (10^6 cells/ml) were incubated with monocytes or B cells (2 X 10^5 cells/ml) in 24-well Falcon plates.

Reagents

Human recombinant IL-2 was kindly provided by Dr. D. Bron (Institut Bordet, Brussels, Belgium). IL-4 and soluble CD40L was a gift from Immunex (Seattle, WA), IL-10 was received from Dr. K. Moore (DNAX, Palo Alto, CA), IL-12 was a generous gift from Dr. M. Gately (Hoffmann-La Roche, Nutley, NJ) and used at 40 pM. Endotoxin-free (<15 pg/ml as determined by the chromogenic Limulus amebocyte lysate, QCL-1000, BioWhittaker Inc., Walkersville, MD) affinity-purified sCD23 was prepared in our laboratory from CSN of CH0 cell line transfected with human cDNA encoding for aa 148 to 321 of the CD23 molecule. The concentration of 25 ng/ml sCD23 used throughout this study was selected on the basis of previously reported dose-response curves. Recombinant TNFα was kindly provided by Dr. W. Fiers (State University, Ghent, Belgium). B6H12 mAb was purchased at the ATCC (clone HB-9771: U.S. Pat. 5,057,604).

Cytofluorimetric analysis

Immunofluorescence was performed on various cells and cell lines according to standard techniques using both anti-CD47 mAbs in the presence of normal human Igs (150 µg/ml). PE-conjugated streptavidin were obtained from Ancell and biotinylated goat anti-human IgG + IgM was purchased from Tago. After staining, cells were analyzed with a FACScan (Becton Dickinson & Co.).

Lymphokine determinations

IFN-γ and IL-10 were measured by a sandwich solid-phase RIA. Anti-IFN-γ mAb (clone 42.25) was used to coat the solid phase and ^{125}I-labeled anti-IFN-γ mAb (clone KM48, purchased from Dimension Labs. Inc., Mississauga, Ont., Canada) as detecting probe; anti-IL-10 clone 9D7 for coating and anti-IL-10 clone 12G8 for labelling. TNF-α was assessed using a sandwich ELISA employing mouse mAb to human TNF-α (clone T144.B, kindly provided by Dr. T. Nakajima, St. Marianna University School of Medicine, Kawasaki, Japan) and a polyclonal rabbit anti-TNF-α received from Dr. J. Tavernier (Roche Research Institute, Ghent, Belgium). IFN-γ, IL-10 and TNF-α assays were calibrated against international standards obtained from the National Institute of Biological Standards and Control (Hertfordshire, England). The detection limit for the IFN-γ and IL-10 RIA is 30 pg/ml and is 45 pg/ml for the TNF-αELISA. IL-1β was measured by ELISA kits purchased from R & D Systems. IL-12 p40 and IL-12 p75 were measured by a two-site sandwich ELISA employing clone 2.4 A1 or clone 20C2 as capture mAbs and clone 4D6 as second mAb. Samples were analyzed in serial 5 fold dilutions in duplicate; the sensitivity of the assay is 10 pg/ml.

Immunoglobulin determinations

IgE was measured by sandwich radioimmunoassays (RIA). Clone 89 (mAb anti-IgE) was used as coating mAb and ^{125}I clone 4.15 (anti-IgE) mAb as detecting probe; sensitivity of the assay was <150 pg/ml.

Expression cloning of molecule recognized by 10G2 mAb

A cDNA library was prepared from Jurkat T cell line, that expressed high level of 10G2 epitope, according to the method described by Seed et al (Proc.Natl.Acad.Sci. USA (1987) 84:3365-3369). Briefly, the cDNA library was

used to transfect COS cells by DEAE-dextran method. After 3 days transfection, COS cells were harvested and incubated with 10G2 mAb at 4°C for 1 h. Unbound mAb was removed by washing and the COS cells were incubated in petri dishes coated with goat anti-mouse IgM antibodies. After 2 hrs, the unbound cells were extensively washed with PBS; the cells adhering to the plates were lysed and the episomal DNA was prepared. The cDNA was used to transform bacteria. The antibiotics resistant colonies were amplified and pooled. Plasmids were prepared from pools of 50 colonies and used to transfect COS cells. Single colonies from the positive pools were amplified and their plasmids were tested for their ability to transfer 10G2 epitope into COS cells. The positive clone was subsequently cloned and cDNA was sequenced.

Statistical analysis

Paired Student's t test have been used to assess level of significance (*<0.05; **p<0.01, ***p<0.001)

Explanation and significance of the foregoing results are as follows:

Clone 10G2 mAb is directed against CD47 antigen.

It has been previously reported that soluble CD23 activated monocytes to contribute to the antigen-independent stimulation of T cells (2). During the screening for mAbs that might regulate this bystander T cell response, the applicant found clone 10G2, this clone secreting antibodies having anti-inflammatory properties. Using mammalian vector expression cloning method and 10G2 mAb, the cDNA encoding CD47 Ag was cloned from Jurkat T cell line cDNA library. The CD47 cDNA was transiently expressed in COS 7 cell line. As shown in Fig. 1,

10G2 mAb strongly reacted with CD47-transfectants with no staining of untransfected cell lines. Next, the applicant prepared stable CD47 transfectants in CHO cell line and found similar pattern of staining with clone 10G2 and B6H12 mAb (a commercially available CD47 mAb binding to CD47 Ag) mAbs establishing that 10G2 recognized CD47 Ag (Fig. 2).

Anti-CD47 mAbs suppressed IL-2 and IL-15-induced IFN-γ production in the presence or absence of sCD23.

As shown in Fig. 3-5, anti-CD47 mAbs (clone 10G2 and B6H12), inhibited IL-2 and IL-15 stimulated IFN-γ response not only in the presence but also in the absence of sCD23. The inhibitory effect of anti-CD47 mAbs is dose-dependent (Fig. 6). Interestingly, thrombospondin, the natural ligand of CD47, similarly suppressed IL-2-induced IFNγ production in T/monocytes cocultures (Table II). The applicant has previously demonstrated that the IL-2 or IL-15 induced IFN-γ production was strictly dependent on CD40-CD40L interactions and on endogenous IL-12 production as shown by the inhibitory effects of both anti-IL-12 and anti-CD40L Abs on IFN-γ. production and of anti-CD40L mAb on IL-12 release (3). The applicant therefore examined the effect of these anti-CD47 mAbs on IL-12 secretion. The data in Fig. 7-9 indicated that, like anti-CD40L mAb, both anti-CD47 mAbs strongly suppressed IL-2 or IL-15-induced IL-12 production; however, by contrast to anti-CD40L mAb, addition of exogenous IL-12 failed to restore IFN-γ production (Fig. 10). Taken together, these data indicated that anti-CD47 mAbs inhibited IFN-γ production not only by reducing IL-12 release but also by diminishing T cell response to IL-12.

To further analyze the mechanisms of inhibitory activity of anti-CD47 mAbs in T cells/monocytes cocultures, the applicant prepared $F(ab')_2$ and monovalent Fab

fragments of CD47 mAb. As shown in Fig. 11, divalent or monovalent fragments of CD47 mAb significantly suppressed IFNγ response suggesting that the anti-CD47 mAb mediated its activity by either delivering a negative signal to the cell through the cross-linking of CD47 Ag via its divalent Fab (not via its Fc fragment bound to FcγR) or by inhibiting the interactions between CD47 and its natural ligand, the thrombospondin-derived macrophages.

Cellular distribution of Ag binding to AIM mAbs.

The applicant next examined a panel of cell lines for their reactivity to clone 10G2. As shown in Table I, clone 10G2 reacted to most of the cell lines (T, B, monocytic and erythroleukemia cell lines) with the exception of THP 1 monocytic cell line exclusively stained by BH612 mAb and not by 10G2 mAb (Fig. 12). Both anti-CD47 mAbs stained all leukocytes (T, B and macrophages) (Table I); erythrocytes (Fig. 13a) and dendritic cells (Fig. 13b) are reacting with BH612 but not with 10G2 mAb. All together, these results strongly suggested that both mAbs reacted with different epitopes or different molecular forms of a common antigen.

Anti-CD47 mAbs inhibit inflammatory mediators release by monocytes.

IFN-γ and TNF-α are directly implicated in the pathogenesis of chronic inflammatory disease as shown by *in vivo* studies using neutralizing mAbs. Monocyte-dependent T cell IFN-γ production involved TNF-α and IL-12 production as well as interactions between costimulatory surface molecules (CD40-CD40L; LFA3-CD2, B7-B7 ligands). The applicant therefore examined the regulatory activity of anti-CD47 mAbs on monokine release by purified monocytes. Bacterial stimuli (i.e. lipolysaccharides (LPS) or staphylococcus aureus Cowan 1 (SAC) or sCD23 were used to trigger TNF-α, IL-1β or IL-12 production. The data in Fig. 14 and table IV indicated that anti-CD47 mAbs strongly inhibited sCD23

induced TNF-α release, without affecting LPS induced TNF-α production. Similarly, sCD23-induced IL-1β and PGE2 production were suppressed by anti-CD47 mAbs. Although sCD23 did not trigger IL-12 release by purified monocytes, it costimulated IL-12 production in T cells/monocytes cocultures system. As shown in Fig. 15, CD47 mAb strongly decreased sCD23 costimulatory activity on IL-12 secretion. Most strikingly, CD47 mAb also suppressed IL-12 production by purified monocytes stimulated by T-cell independent (i.e. SAC) or dependent signals (i.e. sCD40L) (Fig. 16). Of interest, thrombospondin significantly reduced SAC and IFNγ-activated IL-12 p70 release by monocytes (Table III). As reported for LPS-induced TNFα, CD47 mAb failed to inhibit SAC plus IFNγ induced TNFα release (Fig. 17). The SAC-induced secretion of other monocyte products (i.e. IL1β, IL-6, IL-10) remained largely unaffected.

Taken together, these results indicated that anti-CD47 mAbs displayed potent suppressing activity on inflammatory mediators release underlying their inhibitory effect on IFN-γ production.

Anti-CD47 mAbs suppress IL-12 and anti-CD3-induced IFN-γ production and allogeneic mixed lymphocyte reaction.

The applicant next examined the biological activity of anti-CD47 mAbs on Ag-dependent T cell stimulation. As depicted in Fig. 18, anti-CD47 mAbs inhibited anti-CD3-induced T cell proliferation and IFN-γ production by purified T cells. Similar selective suppression of anti-CD3-induced T cell response to IL-12 were obtained using purified CD4 or CD8 subpopulations (not shown). Of interest, pokweed mitogen-induced IFN-γ is also suppressed by anti-CD47 mAbs (data not shown). The inhibitory activity by anti-CD47 mAbs of Ag-dependent T cell activation was also observed in mixed lymphocyte reaction (MLR). Irradiated

allogeneic non-T cells enriched populations or purified dendritic cells were used as allostimulators of adult peripheral purified $CD4^+$ T cells. As shown in Fig. 19, anti-CD47 mAbs inhibited primary mixed lymphocyte reaction as measured by ^3H thymidine uptake; the applicant also tested the effect of mAbs on secondary stimulation, and found that triggering of CD47 Ag in primary cultures lead to a state of hyporesponsiveness of T cells in secondary cultures (not detailed).

Anti-CD47 mAbs specifically suppress IgE synthesis without significantly affecting B cell proliferation.

Since resting B cells strongly express CD47 Ag, the applicant examined the effect of CD47 ligation on B cell proliferation and differentiation. Purified tonsillar B cells were stimulated by trimeric soluble CD40L (sCD40L) in the presence of IL-4. As shown in Fig. 20 and table V, anti-CD47 mAbs did not interfere with B cell proliferation as measured by ^3H thymidine uptake at day 5, while they strongly inhibited in a dose-dependent manner (not shown) IL-4-induced IgE synthesis. Moreover, anti-CD47 mAbs were likely to block IgE-class switching since they also suppressed IgE synthesis by IL-4-stimulated naive ($sIgM^+$ $sIgD^+$) B cells and inhibited the expression of germ line ϵ transcripts (not shown). The inhibitory effect was not reversed by addition of neutralizing mAbs to TGF-β, a potent inhibitor of Ig synthesis nor by IL-6 (not shown). Taken together, anti-CD47 mAbs appeared as strong anti-inflammatory agents since they also interfered with the humoral response of the allergic reaction.

IL-12 is a potent proinflammatory and immunoregulatory cytokine which plays a crucial role in innate and adaptive Th1 response. IL-12 is released during the early stage of infection caused by a large variety of bacteria, intracellular pathogens, fungi and certain viruses. IL-12 is also produced in the absence of

infection, following interaction of $CD4^+$ T and dendritic cells/monocytes. It has been reported that Th1 response associated with chronic or autoimmune disease remains IL-12-dependent. Indeed, IL-12 neutralization (by anti-IL-12 Ab) reveals to be an effective treatment of experimental bowel disease, auto-immune encephalitis and insulin-dependent diabetes.

Therefore, the inhibition by CD47 ligation of proinflammatory cytokine (including IL-12) production by monocytes and IL-12 responsiveness by T cells could permit to downregulate inflammatory response on which new therapeutic strategies to chronic disorders could be based.

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Table 1 : Cellular distribution of 10G2 antigen on human cell lines

Freshly isolated human leucocytes			Human cell lines		
	sCD23	10G2		sCD23	10G2
T cells	+++	++++	T cell lines (Jurkat, CEM, HUT 78)	++	++++
B cells	++	++	B cell lines (RPMI 8226, Raji, WIL-2, Daudi)	++	++
Monocytes	+	+	Monocyte cell lines (U937)	++	++
			THP-1	-	-
Erythrocytes	-	-	Erythroleukemia cell lines (K562 / K562-CR2)	+	+

TABLE II

EFFECT OF THROMBOSPONDIN ON IL-2-INDUCED IFNγ PRODUCTION IN T CELLS/MONOCYTES COCULTURES SYSTEM.

	IFNγ (ng/ml)		
Exp. No.	**T + Mono + IL-2**	**T + Mono + IL-2 + TSP**	**% INH**
1	18.4	3.7	79.6
2	11.2	7.0	37.5
3	26.0	10.6	59.0
4	10.5	1.0	89.5
5	13.3	5.5	58.3

TABLE III

SELECTIVE INHIBITION OF IL-12 P70 RELEASE BY THROMBOSPONDIN.

	IL-12 (pg/ml)			TNFα (ng/ml)		
	Medium	TSP	(% INH)	Medium	TSP	(% INH)
Exp. 1	168	79	53	120	147	0
Exp. 2	1,020	746	27	64	83	0
Exp. 3	2,120	1090	49	87	105	0

Monocytes ($1x10^6$/ml) were stimulated overnight with SAC (0.101%) and IFNγ (500 U/ml) in the absence or presence of thrombospondin (10 μg/ml). Three representative experiments out of six.

TABLE IV (Appendix to Fig. 14a)

Effect of 10G2 and B6H12 mAbs on sCD23-induced TNFα production by purified monocytes.

	TNFα (pg/ml)	
	Cont mAb	**10G2**
Exp. 1	993	347
Exp. 2	1392	483
Exp. 3	396	110
	Cont mAb	**B6H12**
Exp. 4	845	347
Exp. 5	1177	166
Exp. 6	1152	252

Monocytes were stimulated overnight with sCD23 (25 ng/ml) in the presence of anti-CD47 mAbs (clone 10G2 or B6H12) or isotype-matched cont mAbs. TNFα was measured in the CSN by specific ELISA.

29

TABLE V (Appendix to Fig. 20)

Effect of 10G2 and B6H12 mAb on IL-4-induced IgE synthesis by CD40-activated B cells.

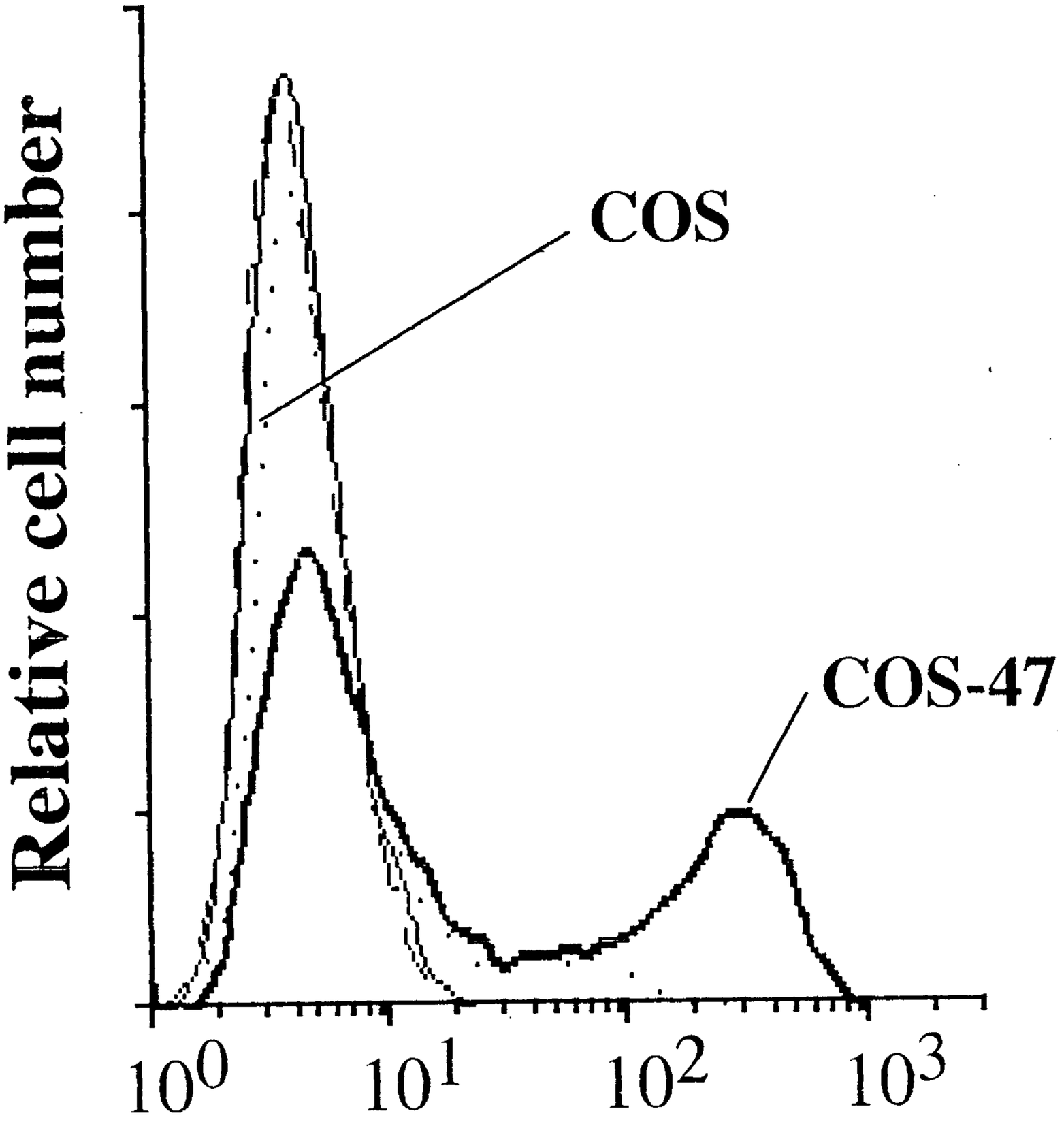
	^{3}H-Thymidine Uptake (X10^3 CPM)		IgE (ng/ml)	
A	**Cont mAb**	**10G2**	**Cont mAb**	**10G2***
Exp. 1	2.8	3.9	51	26
Exp. 2	33	42	31	15
Exp. 3	47	48	79	28
B	**Cont mAb**	**B6H12**	**Cont mAb**	**B6H12***
Exp. 4	4.8	4.5	23	4.5
Exp. 5	87.1	60.4	97	18
Exp. 6	77.1	63.1	80	27.5

* 10G2 mAb and B6H12 mAbs are used at 20 μg/ml and 10 μg/ml respectively.

Fig 1

Fig. 1

30



21

CHO-47

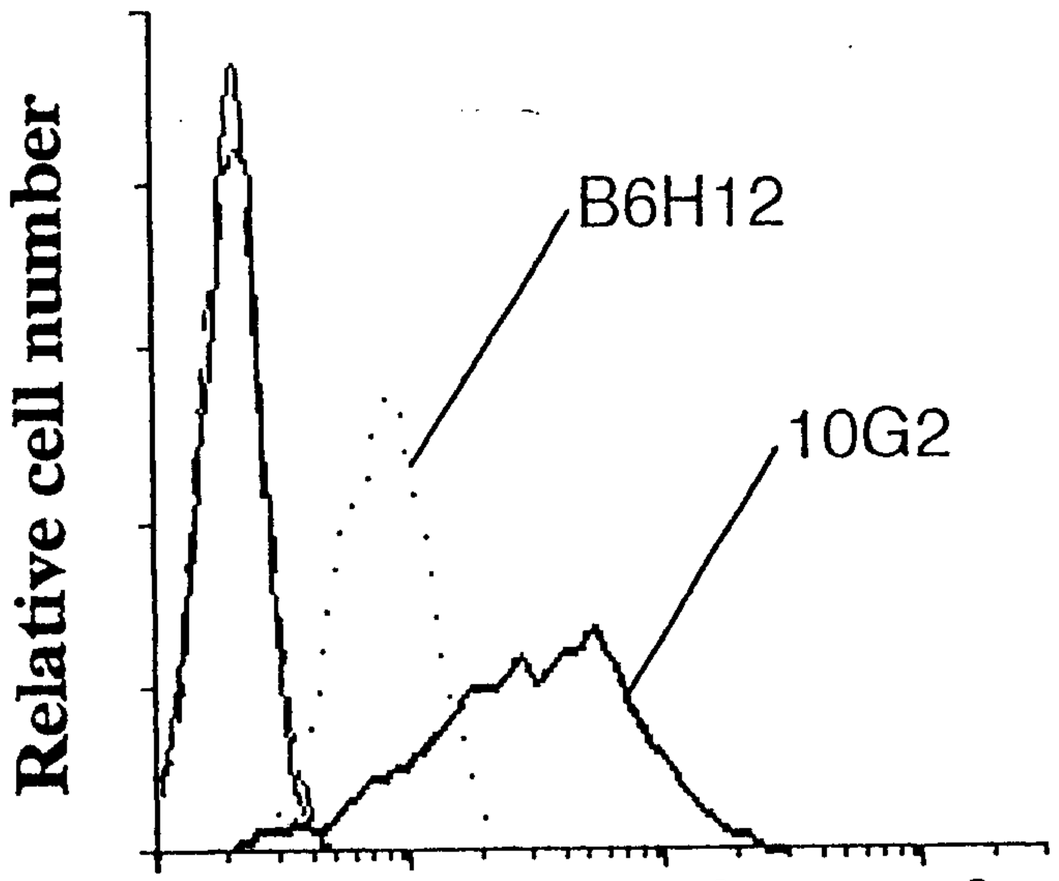


Fig 3

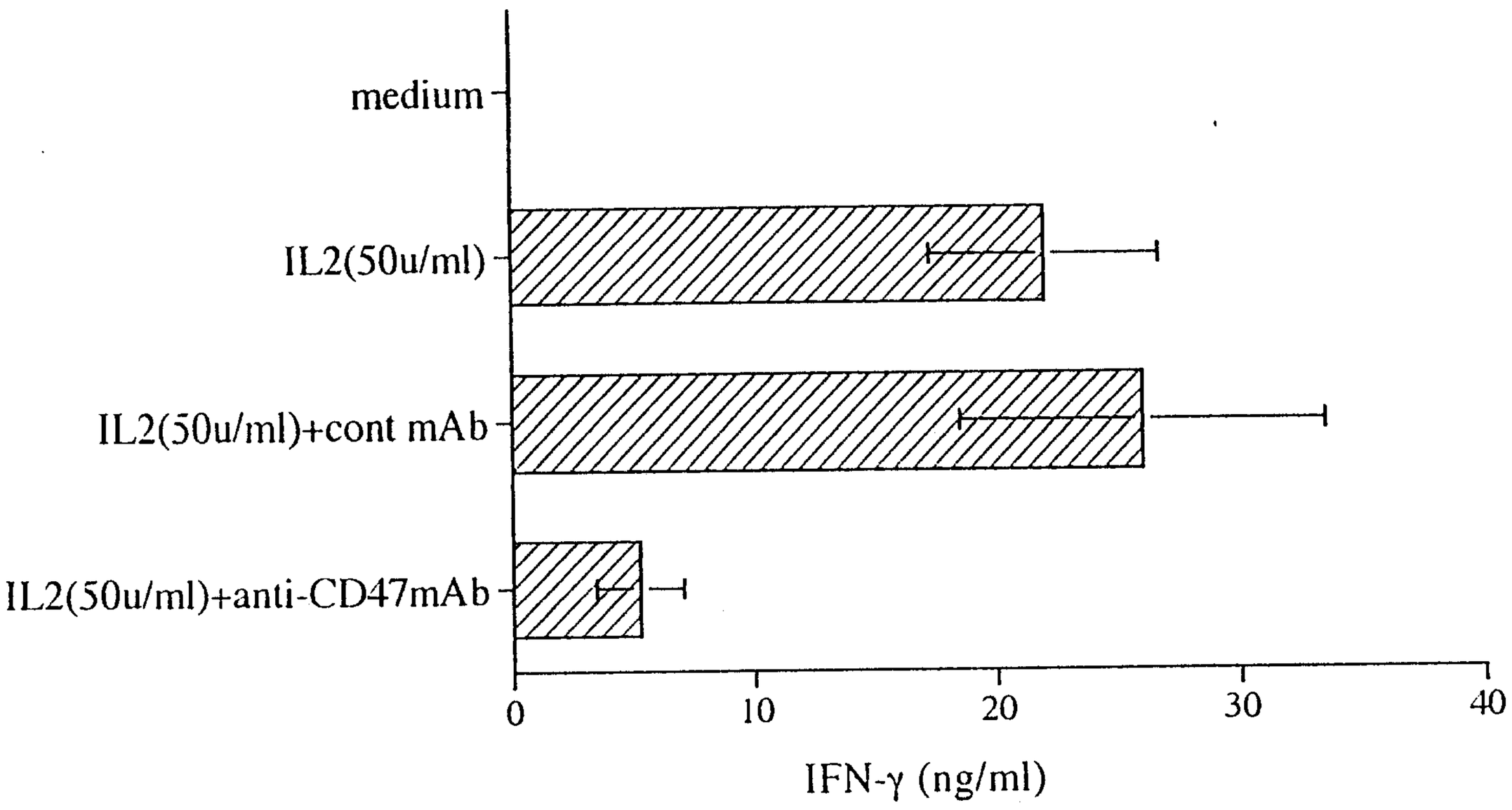
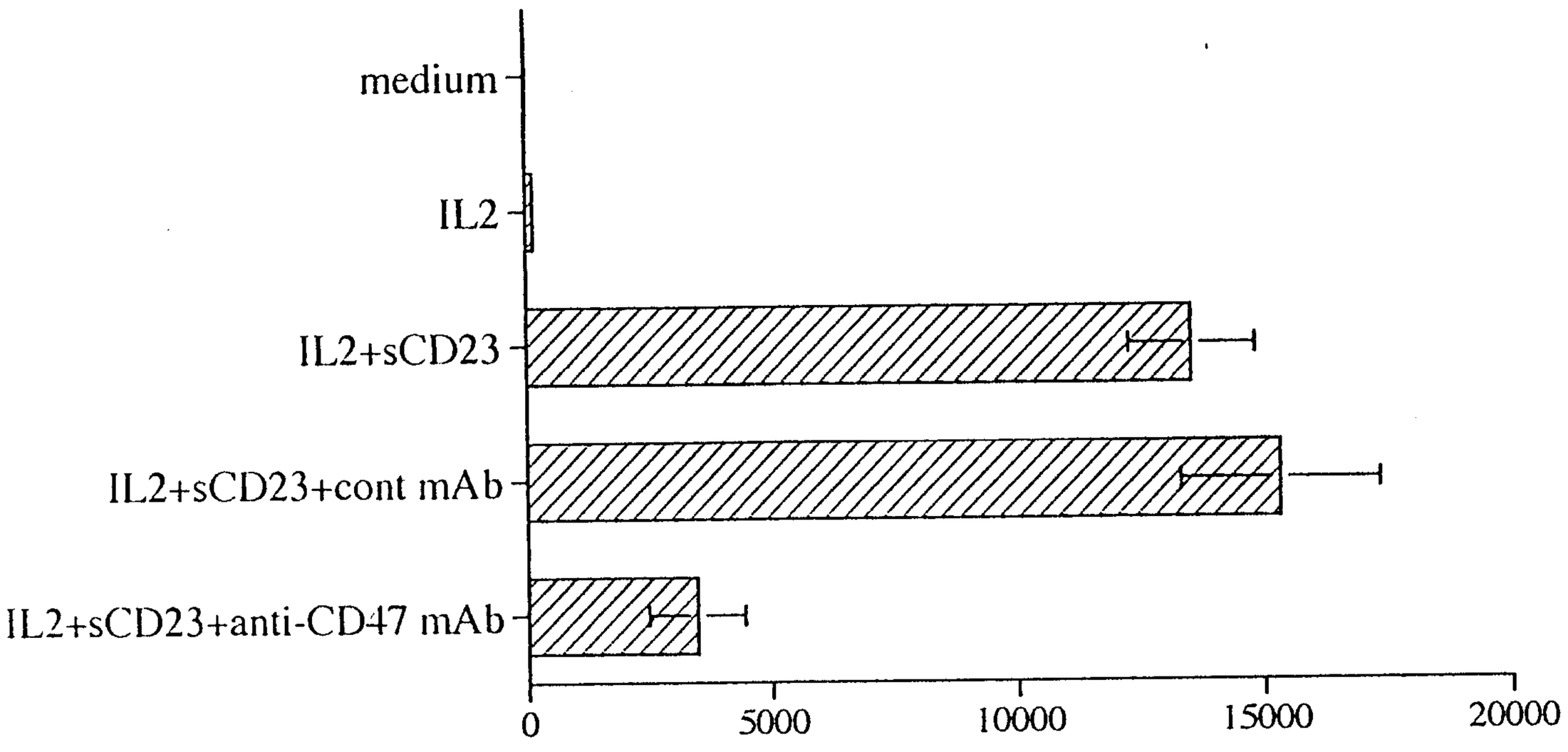

medium
IL2(50u/ml)
IL2(50u/ml)+cont mAb
IL2(50u/ml)+anti-CD47mAb
0
10
20
30
40
IFN-γ (ng/ml)

Fig 4


medium
IL2
IL2+sCD23
IL2+sCD23+cont mAb
IL2+sCD23+anti-CD47 mAb
0
5000
10000
15000
20000
IFN-γ production (pg/ml)

34

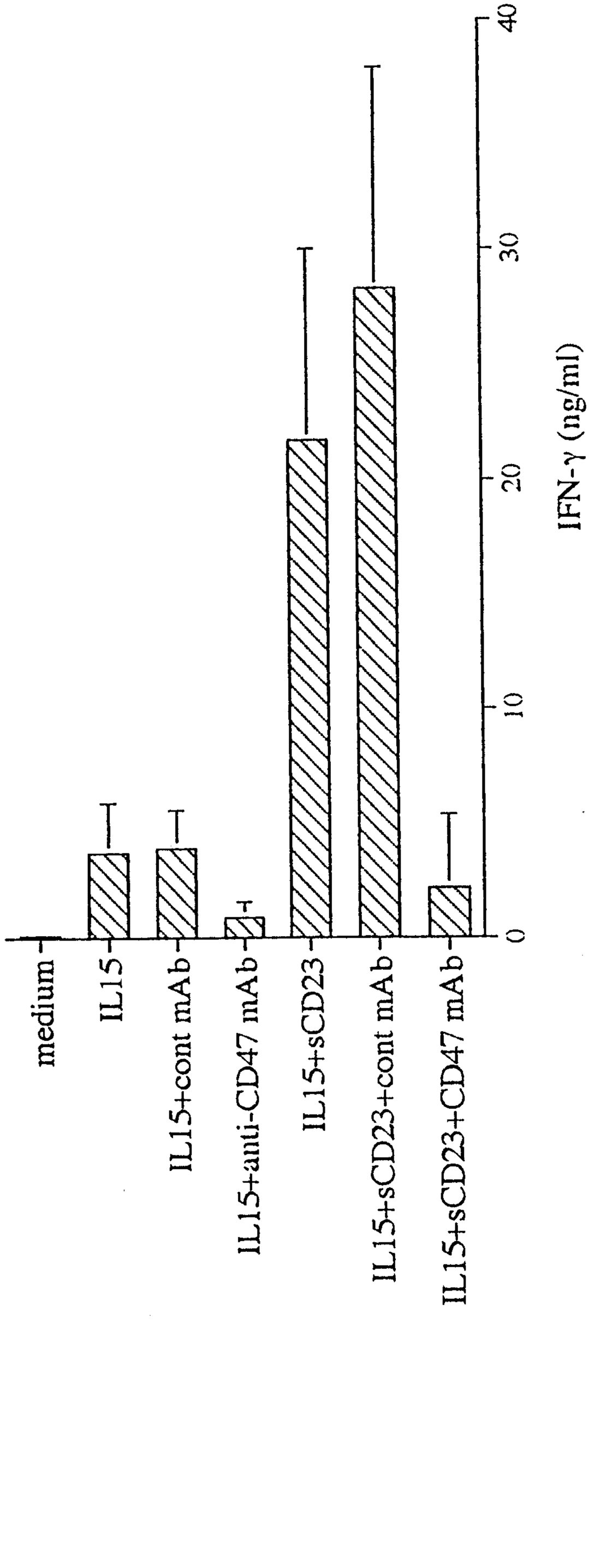

medium
IL15
IL15+cont mAb
IL15+anti-CD47 mAb
IL15+sCD23
IL15+sCD23+cont mAb
IL15+sCD23+CD47 mAb
0
10
20
30
40
IFN-γ (ng/ml)

Fig 6

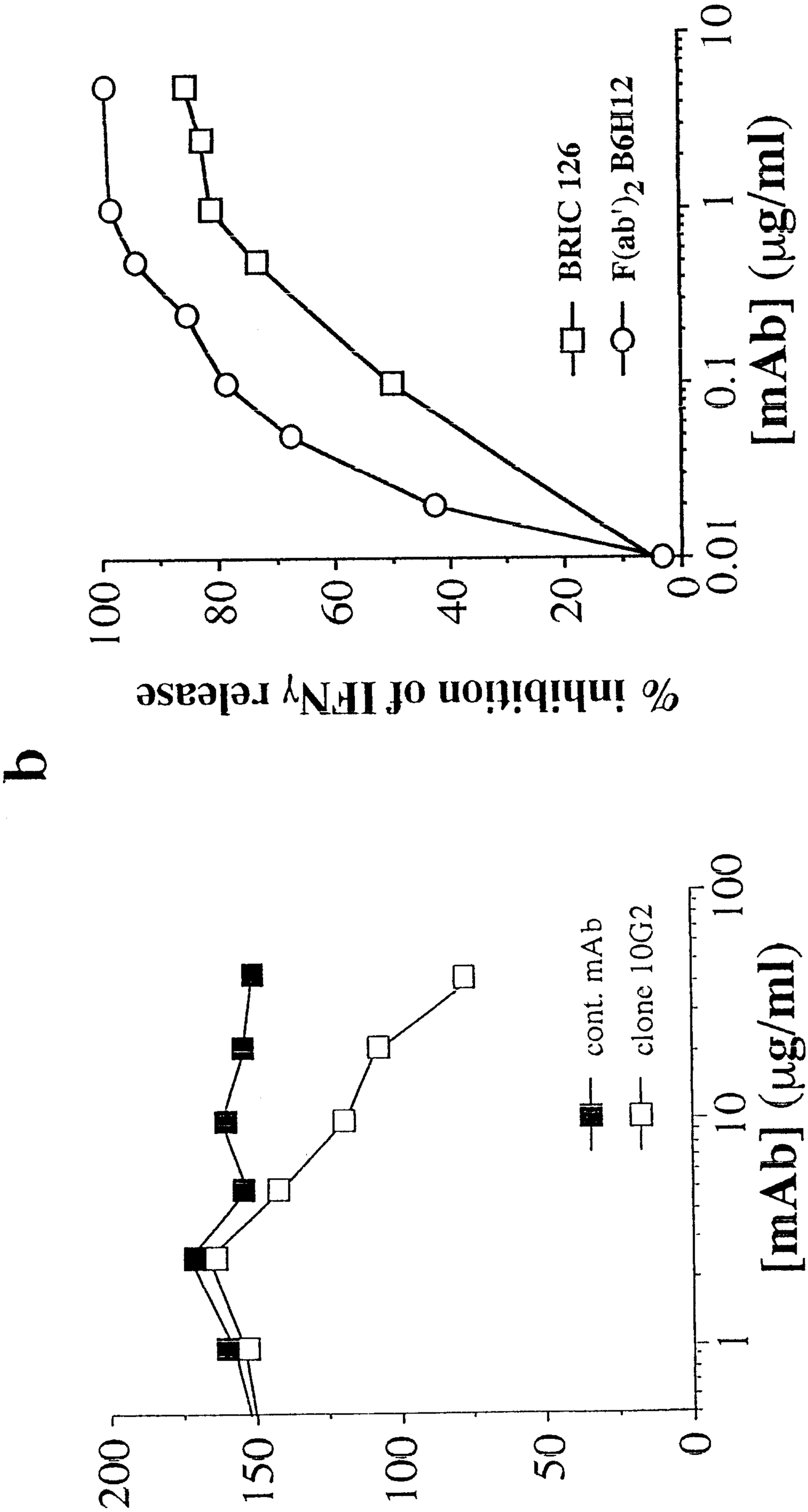
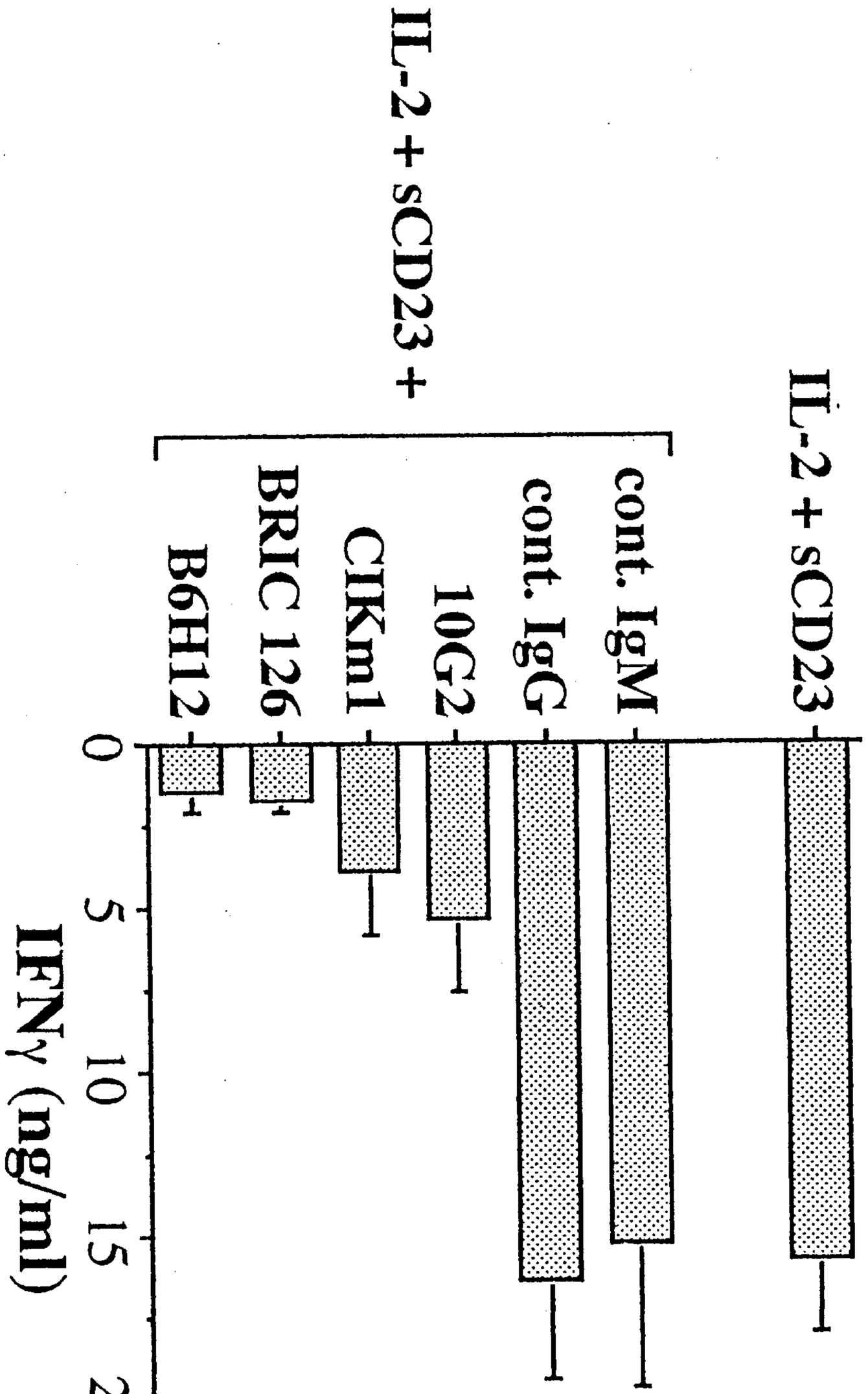

b
% inhibition of IFNγ release
100
80
60
40
20
0
0.01
0.1
1
10
[mAb] (µg/ml)
BRIC 126
F(ab')2 B6H12
a
IFNγ production (ng/ml)
200
150
100
50
0
1
10
100
[mAb] (µg/ml)
cont. mAb
clone 10G2

Fig 6C


IL-2 + sCD23
IL-2 + sCD23 +
cont. IgM
cont. IgG
10G2
CIKm1
BRIC 126
B6H12
IFNγ (ng/ml)
0
5
10
15
20

37

Fig 7

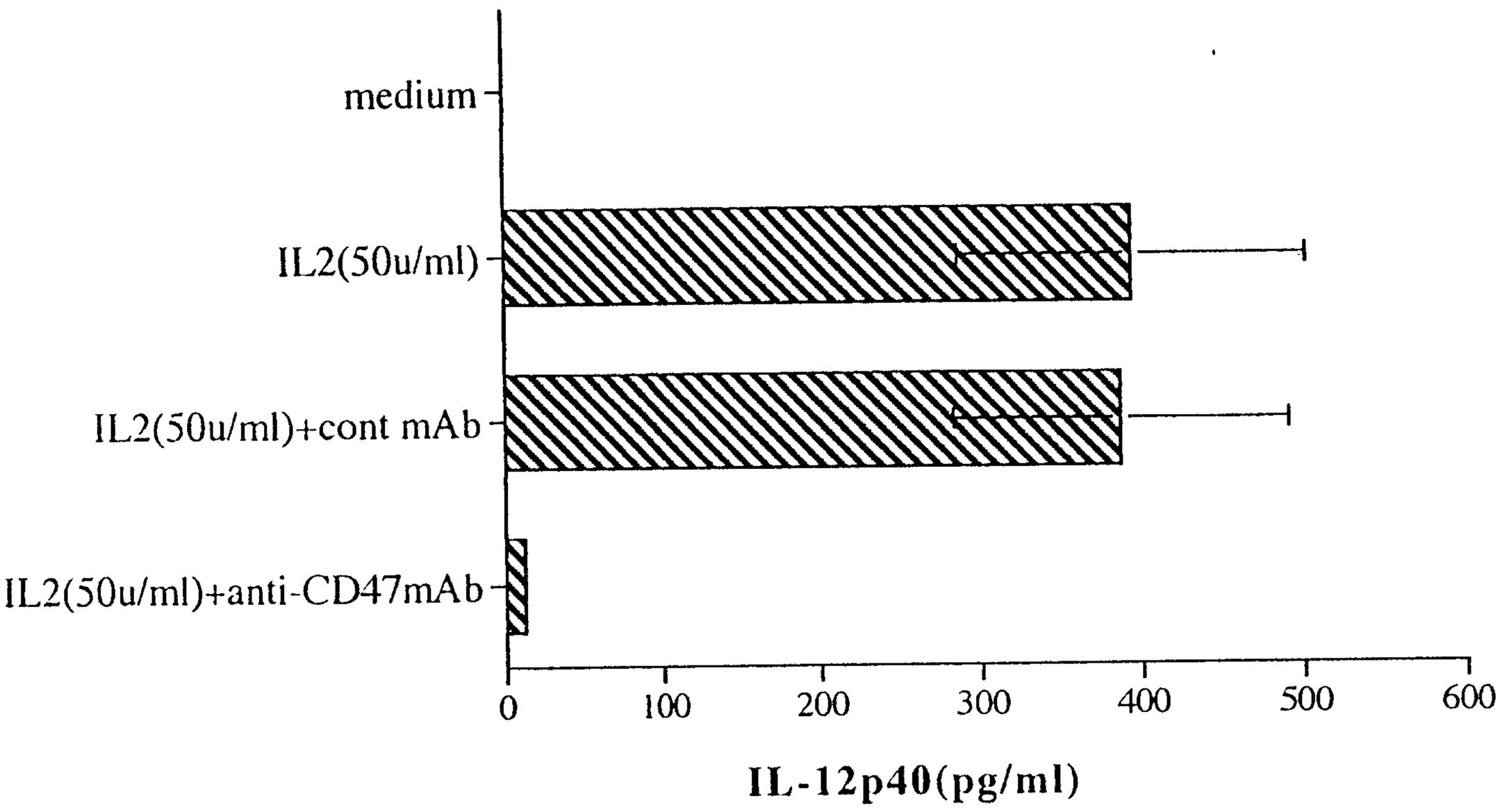

medium
IL2(50u/ml)
IL2(50u/ml)+cont mAb
IL2(50u/ml)+anti-CD47mAb
0
100
200
300
400
500
600
IL-12p40(pg/ml)

Fig 8

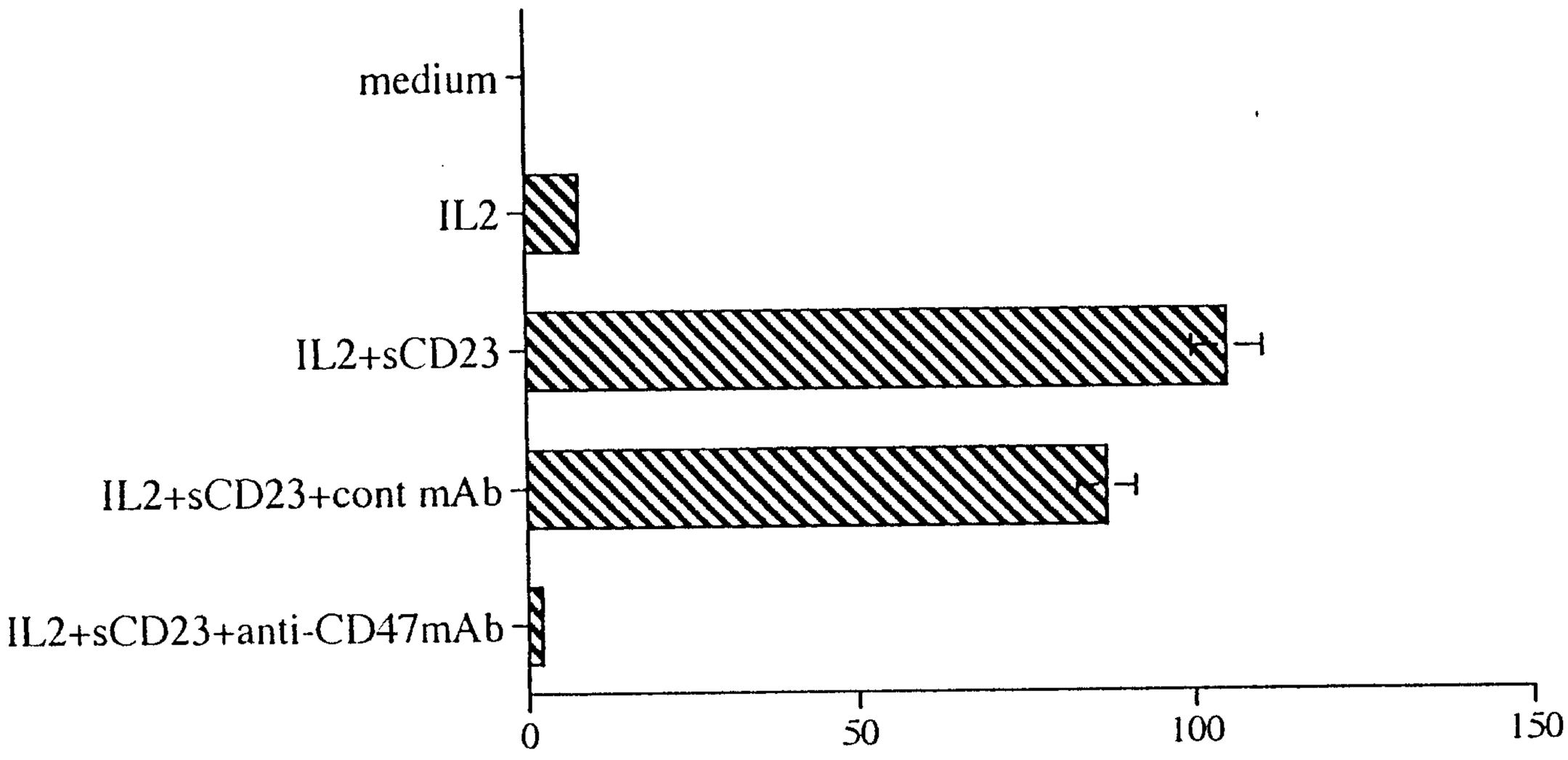
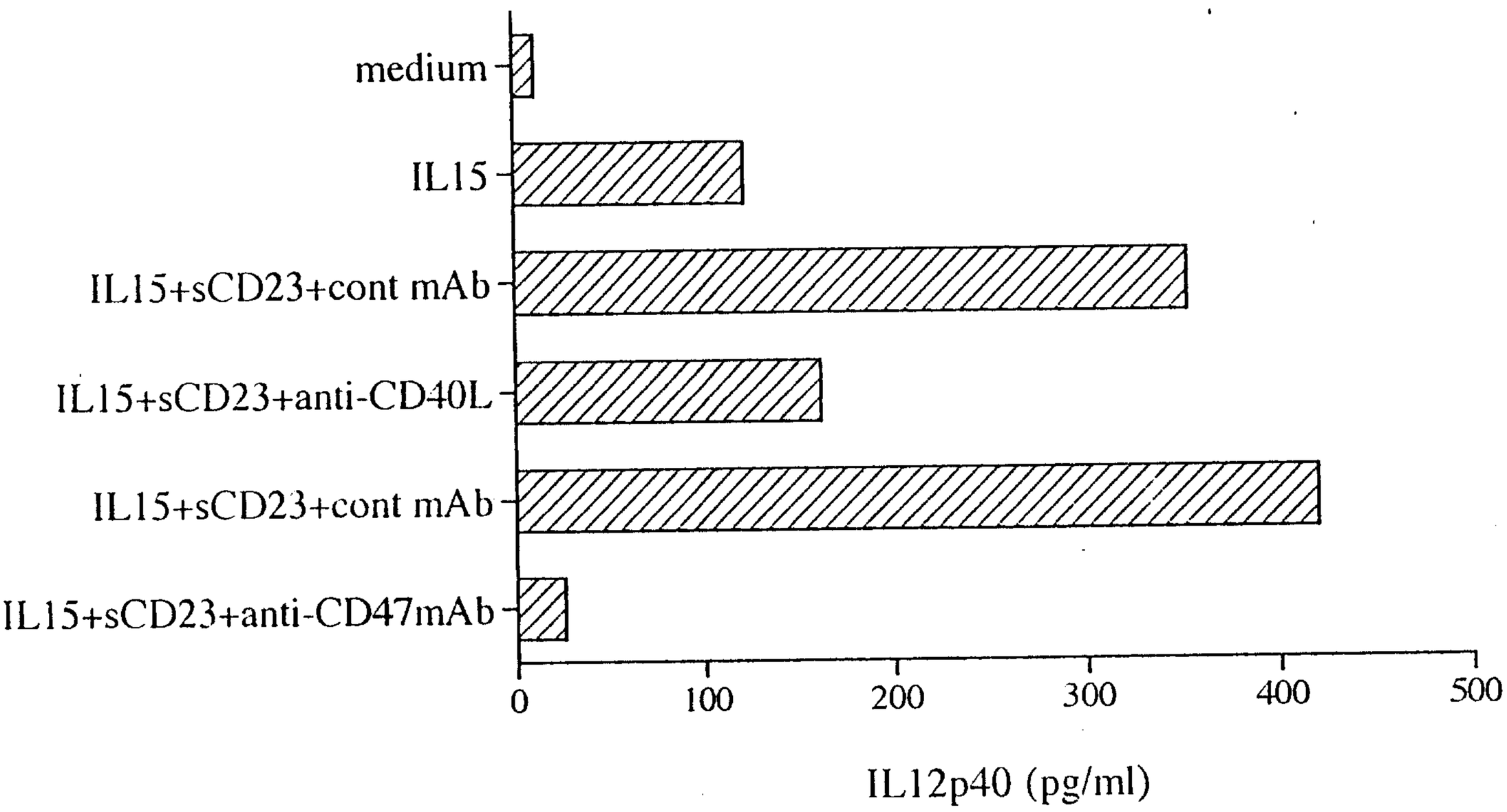

medium
IL2
IL2+sCD23
IL2+sCD23+cont mAb
IL2+sCD23+anti-CD47mAb
0
50
100
150
IL-12 p40(pg/ml)

Fig 3


medium
IL15
IL15+sCD23+cont mAb
IL15+sCD23+anti-CD40L
IL15+sCD23+cont mAb
IL15+sCD23+anti-CD47mAb
0
100
200
300
400
500
IL12p40 (pg/ml)

40

Fig 10

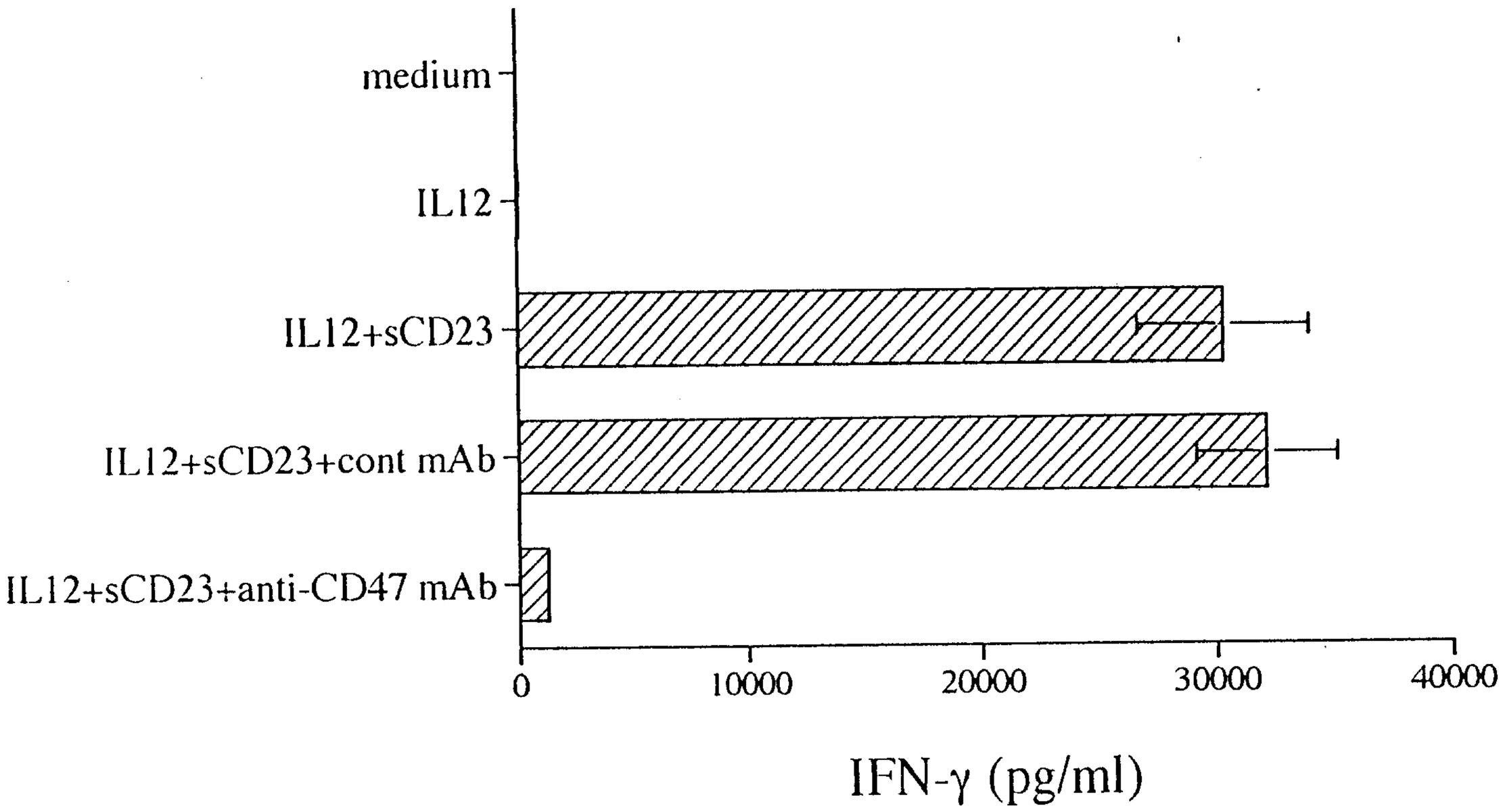

medium
IL12
IL12+sCD23
IL12+sCD23+cont mAb
IL12+sCD23+anti-CD47 mAb
0
10000
20000
30000
40000
IFN-γ (pg/ml)

Fig 11

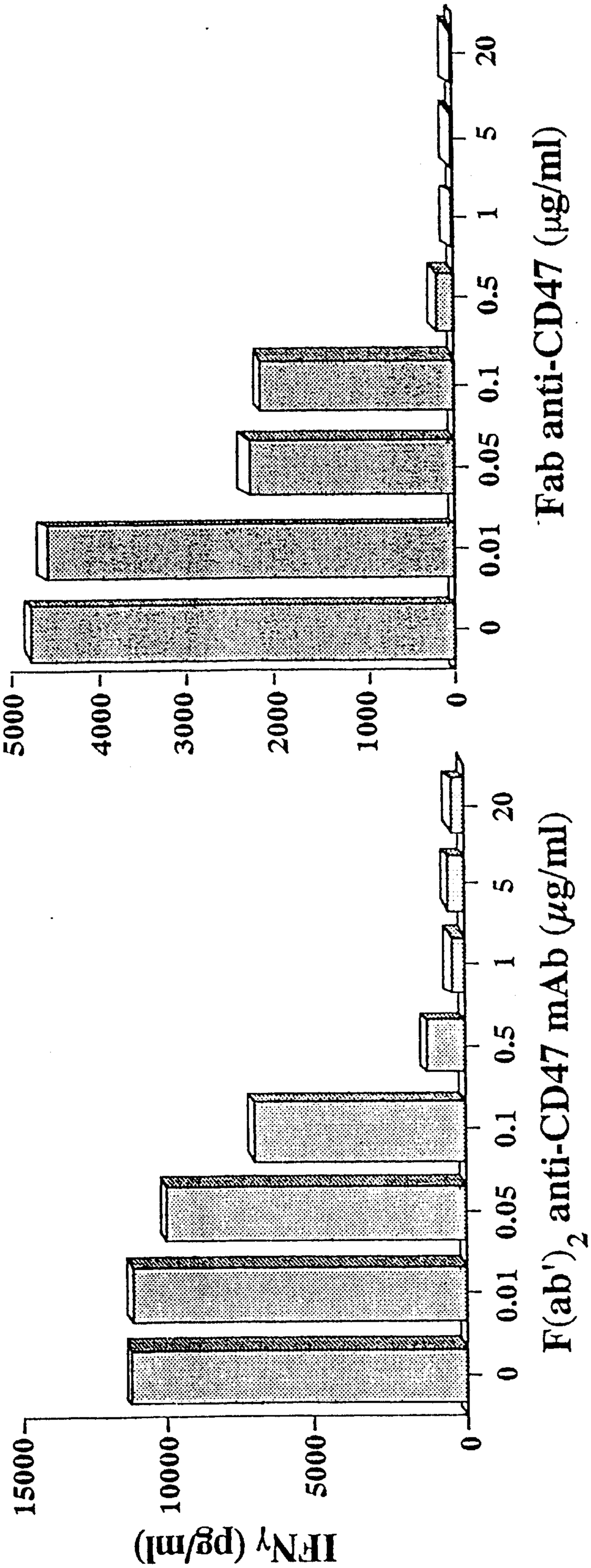

Dose-dependent inhibition of IL-2-induced IFNγ production in T cell/monocytes cocultures by anti-CD47 mAb
IFNγ (pg/ml)
15000
10000
5000
0
0
0.01
0.05
0.1
0.5
1
5
20
F(ab')2 anti-CD47 mAb (μg/ml)
0
0.01
0.05
0.1
0.5
1
5
20
Fab anti-CD47 (μg/ml)

Fig 12

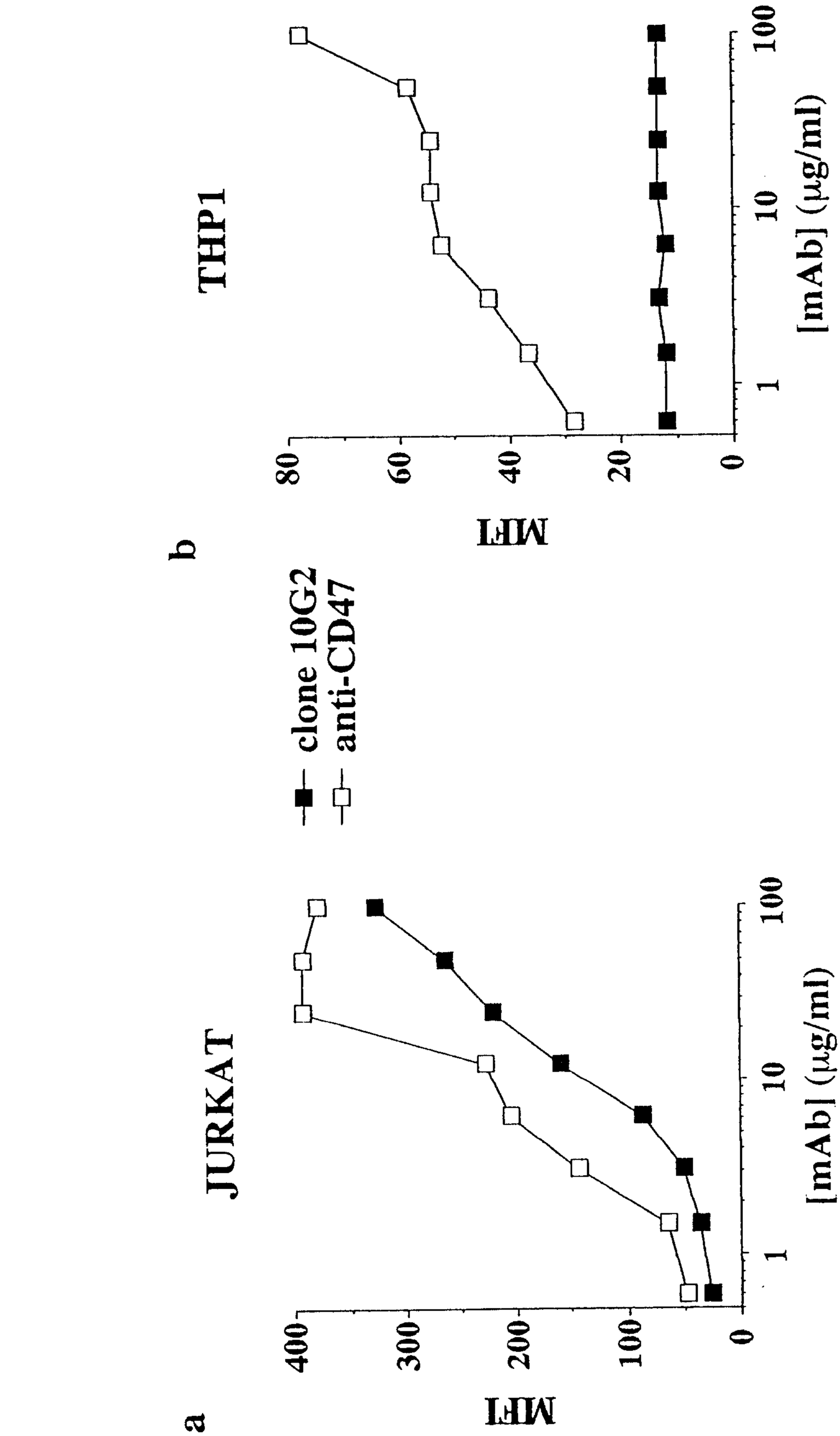
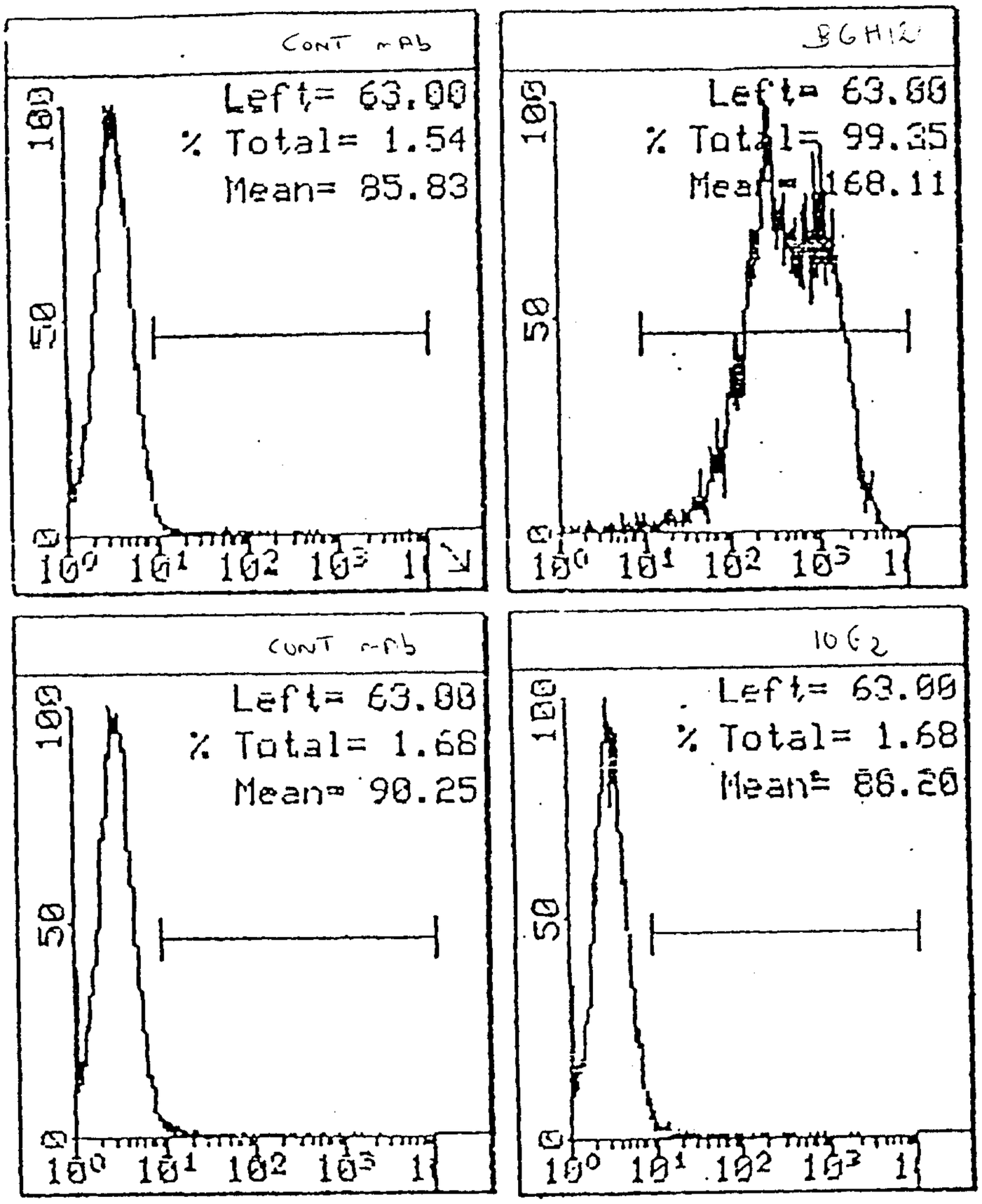

a
JURKAT
MFI
400
300
200
100
0
1
10
100
[mAb] (µg/ml)
clone 10G2
anti-CD47
b
THP1
MFI
80
60
40
20
0
1
10
100
[mAb] (µg/ml)

Fig 13 a

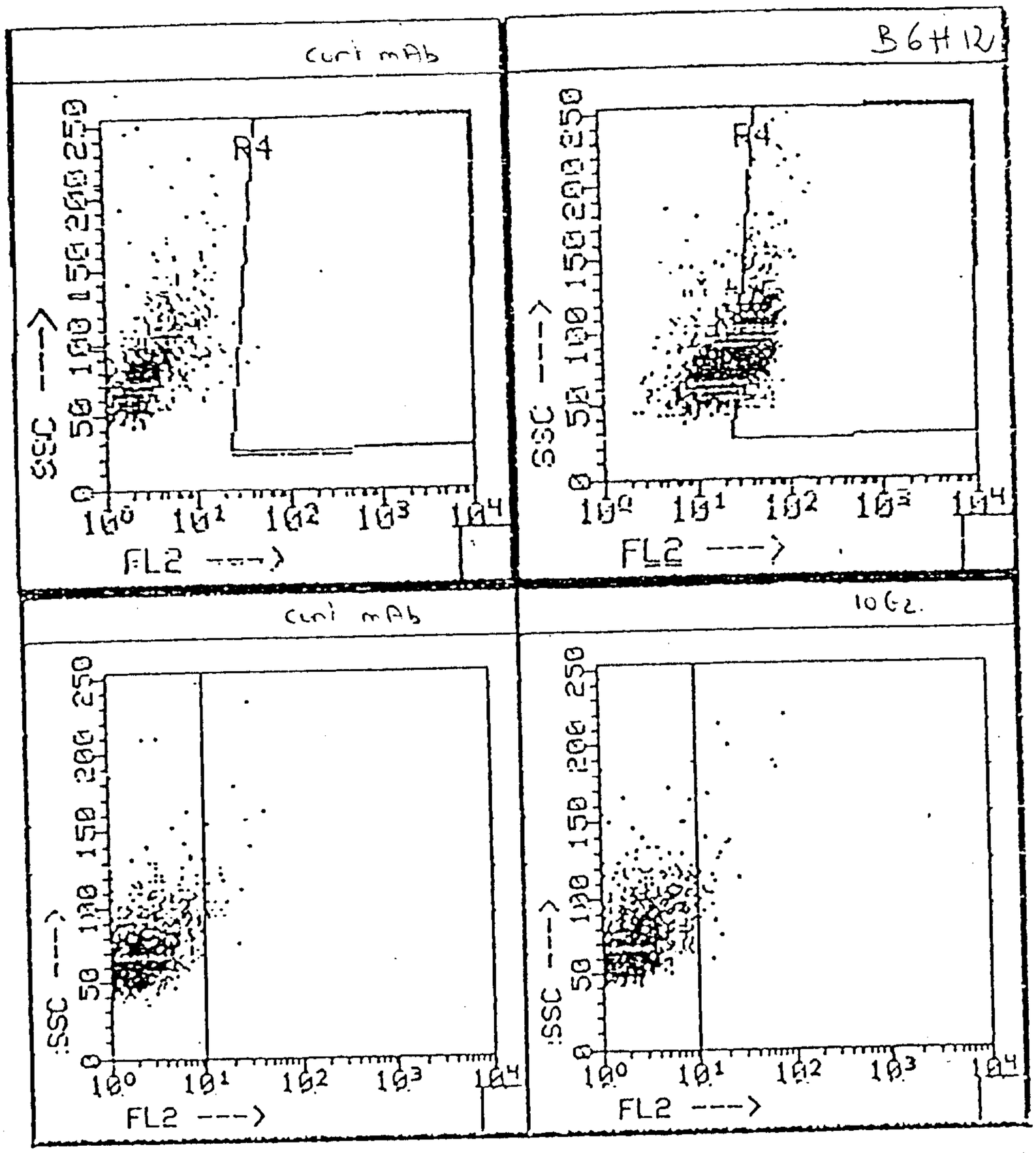
ERYTHROCYTES



44

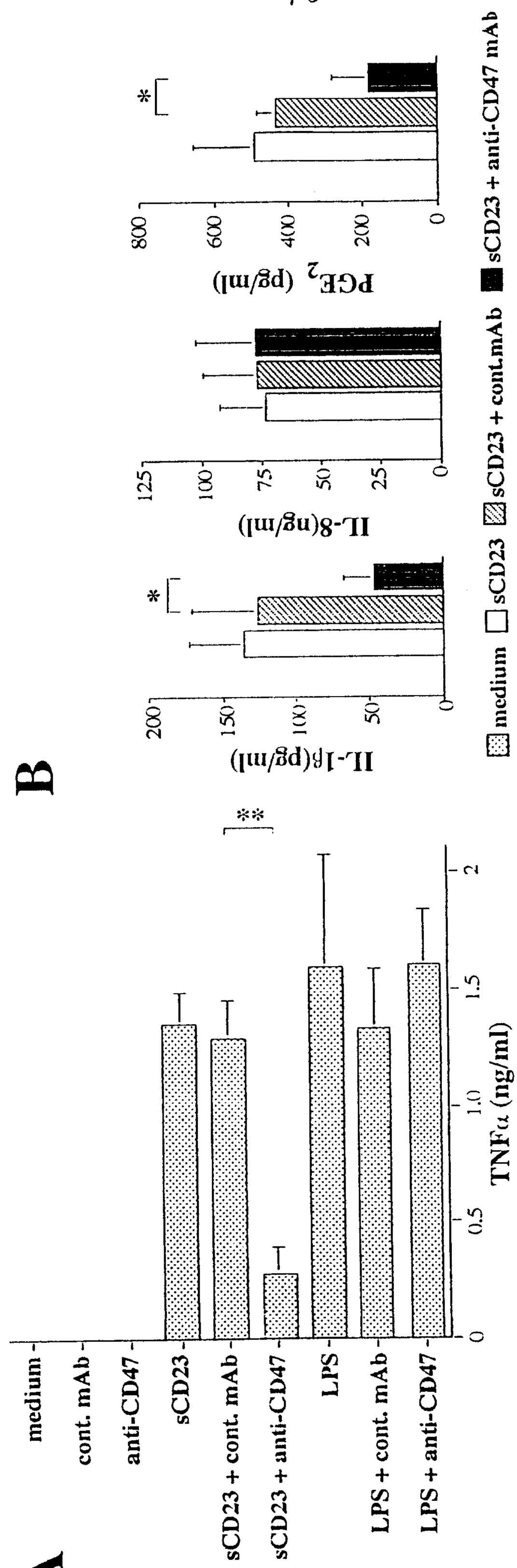
Fig 13 b.

DENDRITIC CELLS



45

Fig 14


A
medium
cont. mAb
anti-CD47
sCD23
sCD23 + cont. mAb
sCD23 + anti-CD47
LPS
LPS + cont. mAb
LPS + anti-CD47
TNFα (ng/ml)
0
0.5
1.0
1.5
2
B
IL-1β(pg/ml)
**
sCD23 + anti-CD47 mAb
medium
sCD23
sCD23 + contLmAb
IL-8(ng/ml)
*
PGE2 (pg/ml)
*

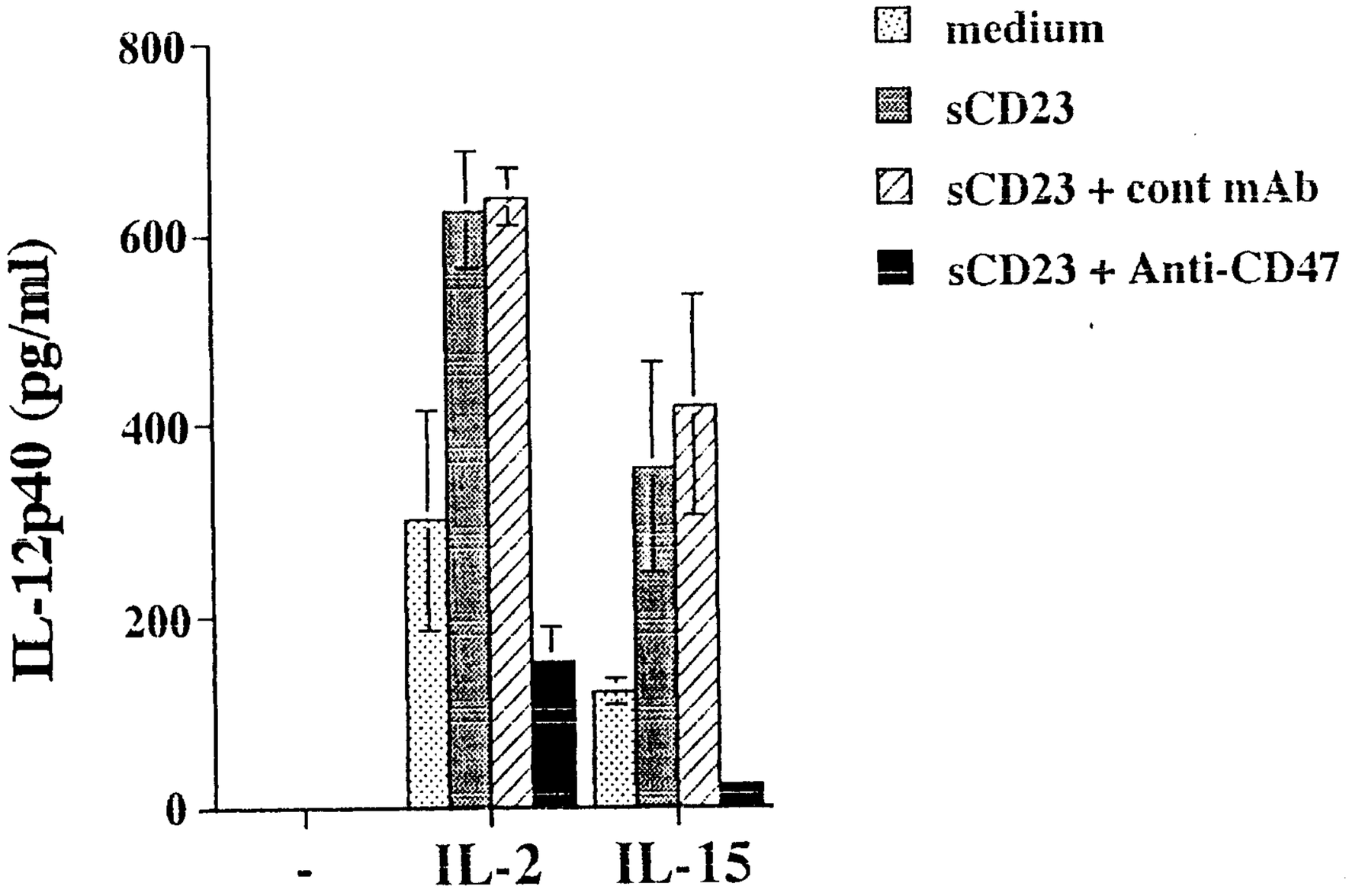
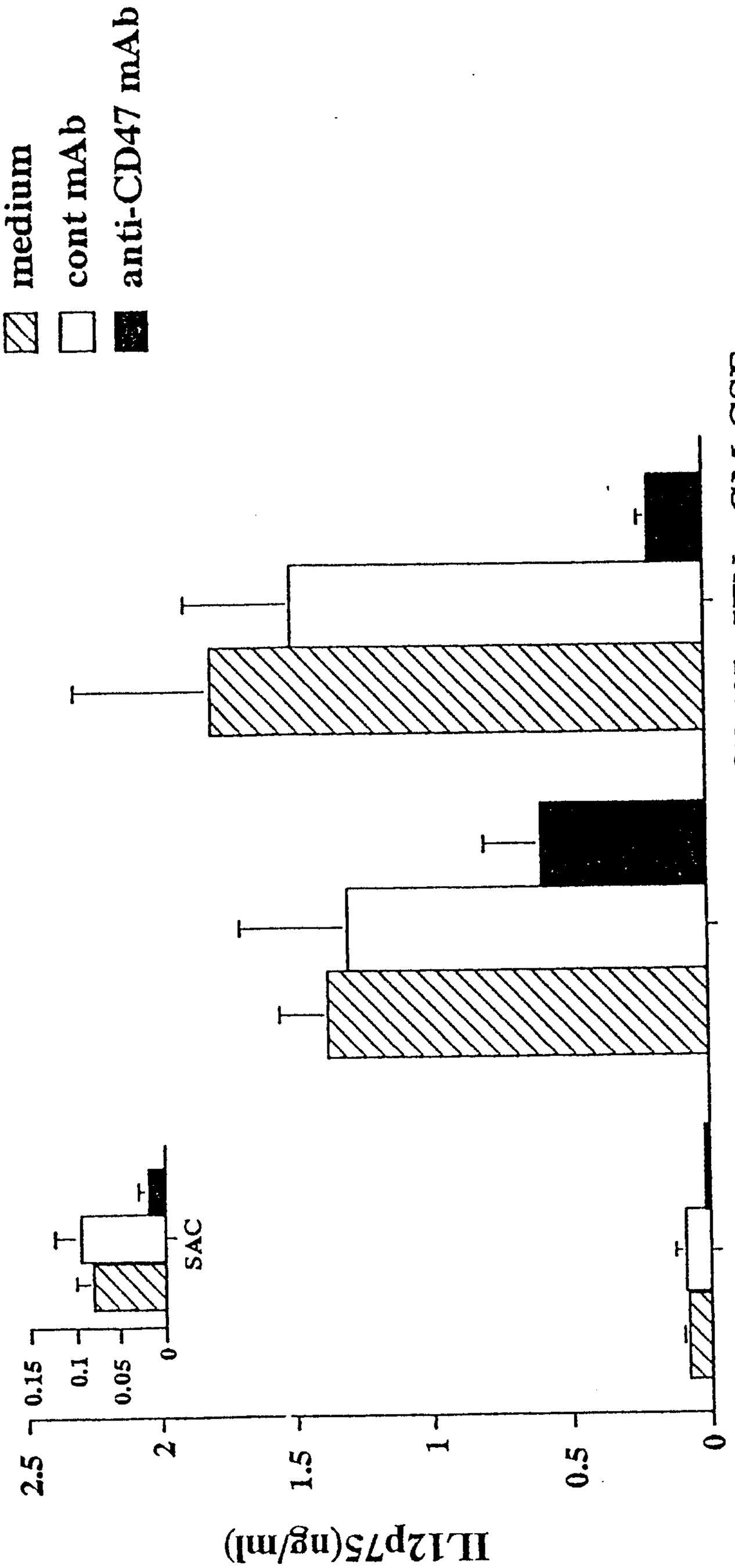

IL-12p40 (pg/ml)
800
600
400
200
0
-
IL-2
IL-15
medium
sCD23
sCD23 + cont mAb
sCD23 + Anti-CD47

Fig 16

Anti-CD47 mAb inhibits IL12p75 prodùction induced by T-independent and T-dependènt stimuli



Effect of anti-CD47 mAb on monkine release

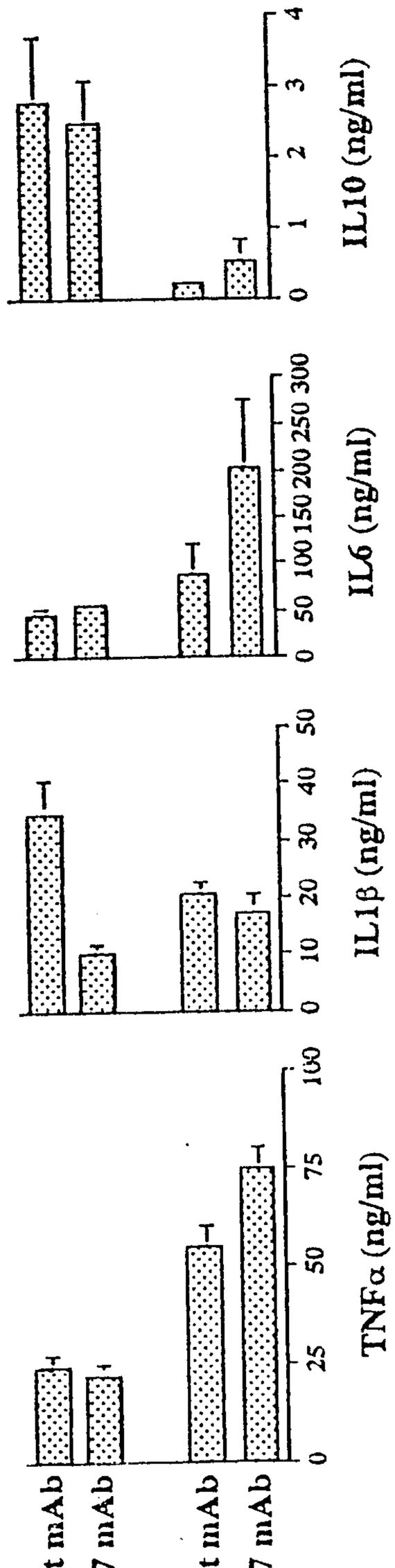


Fig 18

Effect of anti-CD47 mAb on anti-CD3-activated purified T cells

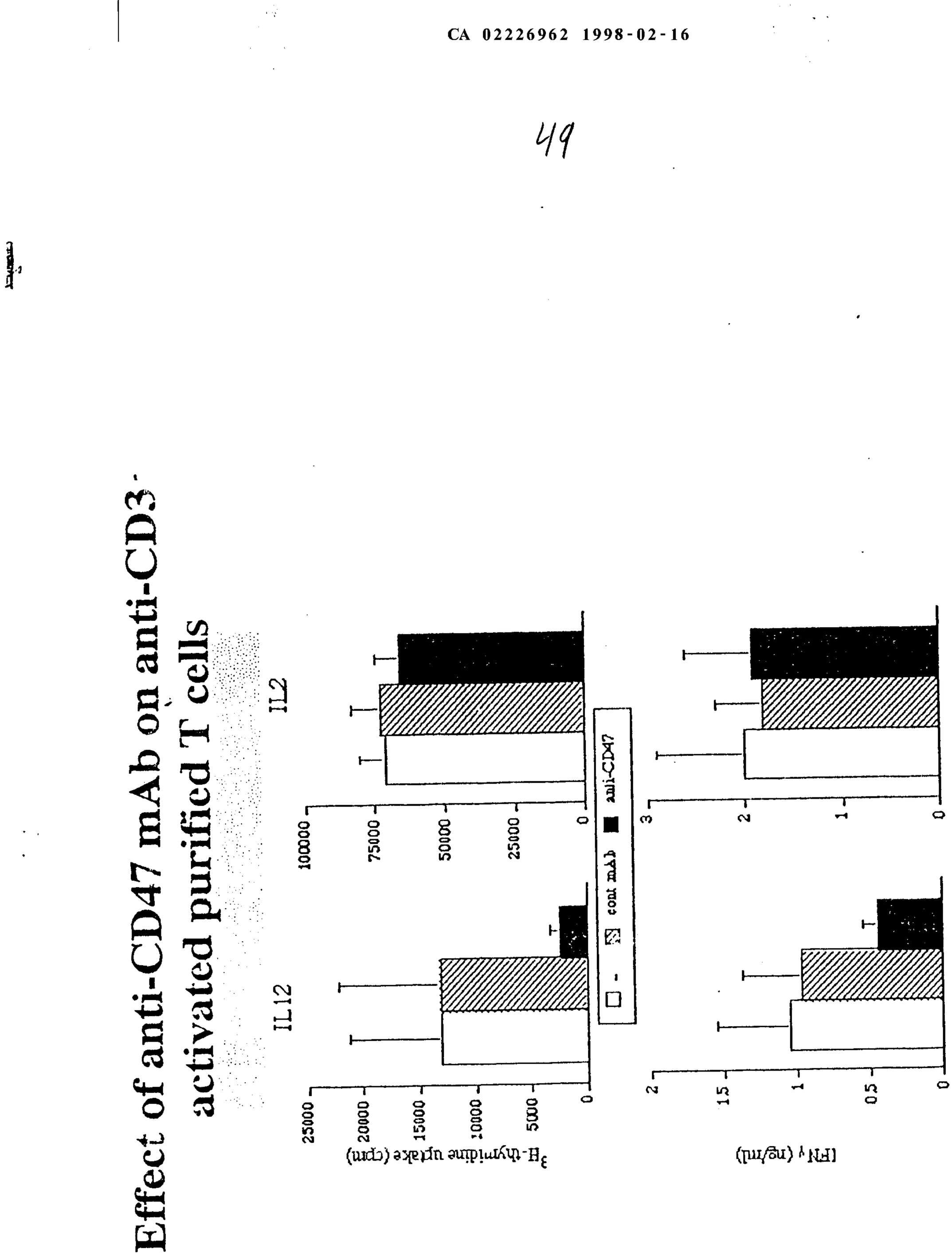
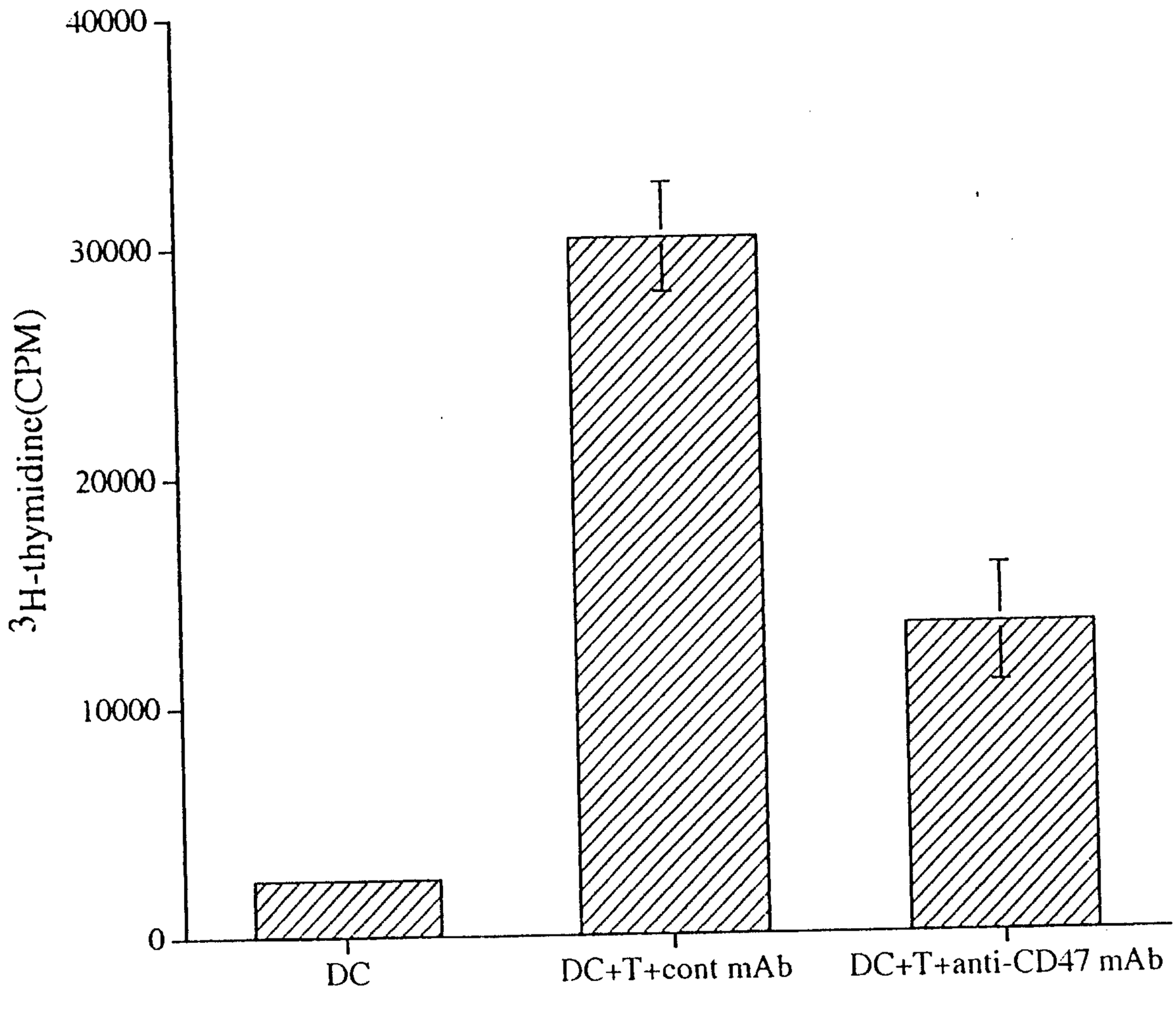
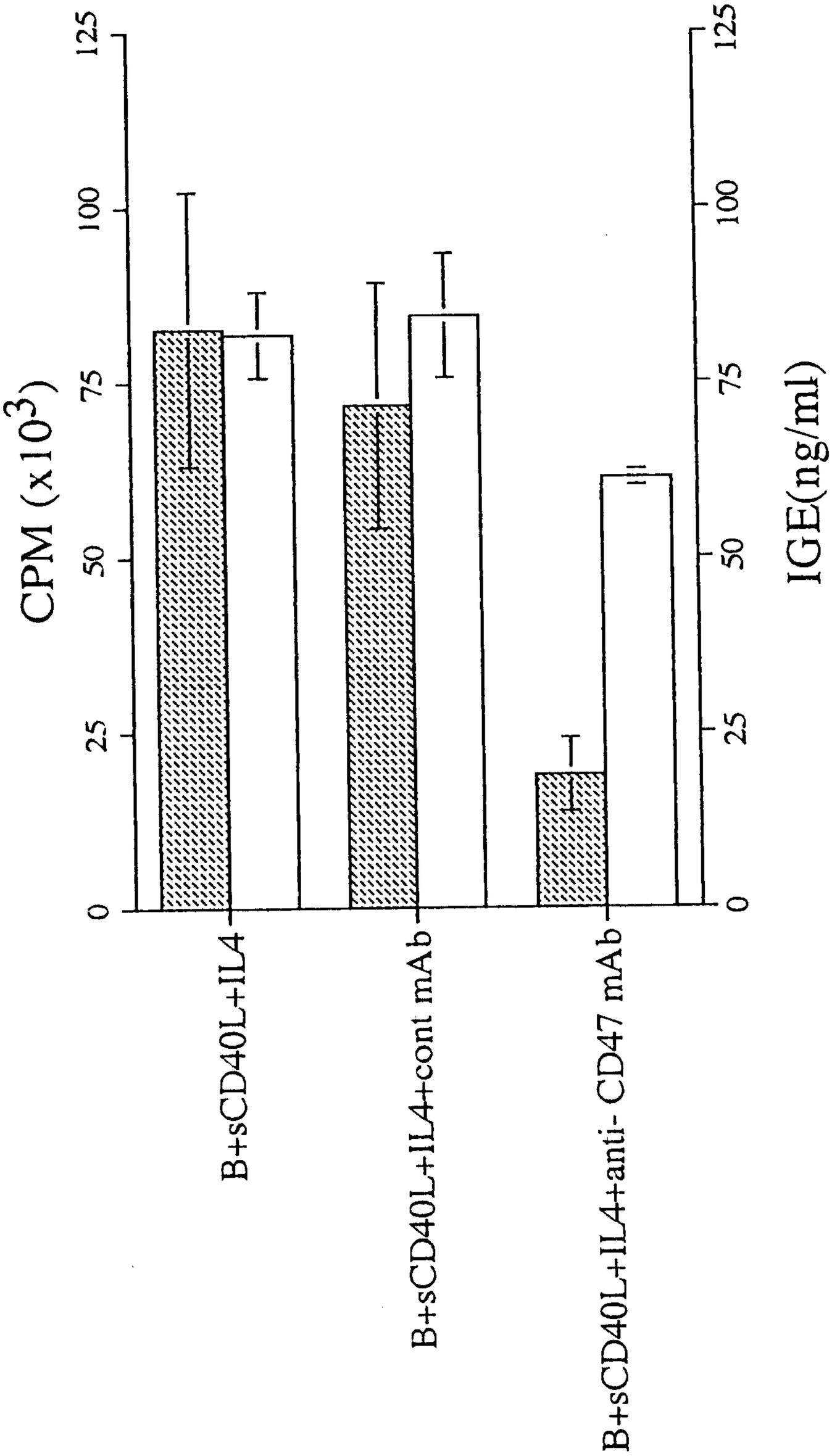


Fig 19


40000
30000
20000
10000
0
^{3}H-thymidine(CPM)
DC
DC+T+cont mAb
DC+T+anti-CD47 mAb

51

Fig 20


CPM (x10^3)
0
25
50
75
100
125
IGE(ng/ml)
0
25
50
75
100
125
B+sCD40L+IL4
B+sCD40L+IL4+cont mAb
B+sCD40L+IL4+anti- CD47 mAb