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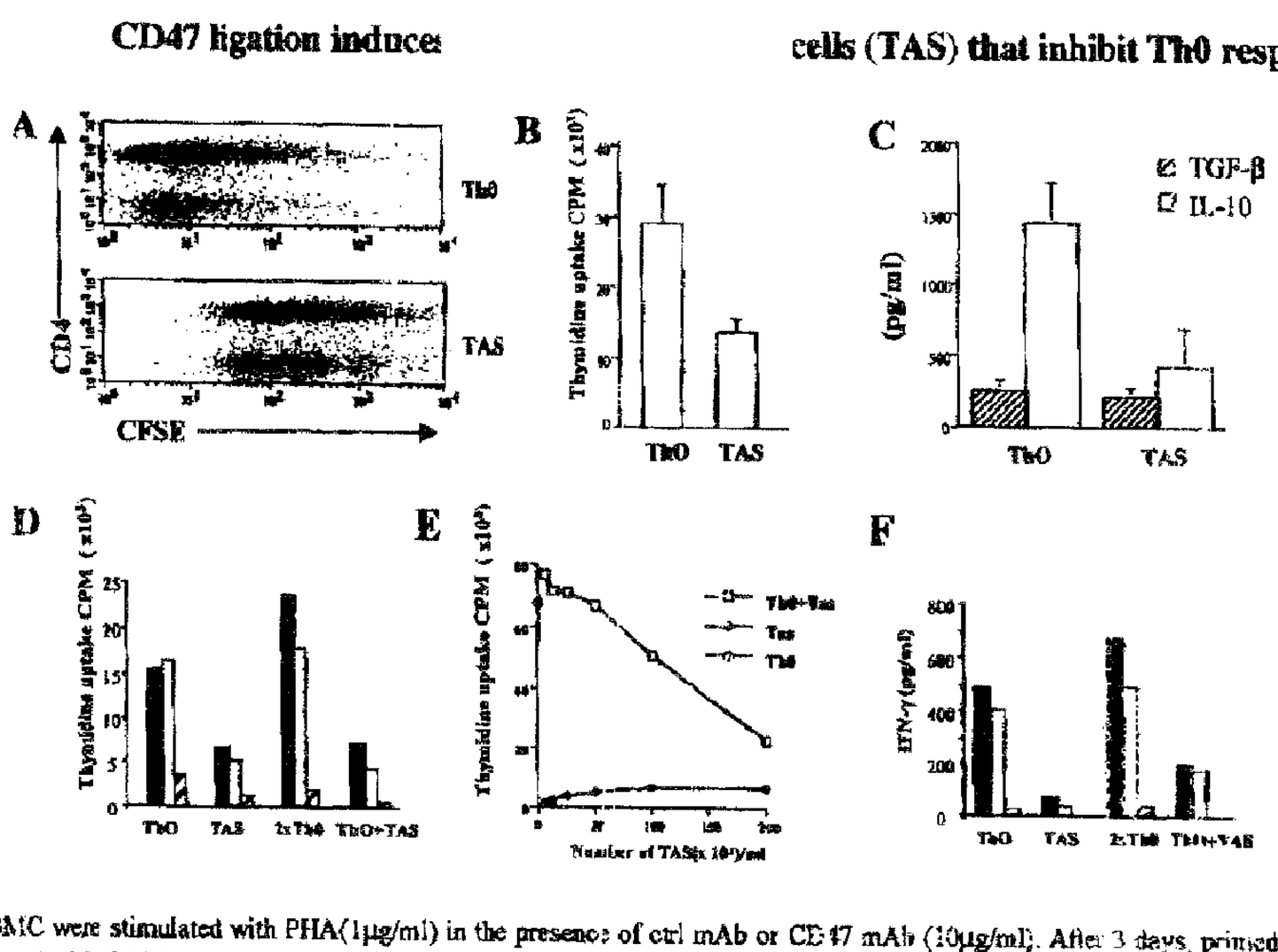
(54) Titre : METHODE POUR L'INDUCTION DE LYMPHOCYTES T REGULATEURS PAR LE BIAIS D'UNE LIAISON AVEC CD47

(54) Title: METHOD FOR INDUCING T CELLS REGULATOR WITH CD47 LIGANDS



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(57) Abrégé/Abstract:
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ABSTRACT

Naturally occurring $CD4^+CD25^+$T regulatory cells (Treg) actively participate to the mechanisms of peripheral tolerance by negatively regulating autoimmunity. However, the molecular mechanisms of *in vivo* induction of T reg remain largely unknown. Regulatory T cells (Tr1) can be generated *in vitro* from human naïve T cells by repetitive stimulation with immature or IL-10-treated DC. We previously reported that CD47 ligation by either CD47 mAb or a peptide of its natural ligand, i.e thrombospondin, induced naïve T cell anergy. Herein, we demonstrate that the CD47 mAb- induced anergic T cells were actively suppressing the proliferation and cytokine production of syngeneic effector T cells. T anergic/suppressors cells (Tas) produced little or no cytokine. The suppressive function required physical contact with the responder T cells and was IL-10 and TGF-β independent. Tas displayed :1) increased expression of CTLA-4, OX40 and GITR; 2) decreased levels of CD40L and CD28 and 3) similar levels of FAS, FASL, CCR4 and CCR7 and CD25 compared to effector T cells. Both $CD4^+$ and $CD8^+$Tas suppressed effector T cells proliferation and none of them regulated DC maturation. Our findings demonstrate that ligation of an endogenous receptor, i.e CD47, on naïve cells generates a distinct type of regulatory T cells (Tas) which produce no IL-10 but share phenotypic and functional features with both Treg and Tr1 described in human and mice. TAS may be re-infused to patients suffering from a large variety of chronic inflammatory diseases, graft versus host disease, graft rejection and allergic diseases. Compounds inducing in vivo the activity of TAS may be used to treat the same patients.

A METHOD FOR THE INDUCTION OF HUMAN REGULATORY T CELLS BY A MONOCLONAL ANTIBODY TO CD47 ANTIGEN

BACKGROUND OF THE INVENTION

The role of the immune system is to maintain the homeostasis of the organism and protect against dangerous components of the environment, mainly micro-organisms. In some situations, the immune response (IR) is undesired and harmful ; for example it may reject allograft or mediate graft versus host disease (GVHD) after allogenic bone marrow transplantation. In other cases, the IR may be aberrant and generate chronic inflammatory diseases. The latter results from a T cell-dependent IR against :1) self-antigens (occurring in conditions like autoimmune diseases, namely multiple sclerosis, type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosous) :2) innocuous and ubiquitous components of the environment such as airborne antigens or food (occurring in conditions like allergic diseases, namely asthma, allergic rhinitis, atopic eczema)
:3) commensal and innocuous bacteria (occurring in conditions like chronic inflammatory bowel diseases).

The failure of normal individuals to respond to self Ag and develop autoimmune disease results from both central tolerance (intra-thymic deletion of auto-reactive T lymphocytes) and peripheral tolerance (clonal deletion, anergy of auto-reactive cells or suppression of their activity by regulatory T cells (Treg). There is increasing evidence, derived mainly from animal models, that Treg also prevent and control allergic diseases and chronic inflammatory bowel diseases. Treg are also involved in long-term transplantation tolerance and prevent experimental GVH.

Regulatory T cells, specialized in the suppression of the immune response are heterogeneous and may belong to the $CD4^+$, $CD8^+$ or NKT cell lineage. Treg control the response to self antigens, tumor antigens, allogeneic cells, innocuous airborne or dietary Ags, commensal or pathogenic bacteria. The most extensively investigated Treg are the naturally occurring $CD4^+$ $CD25^+$ T cells and the induced Tr1 (Type 1 regulatory T cells). Naturally occurring $CD4^+$ $CD25^+$ T cells are generated in the thymus where they are selected following high affinity interaction with still undefined self ligand(s). $CD4^+$

$CD25^+$ T cells constitute 5-10% of the normal peripheral $CD4^+$ T cell repertoire of mice and humans. They are long-lived and display the phenotype of previously activated T cells; for example, human $CD4^+$ $CD25^+$ T cells are $CD45R0^+$ and express increased levels of HLA-DR and CTLA-4. Gene expression analysis of mouse $CD4^+$ $CD25^+$ Treg confirmed that they have been recently activated and revealed that they constitutively express three members of the TNF-R superfamily (i.e.OX40, 4-1BB and GITR) that are transiently expressed on activated T cells. $CD25^+$ $CD4^+$ T cells are anergic, and *in vitro*, they inhibit the response of $CD25^-$ T cells by blocking the transcription of IL-2 gene in $CD4^+$ T cells and the expression of IL-2 R α chain on $CD8^+$ T cells. *In vitro*, the suppression is contact-dependent and not inhibited by neutralizing Abs to IL-10 or TGF-β; however, *in vivo*,the suppressive activity of $CD25^+$ $CD4^+$ T cells is overcome by neutralization of TGF-β or IL-10. Although human and mouse $CD4^+$ $CD25^+$ T cells were reported to express membrane bound TGF-β on their surface, the role of this cytokine in mediating the suppressive activity has been challenged. Naturally occurring Treg may also be $CD25^-$; these are derived from mature naive CD4 T cells outside the thymus (without the help of $CD25^+$ Treg) and display similar phenotypic and functional features as $CD25^+$ Treg.

The term Tr1 was initially used to describe Treg generated *in vitro* after repetitive stimulation of naive T cells in the presence of IL-10. Tr1 cells suppressed *in vitro* and *in vivo* Th1 and Th2 immune responses and their suppressive activity was ascribed to their secretion of IL-10 and TGF-β. Different methods have been developed to generate Tr1 cells *in vitro* including: (1) the addition of IFN-α to T cells activated in the presence of IL-10,(2) the ligation of either CD2, 4C8 or CD46 on TCR-activated T cells in the absence of other co stimulatory signal, (3)T cell activation in the presence of 1,25(0H)$_2$Vit D3 and dexamethasone ,(4) repetitive stimulation of naive T cells by immature allogeneic DCs and(5) naive T cell activation by dendritic cells pre-incubated with the heavy chain of ferritin With the exception of those induced by naïve T cell activation in the presence of immunosuppressive agents and neutralizing vAbs to IFN-γ and IL-4, the Tr1 described in the above studies were anergic and produced variable levels of IFN-γ and IL-5, in addition to IL-10 and TGF-β. In vivo, Tr1 cells may be induced by

infectious agents such as *Bordetella Pertussis, Mycobacterium tuberculosis,* and *Leishmania major.* The lack of pro inflammatory immune response to inhaled protein antigens was ascribed to the development of Ag-specific Tr1 cells. The latter were generated during the interaction between Ag-specific naive T cells and airway DC by mechanisms involving ICOS/ICOSL interactions and IL-10 production by DC. Similarly tolerance to dietary Ags and commensal intestinal bacteria is mediated by a subset of Treg producing high levels of TGF-β known as Th3 cell.

The induction of regulatory T cells presents a clinical potential. Agonistic or antagonistic compounds for such induction will enhance or suppress the activity of Suppressor T cells.

SUMMARY OF THE INVENTION

We have succeeded in developing an *in vitro* or *ex vivo* model allowing the induction of a novel type of regulatory T cells, named TAS. Unlike the naturally occurring $CD25^+$ $CD4^+$ T cells, TAS may be $CD4^+$ or $CD8^+$ and unlike the Tr1 cells they suppress by a contact-dependent but IL-10 and TGF-β-independent mechanism . TAS are generated by engaging the CD47 Ag at the time of T cell activation by mitogens or polyclonal activators.

CD47 Ag, also known as Integrin Associated Protein (IAP), is a widely expressed multispan transmembrane protein, which is physically and functionally associated with αvβ3 integrin, the vitronectin receptor. CD47 has also been implicated in leukocyte transendothelial migration. Its recently described natural ligand, thrombospondin (TSP), is an homotrimeric extracellular matrix protein (ECM) which is produced not only by platelets but also by monocytes and alveolar macrophages. Thrombospondin is transiently expressed at high concentration in damaged and inflamed tissues. We recently suggested a potential role for TSP or CD47 in immune regulation by the finding that they regulate the *in vitro* production and response to IL-12. Engagement of CD47 by either mAb, TSP or 4N1K (a peptide of the C-terminal domain of TSP selectively binding CD47), inhibited IL-12 release by human monocytes. The suppression was selective for

IL-12 and occurred following T cell-dependent or -independent stimulation of monocytes. We have next observed that CD47 engagement in primary cultures of cord blood mononuclear cells inhibits IL-12 driven Th1 cell development, as revealed by the cytokine secretion profile at restimulation and IFN-γ production at the single cell level. Ligation of CD47 does not deviate the phenotype of IL-12-primed cells from Th1 to Th2, however it does not affect IL-4-induced Th2 cell development. CD47 mAb, its Fab'$_2$ fragments or the synthetic peptide 4N1K, corresponding to the CD47-binding site of thrombospondin, all display the same activity and inhibit the production of IFN-γ and IL-2 in primary cultures as well as the expression of IL-12Rβ2 chain. Inclusion of exogenous IL-2 at priming corrects IL-12R expression at priming and at restimulation as well as the inhibition of Th1 cell development.

It is an object of this invention to provide a method for interacting or interfering with the development of a disease or condition involving the activity of Suppressor T cells, in a patient, which comprises the steps of :

- inducing *ex vivo* the number and/or activity of human regulatory T cells by stimulating blood lymphocytes in the presence of a CD47 ligand; and
- reinfusing the induced human regulatory T cells to the patient.

The CD47 ligand may be an agonistic or antagonistic thereof. More specifically, the CD47 ligand that has induced the number and activity of Suppressor T cells is a CD47 monoclonal Antibody, namely the Antibody produced by the hybridoma deposited at the ATCC under accession number B6H12/Hb9771 .

This method will result in the treatment of diseases or conditions wherein Suppressor T cells activity is to be enhanced or inhibited. In the first case, auto immune diseases, allergic diseases and inflammatory diseases would benefit from such inhibition. Enhancing the activity of Suppressor T cells would also result in recipient tolerance to a grafted tissue. In the latter case, inhibiting the activity of Suppressor T cells would result in greater neutralisation of infectious agents.

Another object of this invention is a method of screening a variety of compounds, in order to find agonistic and antagonistic compounds capable of enhancing or suppressing the activity of Suppressor T cells. Agonistic and antagonistic compounds would represent alternatives to the monoclonal Antibody to CD47, for example.

DESCRIPTION OF THE INVENTION

This invention will be described hereinbelow, by reference to specific examples, enbodiements and figures, the purpose of which is to illustrate the invention rather than to limit its scope.

BRIEF DESCRIPTION OF THE FIGURES

In fig.1A, cells were recovered after 3 days of stimulation, washed , stained with the fluorescent vital dye CFSE and cultured for another 5 days in culture medium supplemented with IL-2. Cells were then washed and analyzed by flow cytometry . As seen, anti-CD47-treated cells (TAS) proliferate much less than the control cells (Th0) activated in the presence of control antibody. Tas and Th0 are comprised of >95% CD3+ T cells, of which 60% are $CD4^+$ and 40% $CD8^+$.

In fig.1B and C, TAS and Th0 were expanded in IL-2-supplemented medium for 5 days, washed and restimulated with anti-CD3 mAb immobilized on L cells cotransfected with human CD32 B7 antigens. After 3 days of culture,the proliferative response was measured by determining the tritiated thymidine uptake, whereas the secretion of IL-10 and TGF-β were measured by ELISA. As seen, TAS proliferate and secrete much less IL-10 than Th0 cells. TAS and Th0 produce similar and very low levels of TGF-β.

Fig.ID, TAS were co-cultured with Th0 cells at a 1/1 ratio and the mixture was stimulated for 3 days with graded doses of anti-CD3 mAb (100, 50 and 10 ng/ml) immobilized on CD32 B7 L transfectants. As seen, TAS potently inhibit the proliferation and the production of IFN-γ by Th0 cells.

Fig.1E shows that the suppressive effect of TAS is dose-dependent.

Fig.2 demonstrates that TAS inhibit the proliferative (tritiated thymidine uptake) and the cytokine production by Th2 cells (IL-4) (Fig.2A). and Th1 cells (Fig.2B and C). Fig.2B and C also show that the suppressive activity of TAS is not observed if TAS are physically separated from their target Th0 or Th1 by a semi-permeable membrane.This indicates that the suppressive activity of TAS is contact-dependant and not mediated by soluble factors. Moreover the suppressive activity of TAS is not overcome by blocking anti-IL10 and anti-TGF-β mAbs (not shown)

Fig.3 shows the phenotype of TAS compared to that of Th0 as determined by by 2 to 4 color analysis by means of a FACSCALIBUR. TAS and Th0 cells display similar levels of CD25, CD45RO,CD3, CD4, CD8, CD28 and CD47" TAS express higher levels of OX40, GITR and CTLA-4. The proportion of cells expressing membrane bound TGF-β is slightly higher in TAS. About 50% of TAS co-express OX40, GITR and CTLA-4 as compared to about 30% of Th0 cells on which these molecules are also expressed at lower levels.

In the experiments summarized in figures 1 to 3, umbilical cord blood mononuclear cells were stimulated in standard conditions with a polyclonal activator such as Phytohemaglutinin (PHA) in the presence of a monoclonal antibody to CD47 or a control antibody.

EXAMPLES

Example 1. TAS are prepared from the peripheral blood lymphocytes of a patient with one of the above mentioned inflammatory diseases. The cells are prepared and cultured using conditions similar to those described in Avice et al. (J. Immunol.,2001, 167 : 2459-2468). 10^7 to 10^9 TAS are then re-infused intravenously in the patient. In this example, peripheral blood mononuclear cells are isolated from the patient by

conventional fractionation method , Cells are then activated for a few days with a classical T cell activator, such as PHA in the presence of a CD47 ligand, such as for example anti CD47 mAb; cells are then expanded in IL-2 supplemented medium to obtain sufficient number of cells. TAS so prepared are washed and resuspended in a physiological solution before being infused into the patient. The procedure may be repeated as often as necessary.

Example2. The molecular signature of TAS cells, i.e. the genes specifically expressed in these cells and induced by CD47 ligand ,are used to screen any compound with potential application in the treatment of inflammatory disease, GVHD, graft rejection or cancer. The genes selectively expressed in TAS cells will be identified on highly purified preparations TAS by means of conventional DNA microarrays.

If a given compound activates a signature gene of TAS, the compound will be examined and tested for its activity in inflammatory diseases (see above), GVHD and graft rejection. Such a compound should not be used for the treatment of cancer patient as it will increase the activity of Treg inhibiting the anti-tumor immune response. Compound inhibiting the expression of TAS signature gene may tested and used as immunostimulant to enhance the immune response against cancer cells or any vaccine.

The TAS may be used in assays as they are in a whole blood sample, or in a purified or semi-purified form e.g. blood cells of a sample would be enriched in CD47 cells, by induction and/or by retention on an affinity medium. Such techniques well known in the art, are easily adaptable toCD47 cells. Recombinant cells engineered to express CD47 could also be used to screen ligands simply having affinity to CD47. A potentially clinical compound will be developed for its capacity to be an agonistic or an antagonistic to CD47 cell activity.

The invention being hereinabove described, it will be obvious that the same be varied in many ways. Those skilled in the art recognise that other and further changes and modifications may be made thereto without departing from the spirit of the invention, and

it is intended that all such changes and modifications fall within the scope of the invention, as defined in the appended claims.

CLAIMS

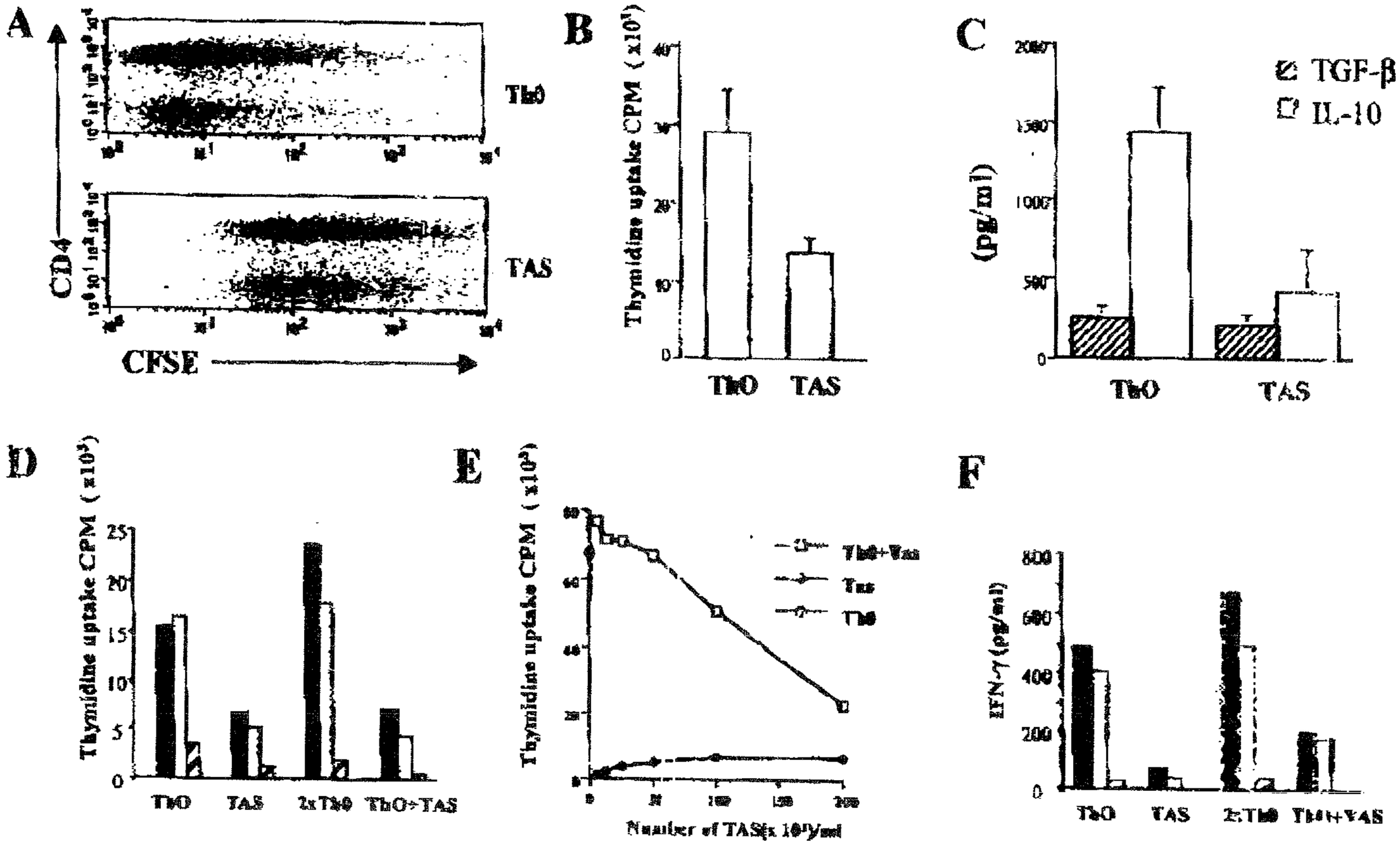
1. A method for interfering with development of a disease or condition involving the activity of Suppressor T cells, in a patient, which comprises the steps of :
- inducing *ex vivo* the number and/or activity of human regulatory T cells by stimulating blood lymphocytes in the presence of a CD47 ligand; and
- reinfusing the induced human regulatory T cells to the patient.

2. The method of claim 1, wherein the ligand is an agonist of Suppressor T cells activity.

3. The method of claim 2, wherein the ligand is a CD47 monoclonal antibody.

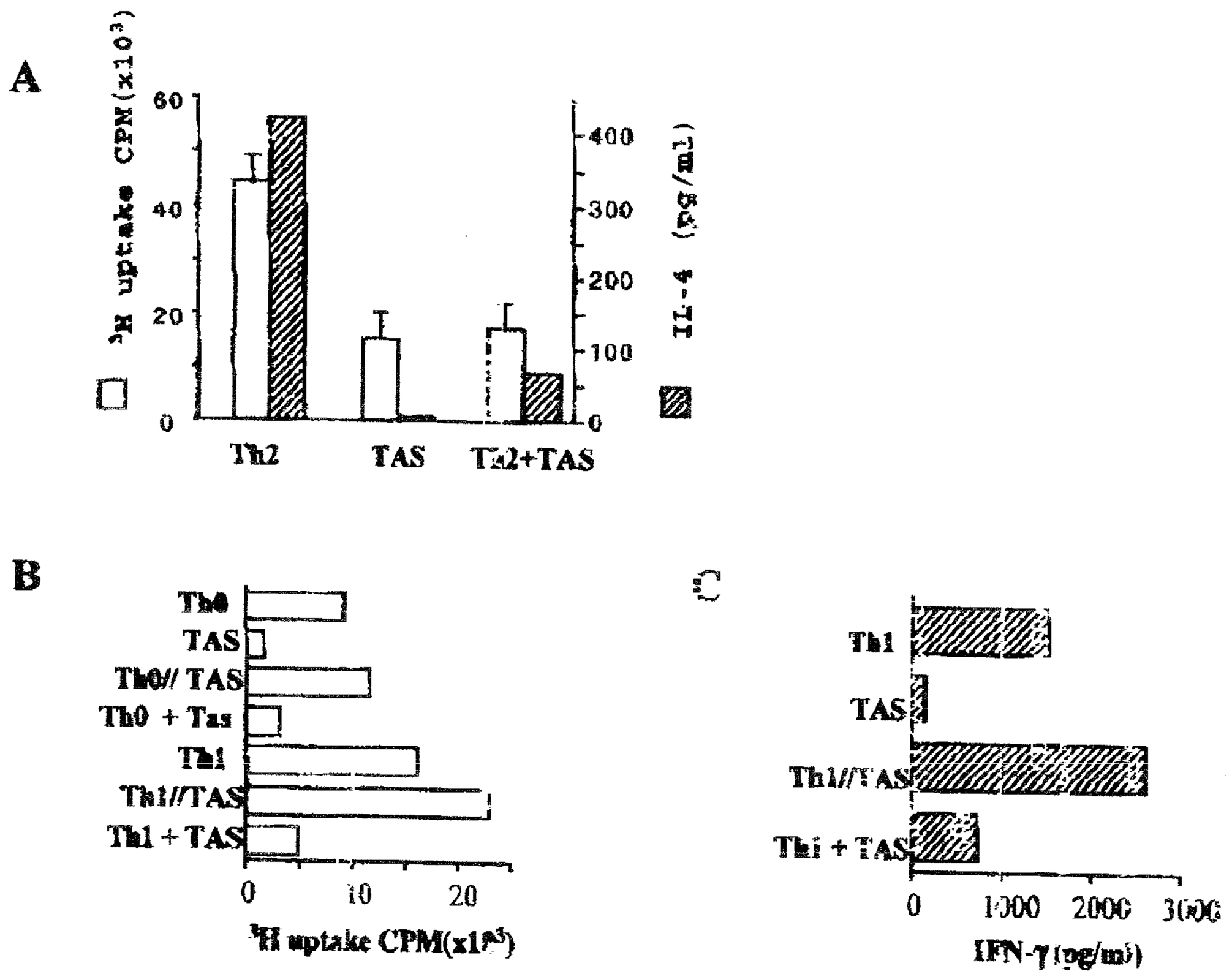
4. The method of claim 3, wherein the antibody is one produced by the hybridoma deposited at the ATCC under accession number B6H12/Hb9771.

Fig.1 CD47 ligation induces cells (TAS) that inhibit Th0 response



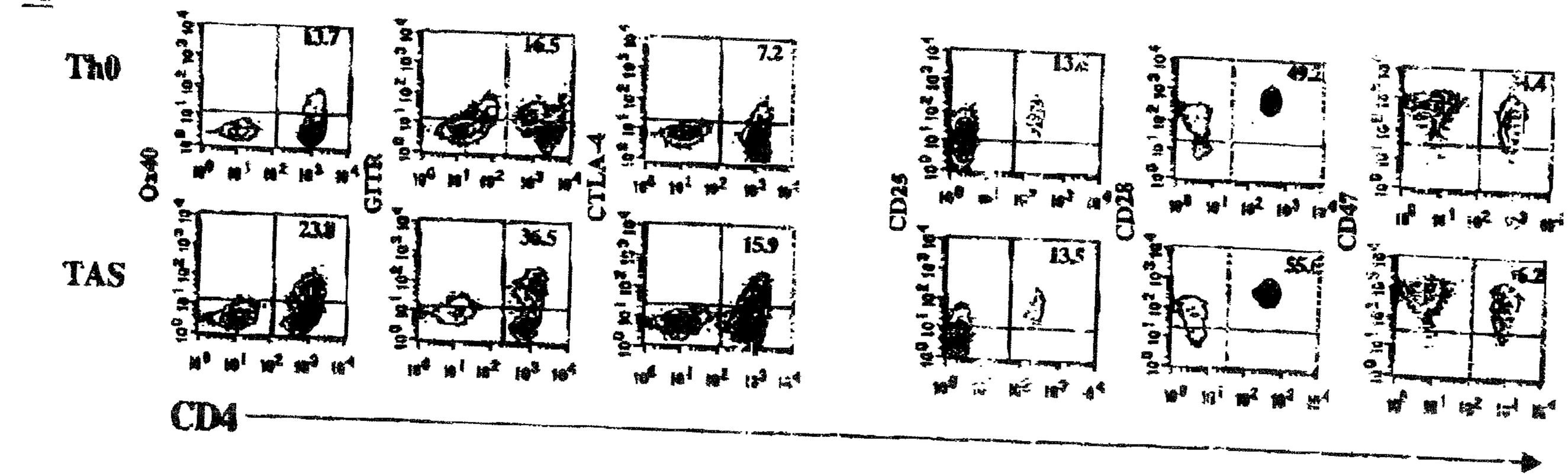
CBMC were stimulated with PHA(1µg/ml) in the presence of ctrl mAb or CD47 mAb (10µg/ml). After 3 days, primed T cells were labeled with CFSE (5 min 37 °C) and expanded in IL2. Staining CD4 of CFSE-labeled cells after 5 days (Panel A). One representative experiment out of 3. At day 9, cells are washed and restimulated at (10^6/ml) in HB101 serum free medium with anti CD3 mAb (200 ng/ml) immobilised on 2x 10^5 L-transfectants (B7/CD32). Cell proliferation (^{3}H thymidine uptake) (Panel B) and IL10 and TGFβ (Panel C) are measured at day 3 . Shown is the mean ±SD of 4 experiments. Suppressive activity of TAS on Th0 is shown in Panel D to F. Target (Th0) and TAS are cultured alone (2x 10^5 /ml)or cocultured at 1:1 ratio with anti-CD3 mAb (30 (hashed column), 100 (white column),200 (black column)ng/ml) immobilised on 2x 10^5 L-transfectants (B7/CD32) . Cell proliferation (panel D) and IFNγ production (Panel F) are measured at day 3. Panel E shows the addition of various number of TAS to 2x 10^5 /ml Th0. One representative experiment out of 4.

Fig.2 TAS suppress Th0, Th1 and Th2 response via cell-cell contact.



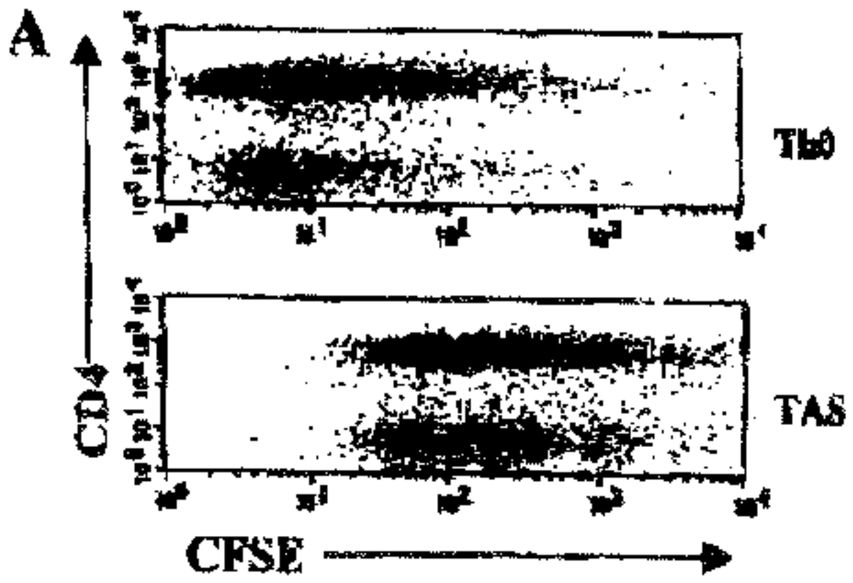
TAS are generated as in fig 1. Target (Th2) and TAS are cultured alone (2x 10^5/ml)or cocultured at 1:1 ratio with anti-CD3 mAb (100 ng/ml) immobilised on 2x 10^5 L-transfectants (B7/CD32). Cell proliferation and IL4 production (Panel A) measured at day 1. Shown is the mean ±SD of 4 experiments. Target (Th0 or Th1) and TAS are cultured alone (2x 10^5/ml)or cocultured at 1:1 ratio either together (Th+Tas) or separately (Th//TAS) using a transwell culture system and stimulated as above. Cell proliferation (Panel B) and IFNγ production (Panel C) are measured at day 2. One representative experiment out of 3.

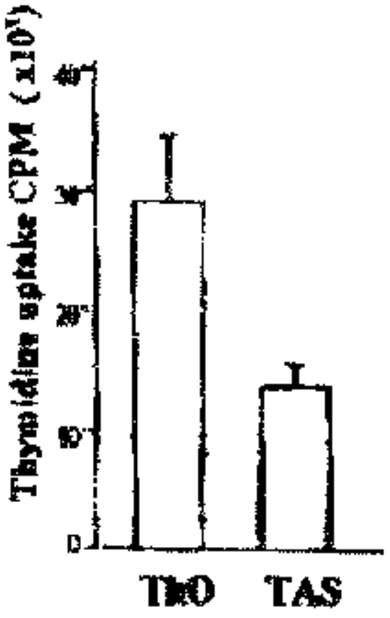
Fig.3 Phenotype of TAS

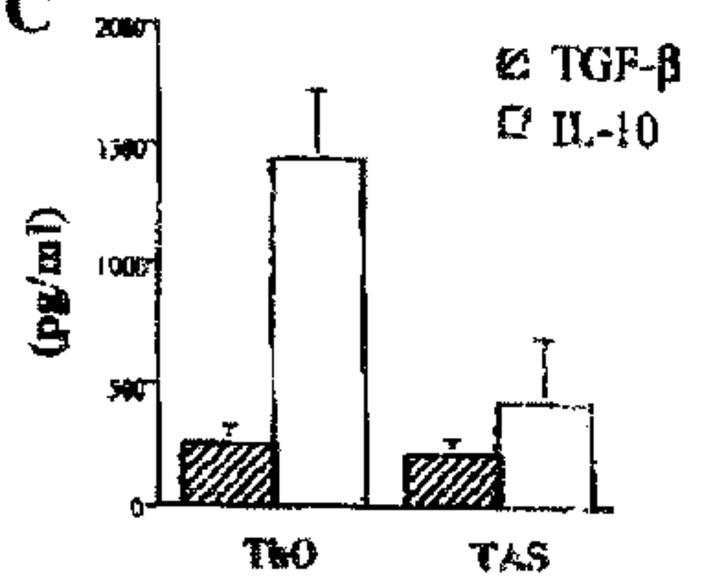


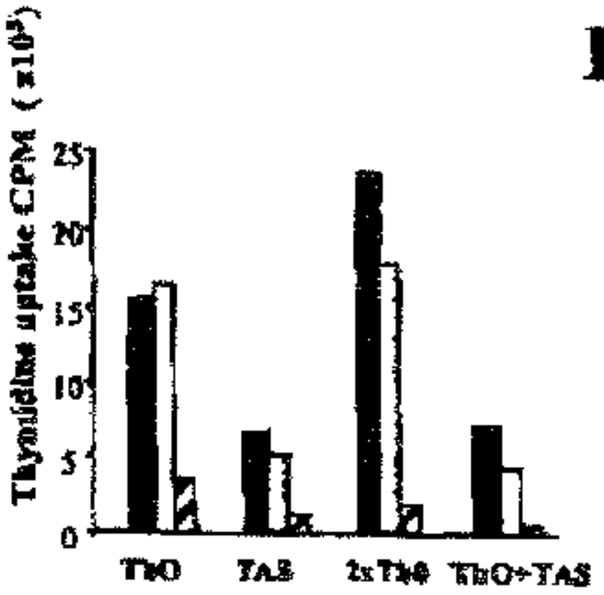
Phenotype of Th0 and TAS is shown at the end of the 9 days expansion in IL2. Double staining CD4 in combination with OX40, GITR, CTLA-4, CD25, CD28, CD47 (Panel A).Quadruple labeling with CD4, OX40, GITR and CTLA-4 Shown is the analysis of double positive cells after gating on CD4+ cells (Panel B).% positive cells are indicated in the quadrants. One representative experiment out of 2.

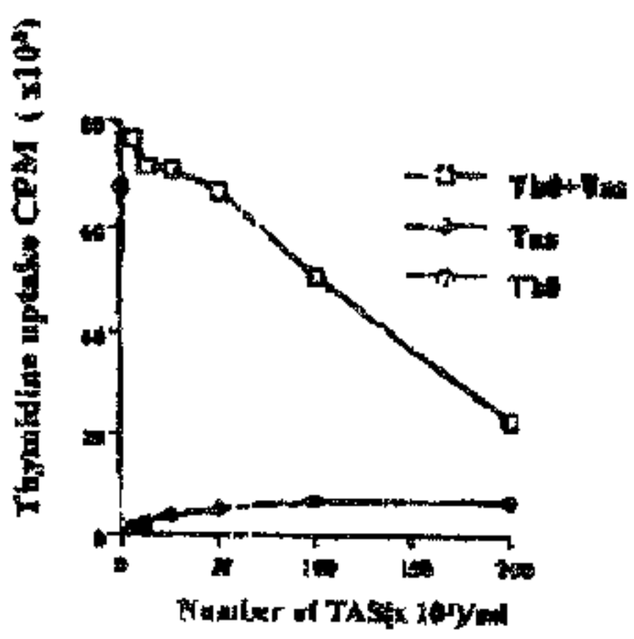
CD47 ligation induces cells (TAS) that inhibit Th0 response

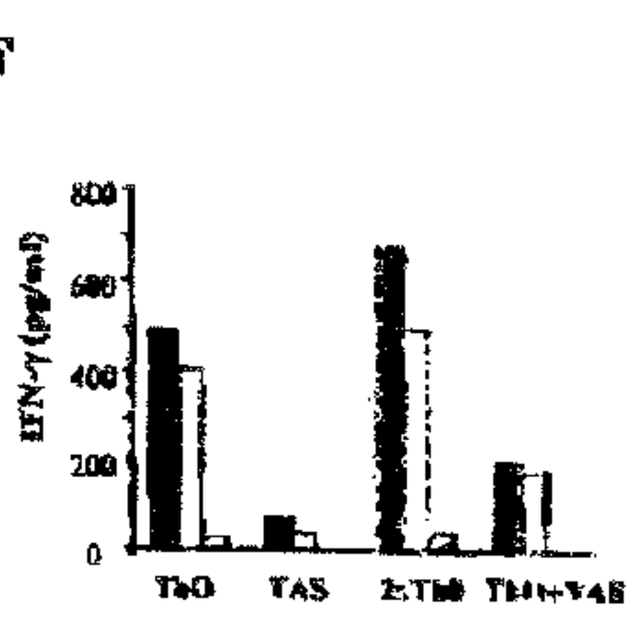












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