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(54) Title: COMPOSITIONS AND METHODS TO MODULATE IMMUNE AND INFLAMMATORY RESPONSES

(57) **Abrégé/Abstract:**

The present invention is directed to compositions and methods to modulate inflammatory and immune responses in a subject, which includes immune activation and inhibiting inflammatory responses, by modulating the regeneration and tolerance factor (RTF).



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(54) Title: COMPOSITIONS AND METHODS TO MODULATE IMMUNE AND INFLAMMATORY RESPONSES

(57) Abstract: The present invention is directed to compositions and methods to modulate inflammatory and immune responses in a subject, which includes immune activation and inhibiting inflammatory responses, by modulating the regeneration and tolerance factor (RTF).

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SPECIFICATION

TITLE OF THE INVENTION

**“COMPOSITIONS AND METHODS TO MODULATE IMMUNE AND
INFLAMMATORY RESPONSES”**

5

BACKGROUND OF THE INVENTION

The inflammatory process initiates a localized defense against foreign antigens. The process is characterized by immune activation as well as destruction of nearby tissues secondary to involved release of destructive agents. Initially involved tissue

10 assumes a significant role in provoking the inflammatory response at large (Matzinger, P., *The danger model: a renewed sense of self*. Science, 2002. 296(5566): p. 301-5; Medzhitov, R. and C.A. Janeway, Jr., *Decoding the patterns of self and nonself by the innate immune system*. Science, 2002. 296(5566): p. 298-300). One of the primary immunogenic mechanisms triggered by the inflammatory response includes the release

15 of ATP into the extracellular space. ATP is liberated from injured tissue itself—as well as from nearby macrophages, neutrophils, platelets and dying cells—following incidents of stress, infection or other forms of tissue insult (Fredholm, B.B., *Purines and neutrophil leukocytes*. Gen Pharmacol, 1997. 28(3): p. 345-50; Beigi, R., et al., *Detection of local ATP release from activated platelets using cell surface-attached*

20 *firefly luciferase*. Am J Physiol, 1999. 276(1 Pt 1): p. C267-78; Schwiebert, E.M., *ABC transporter-facilitated ATP conductive transport*. Am J Physiol, 1999. 276(1 Pt 1): p. C1-8; Mizumoto, N., et al., *CD39 is the dominant Langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness*. Nat Med, 2002. 8(4): p. 358-65; Dubyak, G.R. and C. el-Moatassim, *Signal transduction via P2-*

25 *purinergic receptors for extracellular ATP and other nucleotides*. Am J Physiol, 1993. 265(3 Pt 1): p. C577-606; Gordon, J.L., *Extracellular ATP: effects, sources and fate*. Biochem J, 1986. 233(2): p. 309-19). Extracellular ATP can then exert a wide variety of effects on surrounding cells, most notably the induction of apoptotic events leading ultimately to cell death in both lymphocytes and macrophages (Di Virgilio, F., *The P2Z*

30 *purinoceptor: an intriguing role in immunity, inflammation and cell death*. Immunol Today, 1995. 16(11): p. 524-8; Gargett, C.E., J.E. Cornish, and J.S. Wiley, *ATP, a partial agonist for the P2Z receptor of human lymphocytes*. Br J Pharmacol, 1997. 122(5): p. 911-7; Buisman, H.P., et al., *Extracellular ATP induces a large nonselective*

conductance in macrophage plasma membranes. Proc Natl Acad Sci U S A, 1988. 85(21): p. 7988-92; Ferrari, D., et al., *ATP-mediated cytotoxicity in microglial cells*. Neuropharmacology, 1997. 36(9): p. 1295-301; Zheng, L.M., et al., *Extracellular ATP as a trigger for apoptosis or programmed cell death*. J Cell Biol, 1991. 112(2): p. 279-88). Diminution of cell integrity then leads to further ATP release, propagating the inflammatory response and continued tissue destruction. Recent data have shown an attenuated effect of extracellular ATP in the presence of the regeneration and tolerance factor (RTF) protein. The present invention discloses compositions and methods to modulate inflammatory and immune responses by regulating the activities of RTF. RTF prevents ATP activation of P2Z, thus preventing the cell from undergoing pro-inflammatory activation and apoptosis.

SUMMARY OF THE INVENTION

The present invention is directed to compositions and methods to modulate inflammatory and immune responses in a subject, which includes immune activation and inhibiting inflammatory responses, by modulating the regeneration and tolerance factor (RTF). Immune activation can be accomplished by inhibiting the RTF activity. In one embodiment, the RTF activity can be inhibited by administering a RTF antagonist, which can be a small molecule, a peptide, or an antibody or a fragment thereof. Inhibiting the RTF activity may result in cell apoptosis, especially cancer cells such as ovarian carcinoma cells.

Inflammatory responses can be inhibited by increasing the level of activity of RTF. In an embodiment, the method of increasing the level of activity of RTF is by administering an effective amount of RTF or a fragment of RTF. The RTF or its fragment can be isolated or purified from mammalian cells such as the T-lymphocyte, the B-lymphocyte, the macrophage, thymus, or the fetalplacental tissue. Alternatively, the RTF can be synthetic or a recombinant RTF.

The present invention is also directed to a method of treating cancer, such as ovarian cancer, by inhibiting the RTF activity. In one embodiment, the method to inhibit RTF activity is to administer an effective dose of a RTF antagonist.

The present invention is further directed to a method of treating or ameliorating an inflammatory disorder or an autoimmune disorder or one or more symptoms thereof by administering an effective amount of RTF or a fragment of RTF. The RTF or its

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fragment can be isolated or purified from mammalian cells such as the T-lymphocyte, the B-lymphocyte, the macrophage, thymus, or the fetalplacental tissue. Alternatively, the RTF can be synthetic or a recombinant RTF. The RTF can be formulated with one or more suitable pharmaceutical excipients for administration by parenteral, intramuscular, topical, nasal, pulmonary, or oral route. The formulation can also be in the form of microparticles or nanoparticles. In an embodiment, the method of treating or ameliorating an inflammatory disorder or an autoimmune disorder or one or more symptoms thereof includes an additional step of administering a TNF- α antagonist, which includes but is not limited to infliximab, etanercept, D2E7, CDP571, CDP870, thalidomide and its analogs, and phosphodiesterase type IV inhibitors.

Additional features and advantages of the present invention are described in, and will be apparent from, the following Detailed Description of the Invention and the figures.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the expression of RTF and P2Z on PBMC and macrophages. Flow cytometric analysis of the expression of RTF and P2Z. Dotted line indicates isotype control antibody. Solid line represents either RTF expression (column 1) or P2Z expression (column 2). The first row represents unstimulated PBMC. The second row represents PBMC stimulated for 72 hours with PHA. The third row represents J774A1 macrophages.

FIG. 2 illustrates that RTF expression is concurrent with P2Z expression on lymphocytes during stimulation. Lymphocytes were stimulated with PHA for 0-72 hours. The expression on RTF and P2Z was measured at time points 0, 24hrs, and 72 hrs as indicated by flow cytometry. The Y axis represent the percent maximal expression of each molecule. The black bars represent RTF expression and the gray bars represent P2Z expression. Error bars represent the standard error of the mean.

FIG. 3 shows that RTF moves to the surface of the membrane in a 50 kDa form during activation. PBMC were stimulated for 0-72 hours and RTF size was examined by western blot and RTF location was examined by confocal microscopy. Panel A: Western Blot: PBMC were stimulated with plate bound CD3 and CD28 and harvested at the time points indicated (in hours), western blotted and probed by 2C1. Row 1 represents proteins of 70 kDa size, and row 2 represents proteins of 50 kDa size. Each lane represents a stimulation time of 0, 18, 48, and 72 hours respectively. Panel B:

Confocal Microscopy: Confocal microscopy images of PBMC taken before activation (left panel) and at 72 hrs of activation (right panel), permeabilized and stained with anti-RTF-FITC and counterstained with anti-CD8-PE.

FIG. 4 demonstrates that surface ATPase activity is inhibited by anti-RTF. PBMC and J774A1 macrophages with anti-RTF antibody (black columns) or isotype control antibody (gray columns) were examined for surface ATPase activity, by adding [32] γ P-ATP and measuring the release of [32]-Pi. The Y axis represents surface ATPase activity as measured in counts per minute [32]-Pi. Error bars represent the standard error of the mean.

FIG. 5 shows that anti-RTF and ATP generate more apoptosis together than either alone. Top row represent flow cytometry histograms of J774A1 macrophages incubated with ATP, anti-RTF, or both together. Bottom row represents J774A1 cells incubated with ATP, anti-RTF, and different concentrations of ATPase as indicated. X axis represents annexin-V staining. Means of four independent experiments with standard error are given for each.

FIG. 6 demonstrates the enhancement of secretion of IL-1 β in THP-1 Macrophages by anti-RTF antibody.

FIG. 7 illustrates the cytotoxic effect of anti-RTF antibody to ovarian carcinoma cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to compositions and methods to modulate inflammatory and immune responses in a subject by regulating the level of activity of regeneration and tolerance factor (RTF). Modulating inflammatory and immune responses includes both immune activation and the inhibition of the inflammatory responses. In a preferred embodiment, the subject is a mammal. In a more preferred embodiment, the subject is a human.

A principle means by which RTF controls the extent of inflammation is via its interaction with P2Z, a cell surface receptor expressed primarily on cells of hematopoietic origin such as lymphocytes and macrophages. P2Z is one of a family of receptors known as purinoceptors which are activated by nucleotides. Extracellular ATP binds P2Z, which triggers the opening of specific calcium channels as well as non-specific transmembrane channels (Dubyak, G.R. and C. el-Moatassim, *Signal*

transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. Am J Physiol, 1993. 265(3 Pt 1): p. C577-606; Gargett, C.E., J.E. Cornish, and J.S. Wiley, *ATP, a partial agonist for the P2Z receptor of human lymphocytes*. Br J Pharmacol, 1997. 122(5): p. 911-7; Zheng, L.M., et al., *Extracellular ATP as a trigger for apoptosis or programmed cell death*. J Cell Biol, 1991. 112(2): p. 279-88). These channels then allow for rapid influx of calcium ions into the cytosolic space. This calcium influx in turn drives forward many specific activation pathways. The activation of P2Z can cause specific activation of NF- κ B, and the maturation and secretion of IL-1 β , a potent inflammatory cytokine. This will subsequently increase both cellular activation and drive the inflammatory response, making P2Z a key mediator in the inflammatory process. It can also cause apoptosis and an inflammatory counterpart to apoptosis, oncosis, inducing the release of further ATP. Recent reports have shown that the absence of the P2Z purinoceptor can attenuate the inflammatory response (Labasi JM, P.N., Donovan C, McCurdy S, Lira P, Payette MM, Brisette W, Wicks JR, Audoly L, Gabel CA., *Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response*. J Immunol, 2002. 168(12)(Jun 15): p. 6436-45).

Therefore it follows that extracellular ATP up-regulates the inflammatory response through its instigation of P2Z activation at the site of inflammation and also the stimulated release of pro-inflammatory cytokines. A key regulatory point at which to reduce the inflammatory response, then, is in the concentration of extracellular ATP. It has been suggested that ecto-ATPases hold important regulatory surface ATPase activity, which attenuates inflammatory as well as immune responses (Labasi JM, P.N., Donovan C, McCurdy S, Lira P, Payette MM, Brisette W, Wicks JR, Audoly L, Gabel CA., *Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response*. J Immunol, 2002. 168(12)(Jun 15): p. 6436-45; Filippini, A., et al., *Ecto-ATPase activity in cytolytic T-lymphocytes. Protection from the cytolytic effects of extracellular ATP*. J Biol Chem, 1990. 265(1): p. 334-40). The present application discloses that RTF is the primary surface ATPase with this function.

RTF is the α -subunit of α 2-isoform of vacuolar ATPase, and that RTF exists as a 70 kDa protein (Toyomura, T., et al., *Three subunit α isoforms of mouse vacuolar H(+)-ATPase. Preferential expression of the α 3 isoform during osteoclast differentiation*. J Biol Chem, 2000. 275(12): p. 8760-5; Nichols, T.C., et al., *Expression of a membrane form of the pregnancy-associated protein TJ6 on lymphocytes*. Cell Immunol, 1994.

155(1): p. 219-29; Boomer, J.S., et al., *Regeneration and tolerance factor's potential role in T-cell activation and apoptosis*. Hum Immunol, 2000. 61(10): p. 959-71). A 20 kDa fragment can be cleaved, leaving the 50 kDa external membrane form (Nichols, T.C., et al., *Expression of a membrane form of the pregnancy-associated protein TJ6 on lymphocytes*. Cell Immunol, 1994. 155(1): p. 219-29). Current data support that RTF is known to have a role in certain forms of apoptosis (Boomer, J.S., et al., *Regeneration and tolerance factor's potential role in T-cell activation and apoptosis*. Hum Immunol, 2000. 61(10): p. 959-71; Boomer, J.S., et al., *Regeneration and tolerance factor is expressed during T-lymphocyte activation and plays a role in apoptosis*. Hum Immunol, 10 2001. 62(6): p. 577-88). It has also been shown equally that anti-RTF antibody can block RTF's ATPase activity, subsequently allowing ATP to bind P2Z and induce apoptosis. It has additionally been shown that RTF works in an antagonistic fashion versus the P2Z surface purinoceptor. United States Patent No. 6,133,434 and International Patent Application (PCT) No. WO 95/33048 disclose that antibodies 15 against P2Z receptors can be useful in treating a variety of diseases and conditions, including epilepsy, cognition, emesis, pain (especially migraine), asthma, peripheral vascular disease, hypertension, diseases of immune system, irritable bowel syndrome and premature ejaculation.

One possible mechanism for RTF to modulate inflammatory and immune 20 responses is by RTF acting as an ATPase to reduce the level of extracellular ATP. ATP, released during injury or stress from tissues, infiltrating cells, and dying cells at the site of inflammation, interacts with the P2Z receptor. This interaction exacerbates the inflammatory response which leads to cell activation and the maturation and secretion of IL-1 β . By controlling the degradation of extracellular ATP, RTF down regulates 25 immune responses, including immune activation and inflammation. Thus, inhibiting the RTF activity leads to immune activation, which may lead to cell death, while enhancing the RTF activity leads to inhibition of inflammatory responses.

Any method that inhibits RTF activity can be employed in the present invention for immune activation. In a preferred embodiment, the method to inhibit RTF is by 30 administering a RTF antagonist. The RTF antagonist can be a small molecule, a peptide, an antibody or a fragment thereof, and the like. The antibody can be either polyclonal or monoclonal. In one embodiment, the antagonist acts by binding to RTF to inhibit its activity. A preferred RTF antagonist is an anti-RTF antibody or a fragment

thereof, and a preferred anti-RTF antibody is a monoclonal RTF antibody or a fragment thereof. A method to produce and purify a monoclonal RTF antibody is disclosed by Boomer et al. (Boomer, J.S., et al., *Regeneration and tolerance factor's potential role in T-cell activation and apoptosis*. Hum Immunol, 2000. 61(10): p. 959-71).

5 In another embodiment, the antagonist acts by inhibiting cellular expression of RTF. An example of such an antagonist includes, but is not limited to, an antisense nucleic acid which binds to the DNA coding for the RTF molecule. Using antisense nucleic acid to bind to DNA to inhibit cellular expression of proteins is well known in the art. Another example of
10 antagonizing RTF is by inducing RNA interference (RNAi). The RNAi process induces the degradation of the target gene mRNA so as to silence the target gene expression. A typical RNAi process includes introducing into cells a double-stranded interfering RNA that comprises a sense RNA having the sequence homologous to the target gene mRNA and antisense RNA having the sequence complementary to the sense RNA. The double-
15 stranded interfering RNA can be a small interfering double stranded RNA (also known as siRNA). The double-stranded interfering RNA can be introduced to the cells exogenously, or it can be expressed intracellular by incorporating into the cells an expression system to express the double-stranded interfering RNA. Intracellular expression is preferred for siRNA.

20 The RTF antagonist can be prepared as a pharmaceutical formulation by including one or more suitable or appropriate excipients. In an embodiment, the pharmaceutical formulation is a nanoparticle or a microparticle. The antagonist can be administered to the subject by any route including but not limited to parenteral, topical, nasal, pulmonary, ophthalmic, bucal, vaginal, transdermal, intrathecal, and oral

25 One of the results of inhibiting RTF activity is induction of cell apoptosis, especially cancer cells, such as ovarian carcinoma cells. Thus, inhibiting RTF can be used as a method to treat cancer. Any of the methods described above which inhibit RTF can then be used as a method to treat cancer. An example of cancer that can be treated in the present invention is ovarian cancer.

30 Since inflammatory responses are the result of immune activation by the injured tissue, it is anticipated that enhancing RTF activity will result in inhibiting the inflammatory responses. Inflammation is known in many disorders which include, but are not limited to, arthritis (including but not limited to rheumatoid arthritis,

spondyloarthropathies, gouty arthritis, osteoarthritis, systemic lupus erythematosus and juvenile arthritis), asthma, bronchitis, menstrual cramps, tendonitis, bursitis, and skin related conditions (such as psoriasis, eczema, burns and dermatitis), certain gastrointestinal conditions (such as inflammatory bowel disease, Crohn's disease, 5 gastritis, irritable bowel syndrome and ulcerative colitis), vascular diseases, migraine headaches, periarteritis nodosa, thyroiditis, aplastic anemia, Hodgkin's disease, sclerodoma, rheumatic fever, type I diabetes, myasthenia gravis, multiple sclerosis, sarcoidosis, nephrotic syndrome, Behcet's syndrome, polymyositis, gingivitis, hypersensitivity, conjunctivitis, swelling occurring after injury, myocardial ischemia, 10 certain central nervous system disorders (such as Alzheimer's disease and dementia), allergic rhinitis, respiratory distress syndrome, endotoxin shock syndrome, atherosclerosis, central nervous system damage resulting from stroke, ischemia, trauma, and the like. Many of the above inflammatory disorders are autoimmune disorders in which the body develops immune responses to its own protein. Thus, enhancing the 15 activity of RTF can be used to treat or prevent inflammatory disorders, including autoimmune disorders, or their symptoms.

Any method that enhances the level of activity of RTF can be used to inhibit inflammatory responses and to treat or prevent inflammation in the present invention. In a preferred embodiment, the method is to administer to the subject an effective 20 amount of RTF or a fragment thereof. In an embodiment, the RTF is an intracellular form which has a molecular weight of about 70 kDa. In another embodiment, the RTF is external membrane form having a molecule weight of about 50 kDa.

In one embodiment, the RTF can be isolated or purified from a mammalian source, such as murine or human, which includes but is not limited to, lymphocyte, 25 macrophage, thymus, fetalplacental tissue, and the like. The lymphocyte can be a T-lymphocyte or a B-lymphocyte. The T- or B-lymphocyte preferably is activated.

In another embodiment, the RTF or a fragment thereof can be synthetic or a recombinant RTF, which can be derived, for example, from a nucleic acid sequence (e.g. cDNA) encoding the amino acid sequence of the RTF or the fragment. The nucleic 30 acid sequence can be incorporated by recombinant techniques into an appropriate host organism for the expression of the RTF or the fragment. Examples of host organisms include, but are not limited to, bacteria, fungi, yeasts, protozoa, transgenic animals, transgenic plants, and the like. Recombinant techniques are well known to those skilled

in the art and many biopharmaceuticals have been successfully produced by such techniques. A method for producing a recombinant RTF in *E. coli* is disclosed by Lee et al. (Lee, G. W., Bommer, J.S., Gilman-Sachs, A., Chedid A., Gudelj, L., Rukavina, D. and Beaman, K. D. *Regeneration and tolerance factor of the human placenta induces*
5 *IL-10 induction*. Eur J Immunol, 2001. 31: p. 687-691).

In a preferred embodiment of the present invention, the RTF or the fragment is formulated with one or more acceptable pharmaceutical excipients for administration to the subject. The RTF or the fragment can also be formulated as microparticles or
10 nanoparticles. The microparticles or nanoparticles can further include ligands to deliver the microparticles or the nanoparticles to a target tissue such as the T- or B-lymphocyte or the macrophage. The RTF or the fragment can be administered to the subject by any acceptable route including, but not limited to, parenteral, topical, nasal, pulmonary, ophthalmic, bucal, vaginal, transdermal, intrathecal, oral and the like.

15 In another embodiment, the method to enhance RTF activity can be a method which enhances the expression of cellular RTF.

In yet another embodiment of the present invention, the method of treating inflammation by enhancing the RTF activity may include an additional step of administering to the subject a TNF- α antagonist. Since both IL-1 β and TNF- α are
20 known to cause inflammation, inhibiting the secretion of IL-1 β by RTF and blocking the TNF- α activity by a TNF- α antagonist should have synergistic effects in treating inflammation. There are many known TNF- α antagonists. Examples include, but are not limited to, infliximab, etanercept, D2E7, CDP571, CDP870, thalidomide and its analogs, and phosphodiesterase type IV inhibitors.

25

EXAMPLES

Example 1: Cell Culture

J774A1 cells were maintained by allowing them to adhere to the bottom of a T75 flask with 25 mls of RPMI 1640 complete media at 37°C in a 5% CO₂ atmosphere.
30 Cells were split into a new flask every 4 days using trypsin and a gentle wash with RPMI.

Example 2: ATPase Assay

PBMC or J774A1 macrophages were suspended in 50 μ l of RPMI at a density of 2.5x10⁵ in a 96 well flat bottom plate and incubated with 10 μ g of either 2C1 (anti-RTF) or an isotype matched control antibody for 45 mins at 37°C in a 5% CO₂ atmosphere.

5 Cells were washed with PBS and re-suspended in 100 μ l of assay buffer (5mM KCl, 2mM CaCl₂, 2mM MgCl₂, and 1.5 μ C of [γ -32P]ATP) in 1.5ml eppendorf tubes. The ATPase reaction was allowed to continue for 10 mins at room temperature. 500 μ l of 20% w/v activated charcoal (in distilled water) was added to scavenge any unused [γ -32P]ATP and allowed to sit for 10 mins on ice. The cells were centrifuged at 3200 x g

10 for 5 mins, pelleting the charcoal. 500 μ l of supernatant containing released γ -32P was removed and added to scintillation vials with 3mls of scintillation fluid and read in the scintillation counter for 1 min/tube (Filippini, A., et al., *Ecto-ATPase activity in cytolytic T-lymphocytes. Protection from the cytolytic effects of extracellular ATP*. J Biol Chem, 1990. 265(1): p. 334-40; Dombrowski, K.E., et al., *Identification and partial*

15 *characterization of an ectoATPase expressed by human natural killer cells*. Biochemistry, 1993. 32(26): p. 6515-22; Dombrowski, K.E., et al., *Antigen recognition by CTL is dependent upon ectoATPase activity*. J Immunol, 1995. 154(12): p. 6227-37).

Example 3: Apoptosis Induction

20 Approximately 2.5x10⁵ PBMC were suspended in 100 μ l of RPMI in a 96 well round bottom plate and a final concentration of either 3mM ATP, 0.75mM ATP, 10 μ g anti-RTF, or 0.75mM ATP + 10 μ g anti-RTF together was added. Cells were incubated in these conditions at 37°C in 5% CO₂ for 6 hours. Cells were washed twice in 0.01% BSA/PBS, and re-suspended in 100 μ l binding buffer (10mM HEPES pH 7.4, 140mM

25 NaCl, 2.5mM CaCl₂) with 5 μ l of annexin-V/FITC (BD/Pharmingen) for 15 mins. Cells were washed in binding buffer and re-suspended in 300 μ l of binding buffer with 3 μ l of 1mg/ml propidium iodide. Apoptosis was measured by quantifying annexin-V binding by flow cytometry by gating on PI-/annexin-V+ cells (Vermes, I., et al., *A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early*

30 *apoptotic cells using fluorescein labeled Annexin V*. J Immunol Methods, 1995. 184(1): p. 39-51).

Example 4: P2Z and RTF Staining

A total of 2.5×10^5 cells were washed twice with 0.01% BSA/PBS for 5 mins and re-suspended in 100ul of staining buffer. 5ul of rabbit anti-P2X₇ (P2Z) (Chemicon) antibody, 5ul anti-RTF-FITC, or 5ul of isotype matched control antibody was added to the appropriate tubes and allowed to react in the dark for 15 mins. Cells were washed again and re-suspended in 100ul of staining buffer (1X PBS, 5% FBS) with 5ul of anti-Rabbit biotinylated antibody (Pharmingen) and allowed to react for 15 mins. A third wash was performed and 2ul of Streptavidin-PE (Pharmingen) was added for 15 mins. After a final wash, the cells were re-suspended in 300ul of staining buffer and run on a BD FACS Calibur flow cytometer.

Example 5: Propidium Iodide and Lucifer Yellow Incorporation

J774 cells were suspended in 100ul of RPMI in a 96 well round bottom plate at a density of 2.5×10^5 in one of the following conditions: 3mM ATP, 0.75mM ATP, 50ug anti-RTF, or 0.75mM ATP + 10ug anti-RTF. Cells were incubated at 37°C in 5% CO₂ for 30 mins in the presence of 1ug PI or LY, washed in PBS, re-suspended in 300ul PBS and analyzed in the BD FACSCALIBUR™ flow cytometer.

For slides, J774 cells were grown in 4 well chamber slides for 2 days. The supernatant was removed and each chamber was incubated in one of the conditions described above for 15 mins in the presence of 1ug PI or LY. The slides were washed in PBS, and incubated with 10u Trypan blue for 5 mins, washed again and examined under a fluorescent microscope.

Example 6: Confocal Microscopy

PBMC were obtained by FICOLL™ separation and 2.5×10^5 cells per well were incubated in a 96 well plate in RPMI 1640 medium in the presence of 2 ug/ml PHA, or RPMI alone. After 72 hours both unstimulated, and stimulated cells were washed twice in PBS, permeabilized with ice cold 70% ethanol for 15 mins, and reacted with 7 ul anti-RTF-FITC, anti-CD8-PE (Pharmingen), or isotype control antibody for 15 mins. Cells were washed again, fixed with Coulter fixative solution, and a CYTOSPIN™ was used to transfer them to a positively charged slide which was mounted with Dapco medium. Slides were then examined by confocal microscopy.

Example 7: RTF Expression Follows P2Z Expression on Lymphocytes

It has been previously shown that RTF expression in lymphocytes is dependent on activation (Boomer, J.S., et al., *Regeneration and tolerance factor's potential role in T-cell activation and apoptosis*. Hum Immunol, 2000. 61(10): p. 959-7). To
5 characterize RTF expression in relation to P2Z expression during the immune response, the surface expression of RTF and P2Z on lymphocytes was measured following stimulation with PHA for 24-72 hours. In resting lymphocytes, RTF expression was $14.42 \pm 0.06\%$ that of maximal expression while P2Z expression was $35.23 \pm 8.5\%$. RTF and P2Z expression increased together on the surface of the cells as stimulation
10 time increased, with RTF and P2Z expression at $88.64 \pm 8.8\%$ and $81.34 \pm 13.2\%$ respectively at 72 hours (Figure 1). Both molecules matched the others percent maximal expression at 0 and 24 hours, reaching maximal expression at 72 hours (Figure 2).

The expression of RTF and P2Z on the J774A1 macrophage cell line has also
15 been examined. Figure 1 shows that each molecule to be expressed in a constitutive manner.

Example 8: RTF Exists as a 50 kDa Protein on the Surface of Activated Cells, and as a 70 kDa Protein Inside of Resting Cells

20 When lymphocytes examined by western blot at the time points stimulated above (Figure 3a), unstimulated lymphocytes expressed a 70 kDa protein of RTF as their major form of RTF. Upon stimulation, expression of RTF fell below detectable levels at a time period of 16-24 hours. At a time period of 24-72 hours after stimulation RTF expression returned to detectable levels, however predominantly in a 50 kDa sized form
25 (Figure 3a).

It is hypothesized that the 70 kDa form represents the intracellular form of RTF, while the 50 kDa form represents the external membrane form. To test this, the activated lymphocytes from Figure 3a were further examined by confocal microscopy at 0 and 72 hours of stimulation (Figure 3b). In this figure RTF (green) is present in the
30 cytoplasm of un-stimulated cells. In cells stimulated with PHA for 72 hrs, RTF has re-localized to the surface of the membrane. Consistent with the western blot shown in Figure 3a, these findings suggest to us that the 70 kDa protein is intracellular in resting lymphocytes, whereas the 50 kDa protein is found on the surface of activated

lymphocytes.

Example 9: RTF Regulates Surface ATPase Activity in Lymphocytes and Macrophages

It is hypothesized that RTF regulates surface ATPase activity and anti-RTF
5 blocks it. To demonstrate that RTF has surface ATPase activity, PBMC and J774A1
macrophages were incubated with either anti-RTF or isotype control antibody for 45
mins, and their surface ATPase activity was measured by [γ 32-P]ATP degradation
(Filippini, A., et al., *Ecto-ATPase activity in cytolytic T-lymphocytes. Protection from
the cytolytic effects of extracellular ATP*. J Biol Chem, 1990. 265(1): p. 334-40;
10 Dombrowski, K.E., et al., *Identification and partial characterization of an ectoATPase
expressed by human natural killer cells*. Biochemistry, 1993. 32(26): p. 6515-22;
Dombrowski, K.E., et al., *Antigen recognition by CTL is dependent upon ectoATPase
activity*. J Immunol, 1995. 154(12): p. 6227-37). The PBMC incubated with anti-RTF
had a 10 fold decrease in surface ATPase activity with $11,758 \pm 3,394$ cpm compared to
15 PBMC incubated with isotype antibody with a cpm of $115,592 \pm 23,891$. ($P < 0.001$;
Figure 4). The J774A1 cells incubated with anti-RTF had a complete inhibition of
surface ATPase activity compared with the isotype control ($45,752 \pm 16,175$ cpm;
 $P < 0.001$; fig 4). Thin layer chromatography confirmed that anti-RTF completely
inhibited the surface ATPase activity (data not shown).

20

Example 10: RTF Prevents ATP from Inducing Apoptosis

Since RTF moves to the surface of the membrane as a 50 kDa size protein after
stimulation in PBMC, and RTF regulates ATPase activity, it is desirable to demonstrate
that RTF can prevent ATP induced apoptosis. For this purpose the macrophage cell line
25 J774A1 is chosen. J774A1 cells were to be used as a model to demonstrate the
interaction of RTF with P2Z because this cell line expresses both molecules
constitutively and is known to undergo P2Z mediated, ATP induced apoptosis (Di
Virgilio, F., *The P2Z purinoceptor: an intriguing role in immunity, inflammation and
cell death*. Immunol Today, 1995. 16(11): p. 524-8; Coutinho-Silva, R. and P.M.
30 Persechini, *P2Z purinoceptor-associated pores induced by extracellular ATP in
macrophages and J774 cells*. Am J Physiol, 1997. 273(6 Pt 1): p. C1793-800). If the
theory that RTF regulates surface ATPase activity and therefore regulates ATP binding
to P2Z is correct, then it follows that anti-RTF and ATP would work together to

generate more apoptosis than either alone by blocking the ATPase activity of RTF. When added together, at the same concentration as both alone, a near maximal amount of apoptosis occurred as measured by annexin-V (Figure 5). When 3mM ATP was added, 84.14 ± 1.63 % of the cells underwent apoptosis, but when the amount of ATP was decreased to 0.75mM, only 13.26 ± 3.6 % apoptosis was seen. When 10 μ g of anti-RTF was added alone, 15.49 ± 4.05 % of the cells were apoptotic. However, anti-RTF and 0.75mM ATP were added together, apoptosis was 83.05 ± 5.09 %. When ATPase was added back, no apoptosis occurred showing that RTF's apoptosis inducing ability is dependent on the presence of ATP (Figure 5). Previously, in initial experiments we demonstrated that anti-RTF had no effect on the ATPase used, negating the possibility of their interaction (data not shown).

Example 11: RTF Prevents P2Z Activation

A standard technique for detecting P2Z activity is used to demonstrate that the ultimate cause of the apoptosis inducing effect of RTF and ATP was due to P2Z activation (Di Virgilio, F., *The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death*. Immunol Today, 1995. 16(11): p. 524-8; Coutinho-Silva, R. and P.M. Persechini, *P2Z purinoceptor-associated pores induced by extracellular ATP in macrophages and J774 cells*. Am J Physiol, 1997. 273(6 Pt 1): p. C1793-800; Steinberg, T.H., et al., *ATP4- permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes*. J Biol Chem, 1987. 262(18): p. 8884-8). When ATP interacts with P2Z, non-specific channels of about 0.9 kDa in size will open in the membrane (Dubyak, G.R. and C. el-Moatassim, *Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides*. Am J Physiol, 1993. 265(3 Pt 1): p. C577-606; Di Virgilio, F., *The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death*. Immunol Today, 1995. 16(11): p. 524-8; Hickman, S.E., et al., *P2Z adenosine triphosphate receptor activity in cultured human monocyte-derived macrophages*. Blood, 1994. 84(8): p. 2452-6; Greenberg, S., et al., *Extracellular nucleotides mediate Ca²⁺ fluxes in J774 macrophages by two distinct mechanisms*. J Biol Chem, 1988. 263(21): p. 10337-43). This allows the testing for P2Z's influence by measuring the influx of molecules smaller than 0.9 kDa, to which the cell is normally not permeable. Propidium iodide (PI) (0.414 kDa) and lucifer yellow (LY) (0.46 kDa) should be allowed through if P2Z is activated, and trypan blue (TB) (0.96 kDa) should

be excluded under all circumstances unless necrosis has occurred (Di Virgilio, F., *The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death*. Immunol Today, 1995. 16(11): p. 524-8; Coutinho-Silva, R. and P.M. Persechini, *P2Z purinoceptor-associated pores induced by extracellular ATP in macrophages and J774*
5 *cells*. Am J Physiol, 1997. 273(6 Pt 1): p. C1793-800; Steinberg, T.H., et al., *ATP4-permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes*. J Biol Chem, 1987. 262(18): p. 8884-8). The same conditions used in the previous experiment were repeated to observe and measure the entry of propidium iodide or trypan blue in J774A1 cells. Thus P2Z influence would be characterized by cells that were PI and LY
10 positive but TB negative.

Using flow cytometry, cells incubated with anti-RTF + 0.75mM ATP had pronounced channel formation judging from their incorporation of PI (Figure 6). This was confirmed by immunofluorescence microscopy (Figure 6). Cells reacted with anti-RTF and 0.75mM ATP together were found to be permeable to PI, but not to trypan
15 blue (Figure 6). In contrast, control cells permeabilized with 70% ETOH were positive for both dyes (data not shown). Cells that were incubated with anti-RTF alone or 0.75mM ATP alone (Figure 6) showed no PI incorporation, while those incubated with both did incorporate PI. These results were confirmed using lucifer yellow (not shown). This demonstrates that anti-RTF and ATP together can induce the opening of P2Z
20 channels >0.9 kDa, and yet shows membrane integrity is still intact.

Example 12: RTF regulates IL-1 β secretion

To show that RTF regulates IL-1 β secretion, THP-1 macrophages were incubated with both 1mM and 3mM ATP, or anti-RTF and assayed for IL-1 β secretion
25 by ELISA. Since anti-RTF abrogates RTF function, our hypothesis was that anti-RTF would induce IL- β secretion, showing that RTF is a regulator for this molecule.

5x10⁵ THP macrophage cells were stimulated with 5ng/ml of LPS and 1x10⁵ PBMC for 2.5 hours. These cells were then washed and incubated in a final concentration of either 0 ATP, 3mM ATP, 1mM ATP, or anti-RTF for a period of 3
30 hours. The supernatants were assayed for IL-1 β by ELISA (R&D systems). pg IL-1 β per well was calculated from the optical densities. Results are expressed as the mean of 3 independent experiments.

As shown in Figure 7, cells incubated with media alone, secreted 61 ± 14 pg IL- 1β , forming the baseline. Cells incubated with 3mM ATP secreted 285 ± 25 pg ($78 \pm 5.1\%$ above baseline). Cells incubated with 1 mM ATP secreted 130 ± 44 pg IL- 1β (49 ± 5.6 above baseline). Cells incubated with anti-RTF alone secreted 114 ± 22 pg IL- 1β (an increase $47 \pm 3.5\%$), about the equivalent of cells incubated with 1mM ATP. This shows that anti-RTF alone can induce secretion, suggesting that RTF is a regulator of IL- 1β .

Example 13: Anti-RTF is cytotoxic to ovarian carcinoma cell

10 A2780 ovarian carcinoma cells were grown in 96 well plates until 100% confluent. Wells were identified, and cells were then incubated with 3mM ATP, 6mM ATP, 20 μ g of anti-RTF and 20 μ g of anti-RTF combined with 3mM ATP and 6mM ATP. Cells were incubated for 18 hours at 37°C in a 5% CO_2 environment. Cells were then harvested and assayed using flow cytometry for Caspase 3 activation as an
15 indicator for cell death. As shown in Figure 8, anti-RTF is significantly more cytotoxic than extracellular ATP at the indicated concentrations. Addition of extracellular ATP did not significantly enhance cytotoxicity.

Example 14. *In vivo* effect of anti-RTF

20 Female athymic nude mice purchased from Harlan Sprague Dawley approximately 11 weeks old. All mice were injected with 200 μ l of a 1:1 slurry of a MATRIGELTM mix containing 5×10^6 A2780 ovarian carcinoma cells. Tumor cells were transplanted subcutaneously in the upper flank of the right leg. Two mice were untreated and kept as a control to measure tumor growth. The remaining six mice were
25 injected with anti RTF antibody. All mice were left untreated for 1 week in order for a tumor and its vascular bed to become established. On the eighth day all mice that were treated were injected with 100 μ g of anti RTF antibody in a volume of 100 μ l of PBS. Injections were given in the tumor bearing flank of the animal. Antibody was not injected directly into the tumor, but was injected into the area of the tumor bed. Mice
30 received 300 μ g of antibody per week using a 28 gage needle. Mice were injected every Sunday, Tuesday and Friday. Injections took place over 5 weeks. The experiment was terminated when the control mice tumor burden became too obstructive for mice to

ambulate.

The median tumor volume of the control animals after 5 weeks without anti-RTF treatment was 8448 mm³. The median tumor volume for the anti-RTF treated animals(n=6) was 364 mm³. In two of the treated animals no measurable tumor was
5 observed (tumor volume range 0-1670). These results clearly indicate the possible usefulness of the antibody and or the inhibition of the RTF associated ATPase complex in tumor therapy

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art.
10 Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its intended advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

WHAT IS CLAIMED IS:

- 5 1. The use of an anti-Regeneration and Tolerance Factor (RTF) antibody
for inducing apoptosis in an ovarian carcinoma cell.
2. The use of claim 1 wherein the antibody is a monoclonal antibody or a
polyclonal antibody.

10

15

FIG. 1

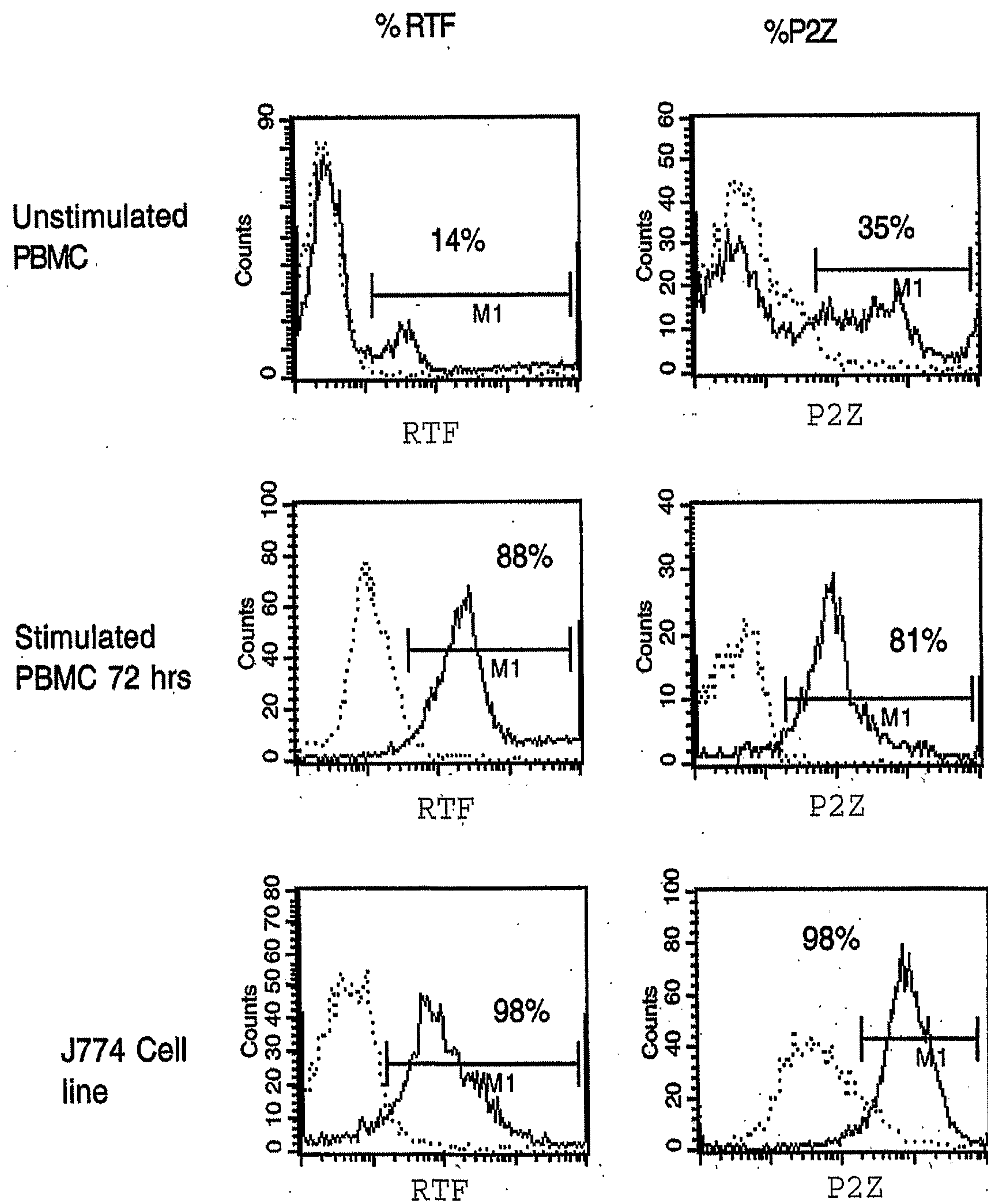


FIG. 2

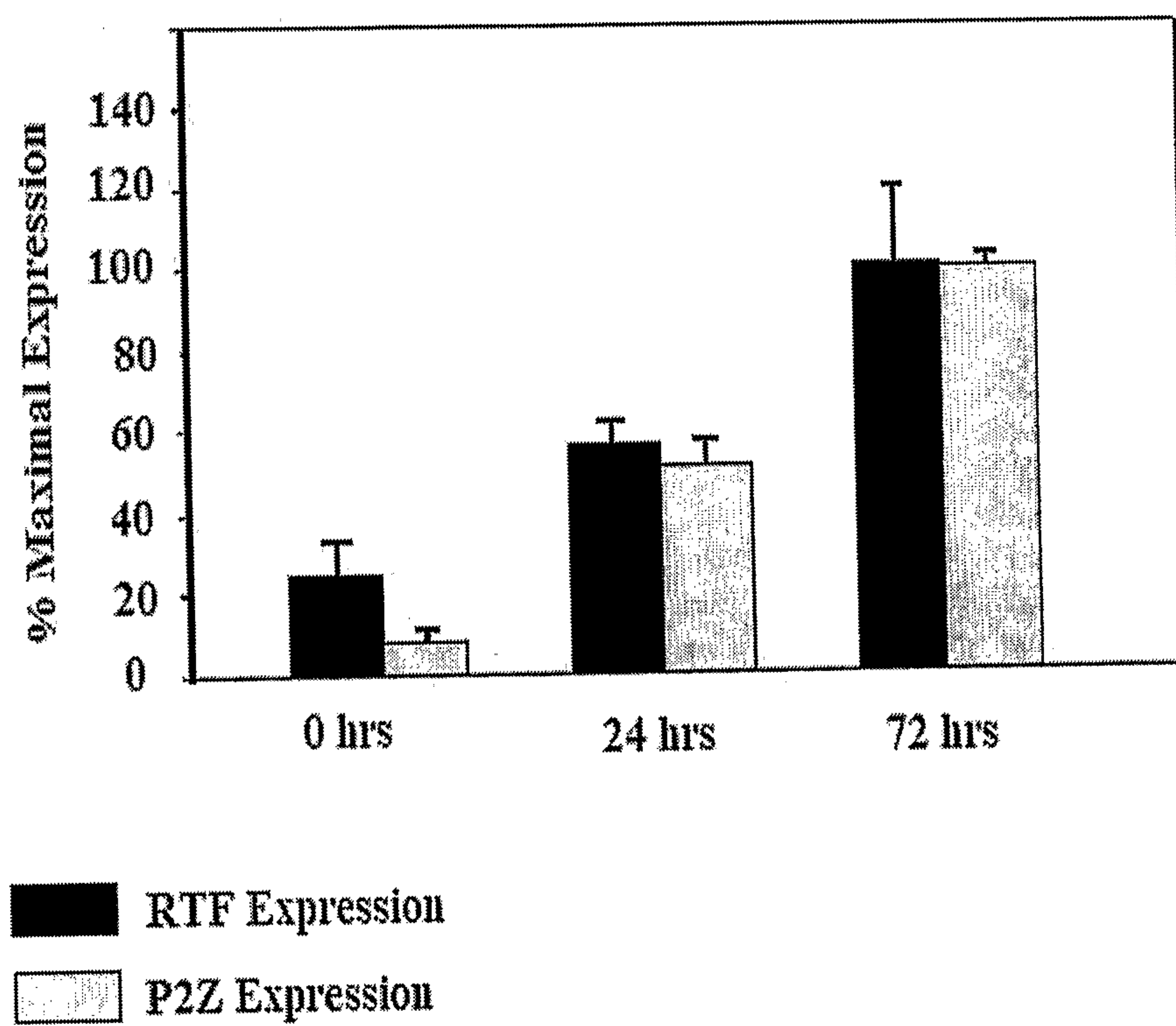


FIG. 3

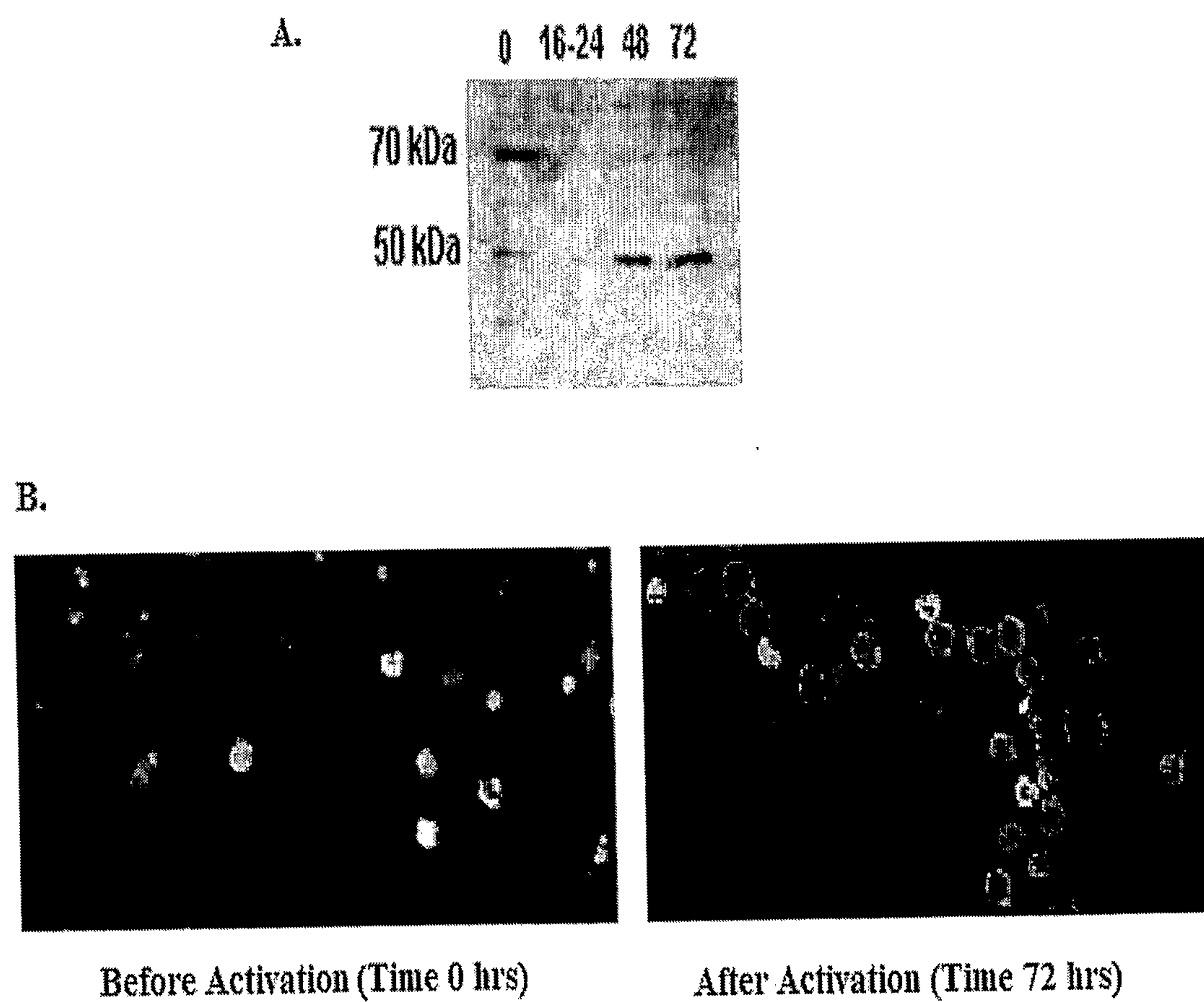
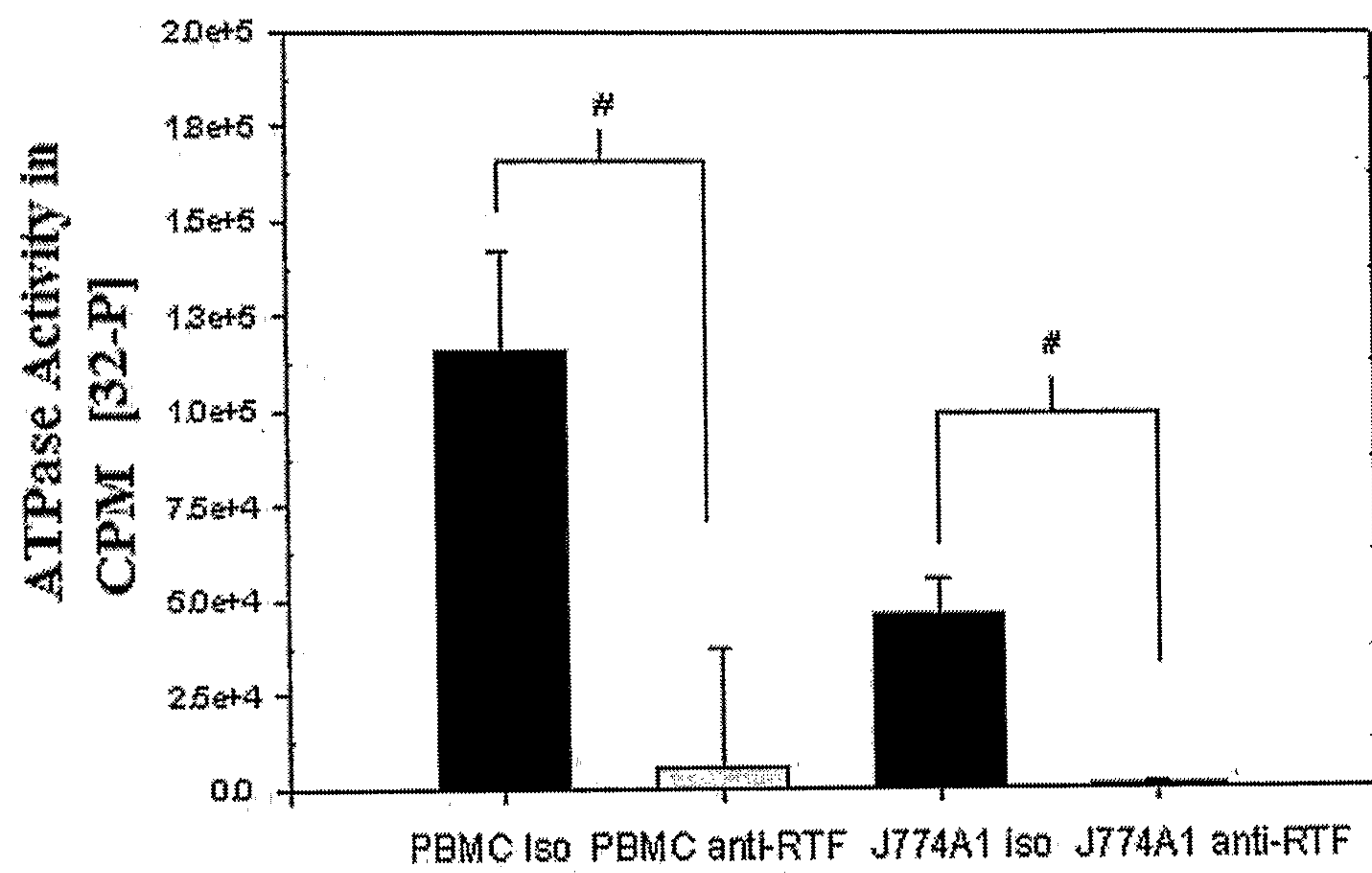


FIG. 4



■ Isotype control

▨ Anti-RTF

= P<0.001

FIG. 5

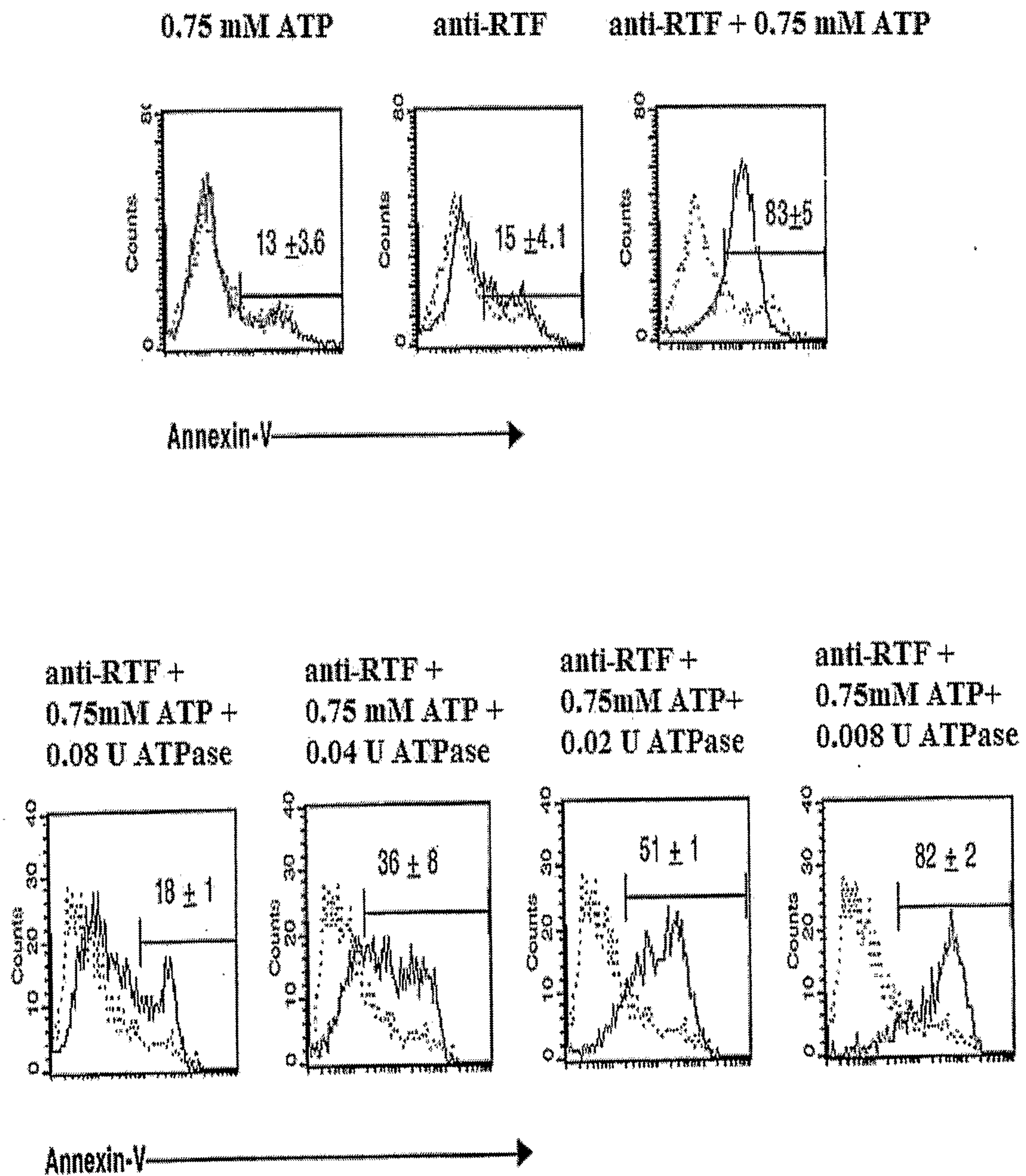


FIG. 6

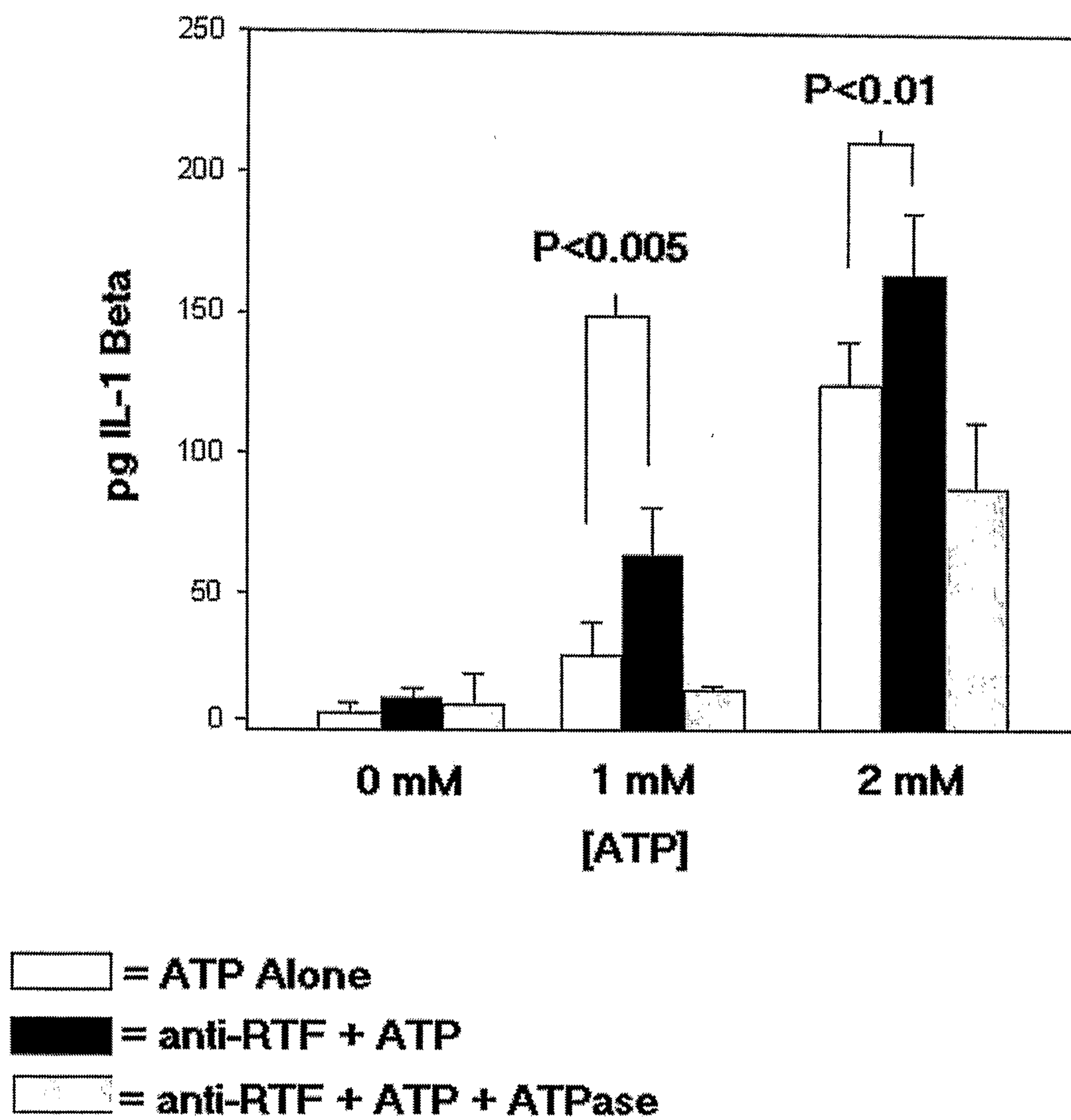


FIG. 7

Anti-RTF Antibody Is Cytotoxic To Ovarian Carcinoma Cells