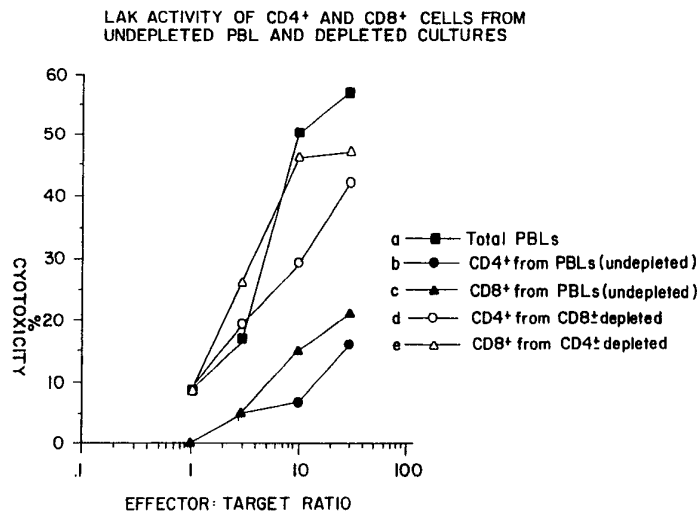




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<p>(21) International Application Number: PCT/US92/02873</p> <p>(22) International Filing Date: 6 April 1992 (06.04.92)</p> <p>(30) Priority data: 681,074 5 April 1991 (05.04.91) US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 681,074 (CIP) Filed on 5 April 1991 (05.04.91)</p> <p>(71) Applicant (for all designated States except US): REGENTS OF THE UNIVERSITY OF MINNESOTA [US/US]; Morril Hall, 100 Church Street, S.E., Minneapolis, MN 55455 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): OCHOA, Augusto, Carlos [CO/US]; 3872 Porter Street, N.W., Apt. F360, Washington, DC 20016 (US). GELLER, Robin, Lee [US/US]; 6920 Dawson Lane, Edina, MN 55435 (US). BACH, Fritz, H. [US/US]; 34 Park Lane, Minneapolis, MN 55416 (US).</p>		<p>(74) Agents: SAXE, Bernhard, D. et al.; Foley & Lardner, 1800 Diatonal Road, Suite 500, Alexandria, VA 22313 (US).</p> <p>(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, RU, SD, SE, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent), US.</p> <p>Published <i>With international search report.</i></p>

(54) Title: METHOD OF ENHANCING THE IMMUNOTHERAPEUTIC ACTIVITY OF IMMUNE CELLS BY DEPLETION/POSITIVE SELECTION OF CELL SUBSETS

**(57) Abstract**

The present invention provides a method for enhancing the immunotherapeutic activity, e.g., cytotoxicity, of immune cells by depleting immune cells of a cell subset that down-regulates the immune response, such as either CD4⁺ or CD8⁺ lymphocytes. The remaining depleted immune cells are then cultured in the presence of an antibody to a lymphocyte surface receptor, preferably an anti-CD3 monoclonal antibody (MoAb) and optionally in the presence of a cytokine, e.g., interleukin-2 (IL-2). The present invention also provides a method of enhancing the immunotherapeutic activity, e.g., cytotoxicity, of immune cells by culturing immune cells in the presence of IL-2 and an antibody to a lymphocyte surface receptor, preferably an anti-CD3 MoAb; separating a cell subset capable of developing immunotherapeutic activity, e.g., cytotoxicity, from the cultured immune cells, e.g., either CD4⁺ or CD8⁺ lymphocytes; and then subculturing the separated lymphocytes in the presence of IL-2.

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**METHOD OF ENHANCING THE IMMUNOTHERAPEUTIC
ACTIVITY OF IMMUNE CELLS BY DEPLETION/POSITIVE
SELECTION OF CELL SUBSETS**

Field of the Invention

5 This invention relates to the stimulation of the
immunotherapeutic activity of immune cells.
Specifically, this invention relates to the stimulation
of antitumor activity upon the depletion or positive
10 selection of specific cells or cell subsets of T
lymphocytes.

Background of the Invention

Peripheral blood mononuclear lymphocytes (PBLs)
can be stimulated to develop lytic activity against fresh
tumor cells, as well as several natural killer (NK)
15 resistant targets, such as Daudi and HL60, after
relatively short-term (3-5 days) culturing in the
presence of recombinant interleukin-2 (IL-2). See, for
example, M. Lotze et al., Cancer Res., 41, 4420 (1981);
C. Grimm et al., J. Exp. Med., 155, 1823 (1982); and E.A.
20 Grimm et al., "The Lymphokine-Activated Killer Cell
Phenomenon: In Vitro and In Vivo Studies," in
Interleukins, Lymphokines and Cytokines, S. Cohen and J.
Oppenheim, eds., Academic Press, New York, p. 739 (1983).
This function has been termed lymphokine activated killer
25 (LAK) activity.

Initial reports suggested that precursors of
cells with LAK activity did not express the T cell
receptor as determined by anti-CD3 binding. See, E.A.
Grimm et al., J. Exp. Med., 157, 884 (1983). More recent
30 reports have demonstrated that effector cells obtained
from short-term culturing with IL-2 (2-5 days) are a CD3⁻
population of cells that express the NK markers CD16
and/or CD56. The CD3⁺ cells from such cultures have low
lytic activity against NK-resistant targets. Thus, the
35 CD3⁻ population, with the NK markers CD16 and/or CD56, is
responsible for the great majority of the LAK activity in

PBL cultures. That is, CD3⁻ cells appear to be the classical NK effector cells. See, for example, J.R. Ortaldo et al., J. Exp. Med., 164, 1193 (1986); S. Ferrini et al., J. Immunol., 138, 1297 (1987); K. Itoh et al., J. Immunol., 136, 3910 (1986); and J.H. Phillips et al., J. Exp. Med., 164, 814 (1986).

Reversible induction of NK activity in cloned cytotoxic lymphocytes in response to IL-2 and interferon (IFN) has been reported. See, C.G. Brooks, Nature, 305, 155 (1983). Furthermore, the generation of large numbers of cells with LAK activity using relatively long-term cultures (10-30 days) of PBLs stimulated with the anti-CD3 monoclonal antibody (MoAb) OKT3, in combination with IL-2 has been reported (CD3-LAK cells or T-AK cells). See, A.C. Ochoa et al., J. Immunol., 138, 2728 (1987). The effector cells in these long-term IL-2 and OKT3 cultures include CD3⁻ cells, as well as a CD3⁺ population that is both CD4 and CD8 negative, and expresses the $\gamma\delta$ chains of the T cell receptor. In contrast, the effector cells in short-term IL-2 and OKT3 cultures (2-5 days) are predominantly CD3⁻ cells.

Numerous studies have shown that very little LAK activity appears to be mediated by the classically described CD4⁺ or CD8⁺ T cells. Furthermore, CD4⁺ or CD8⁺ cells isolated from cultures of mixed PBL populations, which are activated with an antibody to a lymphocyte surface receptor, such as the anti-CD3 monoclonal antibody OKT3, and continuously cultured with IL-2, do not develop significant levels of NK or LAK activity, as determined immediately upon their isolation from the total population. See, for example, A.C. Ochoa et al., Cancer Res., 49, 963 (1989). For example, at an effector to target ratio of about 30:1, i.e., a ratio of the number of T cells capable of mediating cytotoxicity to the number of tumor cell line targets, the cytotoxicity of CD4⁺ or CD8⁺ subsets is no more than about 15-20%.

It has been noted that when PBLs are stimulated in mixed lymphocyte culture (MLC), the CD4⁺ cells are minimally cytotoxic. Furthermore, when the CD4⁺ population is stimulated in MLC in the absence of other
5 T cells, they develop greater cytolytic activity. See, E.L. Reinherz et al., Proc. Natl. Acad. Sci. USA, 76, 4061 (1979). However, this cytotoxicity is antigen specific, and does not involve tumor killing activity.

It has also been recently shown that CD8⁺CD11b⁺
10 cells can develop LAK activity. In this specific situation, the CD8⁺ T cells were isolated from the PBL population before the initiation of culture in the presence of IL-2 alone. However, this method did not involve anti-CD3 MoAb stimulation; however, this method
15 did involve separating the T cells with sheep red blood cells, which in itself can produce a stimulating signal through the CD2 receptor. Thus, NK cells, which express the CD2 receptor, can also be activated. See, U. Dianzani et al., Eur. J. Immunol., 19, 1037 (1989).

20 CD4⁺ and CD8⁺ cells cultured in the presence of IL-2 alone have been shown to express the lytic machinery, but LAK activity was not demonstrated nor was cell growth reported. See, M.J. Smyth et al., J. Exp. Med., 171, 1269 (1990). Finally, it has been shown that
25 tumor infiltrating lymphocytes (TILs), which appear to be effective in the treatment of solid tumors, are primarily CD8⁺. See, for example, S. Shu et al., J. Immunol., 139, 295 (1987); and A. Belldegrun et al., Cancer Res., 48, 206 (1988).

30 The identification of cells that can mediate cytotoxicity, e.g., LAK activity, is important both for an understanding of the interactions of the immune system as well as for the potential development of effective methods of immunotherapy. One of the limitations of
35 current LAK therapies for the treatment of tumors is that LAK cells appear to be transported via the reticuloendothelial system which, in some cases, limits the accessibility of LAK cells to certain tumors. See,

both IL-2 and an antibody to a lymphocyte surface receptor for only the first 48 hours. Thereafter, the culturing preferably occurs in the presence of IL-2 without any additional amount of the antibody to a lymphocyte surface receptor. Alternatively, the depleted immune cell population can be stimulated by an antibody to a lymphocyte surface receptor and thereafter cultured with IL-2; however, in certain situations culturing with IL-2 may not be necessary.

The immune cell population can include all immune cells that are part of an immune system, such as T cells, B cells, NK cells, and macrophages. Any of these cells, or a combination of these cells, can be depleted by the method of the present invention, with a resultant increased immunotherapeutic, e.g., antitumor, effect. As used herein, the "immune cell population" can be, and is preferably, a total, i.e., unseparated or undepleted population as obtained from a whole blood sample; however, the "immune cell population" can be any portion of a total population that contains a cell subset or subpopulation that down-regulates the immunotherapeutic activity of the larger population, or is capable of developing immunotherapeutic activity itself.

Preferably, this method involves the depletion of T lymphocyte populations, e.g., PBL populations, before the initiation of culture with IL-2 and an antibody to a lymphocyte surface receptor. More preferably, the separated cells are CD4⁺ or CD8⁺ lymphocytes, or more specific subsets of each of these populations. The cytotoxicity, or antitumor activity enhanced can be specific or nonspecific lytic activity. Preferably, it is nonspecific lytic, e.g., LAK, activity.

As a result of the removal or depletion of specific cell subsets that inhibit antitumor activity, the remaining immune cells preferably develop increased immunotherapeutic activity as represented by levels of cytotoxicity generally equivalent to that seen in NK

populations in the same cultures. The cytotoxic activity of the cultured NK cells can in some situations also be increased above that of uncultured NK cells by the methods of the present invention. Thus, the remaining
5 immune cell population preferably develops an increased immunotherapeutic activity as represented by an increase in the level of cytotoxicity, i.e., antitumor activity, by a factor of at least about 1.2, and more preferably by about 2.0.

10 To effectuate this enhanced immunotherapeutic activity, e.g., increased antitumor activity, the culturing process of the depleted immune cell populations preferably occurs over a period of at least about two days, and more preferably over a period of at least about
15 ten days. According to specific preferred embodiments of the invention, the culturing process involves: stimulating the depleted immune cell population with an antibody to a lymphocyte surface receptor during the first 48 hours of culturing in a first medium that also
20 contains IL-2; removing the depleted immune cell population from the first medium; and subculturing the depleted immune cell population in a second medium that contains IL-2 without any additional amount of an antibody to a lymphocyte surface receptor. The antibody
25 to a lymphocyte surface receptor is preferably an anti-CD3 MoAb, i.e., an antibody against the antigen receptor complex CD3, such as OKT3.

Furthermore, a method has been developed to enhance the cytotoxicity of immune cells by first
30 culturing an immune cell population to form a cultured immune cell population; separating, i.e., positively selecting, a cell subset, or subpopulation, that is capable of developing immunotherapeutic activity, e.g., cytotoxicity; and separately culturing, i.e.,
35 subculturing, the separated cells in a second medium in the presence of IL-2.

Preferably, by using this method and positively selecting a cell subpopulation, the immunotherapeutic

activity, as represented by the cytotoxicity, of this subpopulation can be increased by a factor of at least about 1.2, preferably by a factor of at least about 2.0. Preferably, the separated cells are CD4⁺ or CD8⁺ lymphocytes, or subsets of each of these populations.

To effectuate this enhanced immunotherapeutic activity, e.g., increased antitumor activity, of immune cell subpopulations, the initial culturing process preferably involves the use of IL-2 and an antibody to a lymphocyte surface receptor to produce what is referred to as CD3-LAK cells or T-activated killer cells (T-AK). The initial culturing process of the unseparated or undepleted, i.e., total, immune cell populations preferably occurs over a period of at least about three days, and more preferably at least about five days. Alternatively, the initial culturing process can involve stimulation by an antibody to a lymphocyte surface receptor and thereafter cultured with IL-2; however, in certain situations culturing with IL-2 may not be necessary. The subsequent subculturing process in IL-2 of each cell subset preferably occurs over a period of at least about three days, and more preferably at least about ten days. These results are shown in Figures 1-5.

Brief Description of the Drawings

Fig. 1 illustrates the LAK activity (% cytotoxicity) of a CD8⁺-depleted PBL population, CD4⁺-depleted PBL population, and unseparated PBLs, stimulated with the anti-CD3 MoAb OKT3 and continuously cultured with IL-2.

Fig. 2 illustrates the LAK activity (% cytotoxicity) of: (a) a CD4⁺-depleted PBL population stimulated with the anti-CD3 MoAb OKT3 and continuously cultured with IL-2; (b) the CD8⁺ cells isolated from the cultured CD4⁺-depleted PBLs; and (c) the remaining CD4⁻ CD8⁻ cell population isolated from the cultured CD4⁺-depleted PBLs.

Fig. 3 illustrates the LAK activity (% cytotoxicity) of: (a) a CD8⁺-depleted PBL population stimulated with the anti-CD3 MoAb OKT3 and continuously cultured with IL-2; (b) the CD4⁺ cells isolated from the cultured CD8⁺-depleted PBLs; and (c) the remaining CD4⁺ CD8⁻ cell population isolated from the cultured CD8⁺-depleted PBLs.

Fig. 4 illustrates the LAK activity (% cytotoxicity) of: (a) an undepleted, i.e., unseparated or total, PBL population stimulated with the anti-CD3 MoAb OKT3 and continuously cultured with IL-2; (b) the CD4⁺ cells isolated from the cultured undepleted PBLs; (c) the CD8⁺ cells isolated from the cultured undepleted PBLs; (d) CD4⁺ cells isolated from a cultured CD8⁺-depleted PBL population; and (e) CD8⁺ cells isolated from a cultured CD4⁺-depleted PBL population.

Figs. 5A and 5B illustrate the LAK activity (% cytotoxicity) of CD4⁺ and CD8⁺ cells, respectively, isolated from cultured (OKT3 + IL-2) undepleted PBL populations and subsequently cultured with IL-2 alone. This data was obtained after five days of culturing the undepleted PBLs, i.e., "day of sort," and after ten and thirteen days of culturing each cell subset in IL-2.

Detailed Description of the Invention

As used herein, LAK activity is defined as the ability of lymphocytes to lyse tumor cells, and to a lesser degree normal cells. This activity in lymphocytes is typically stimulated by lymphokines, such as IL-2. In the examples herein, LAK activity refers to the ability to lyse the human NK-resistant tumor target, HL60. NK activity is defined as the ability to lyse tumor cells, but not normal cells, which does not result from prior stimulation. In the examples herein, NK activity refers to the ability to lyse the human tumor line K562. Since similar results were obtained with both tumor lines, only the LAK results with HL60 are shown.

As used herein, the "immune cell population" can be, and is preferably, a total, i.e., unseparated or undepleted population as obtained from a whole blood sample; however, the "immune cell population" can be any
5 portion of a total population that contains a cell subset or subpopulation that down-regulates the immunotherapeutic activity of the larger population, or is capable of developing immunotherapeutic activity itself. The immune cell population can include all
10 immune cells that are part of an immune system, such as T cells, B cells, NK cells, and macrophages. Any of these cells, or a combination of these cells, can be depleted by the method of the present invention, with a resultant increased immunotherapeutic, e.g., antitumor,
15 effect. The immune cell population can be obtained from many different tissues or fluids including spleen tissue, peripheral blood, tumor tissue, lymphnode tissue, cerebrospinal fluid, pleural effusions ascites, etc.

As used herein, "depleted immune cell
20 population" preferably refers to a total cell population derived from a sample of whole blood with at least one cell subset or subpopulation, which down-regulates the immunotherapeutic activity of the total population, removed therefrom. Alternatively, however, "depleted
25 immune cell population" can be a subpopulation or subset itself, which upon further removal of a cell type can exhibit an enhanced immunotherapeutic activity.

As used herein, "culturing" indicates the process whereby cells are placed in a tissue culture
30 medium comprising nutrients to sustain the life of the cells, and other additives, such as the growth factor IL-2. This process can take place in any vessel or apparatus. The process can involve various stages of culturing and subculturing. Typically, cells are
35 initially cultured and expanded, i.e., increased in size and number. These expanded cells are then counted and divided into groups, or subcultures, for further culturing and expansion. The expanded cells from each of

these subcultures are then divided into additional subcultures, for further culturing and expansion. Each of the culturing or subculturing steps typically lasts about 48 hours, with fresh tissue culture medium used in
5 each culture.

As used herein "immunotherapeutic" refers to any of a variety of immune responses of immune cells. This includes a cytotoxic or antitumor effect. As used herein "cytotoxicity" and "antitumor activity" are used
10 interchangeably and include specific lytic activity, and the nonspecific lytic activity of lymphokine activated killer (LAK) cells and natural killer (NK) cells. In general, the methods of the present invention are preferably directed to enhancing the cytotoxicity, i.e.,
15 antitumor activity, of immune cells, preferably T lymphocytes.

Depletion of Cell Subpopulations

The depletion of at least one cell subset, or subpopulation, such as CD4⁺, or CD8⁺ cells, or specific
20 subsets of either of these cell populations, that is capable of down-regulating (i.e., preventing the effector cells from developing the immunotherapeutic, e.g., cytolytic, machinery) the immunotherapeutic activity of an immune cell population, from the total immune cell
25 population results in the development of enhanced immunotherapeutic activity, as represented by increased cytotoxicity. Specifically, the depletion of a T lymphocyte subpopulation, that inhibits the antitumor activity of a total immune cell population, from the
30 total immune cell population results in the development of high levels of LAK activity in the remaining "depleted" immune cells.

This effect occurs in response to culturing the depleted immune cell population in the presence of
35 interleukin-2 (IL-2) and an antibody to a lymphocyte surface receptor. Preferably, this occurs in response to initial stimulation with an antibody to a lymphocyte

surface receptor and continuous culturing, or subculturing, in the presence of IL-2. That is, preferably the cells are cultured in a first tissue culture medium with IL-2 and the lymphocyte surface receptor antibody. Thereafter, the cells are cultured, or subcultured, in a second tissue culture medium with IL-2 but without any additional amount of an antibody to a lymphocyte surface receptor. Further cultures, or subcultures, in the presence of IL-2 can also occur. Alternatively, the cells can be stimulated with an antibody to a lymphocyte surface receptor, and then optionally cultured with IL-2.

Although the lymphocyte surface receptor antibody is preferably not added to any of the subcultures after the first 48 hours, it can be present in each of the subsequent subcultures if the cultured cells from the first culture are not washed before the addition of a second tissue culture medium containing IL-2 without any surface receptor antibody. Any protocol, however, for culturing immune cells in which the immune cells are in the presence of both IL-2 and an antibody to a lymphocyte surface receptor at any time and for any period of time in the overall course of the culturing process is also within the scope of the present invention. Furthermore, any protocol in which the cells are stimulated with the antibody alone in the tissue culture media without any further IL-2 culturing is within the scope of the present invention.

In the present invention the cells are preferably cultured with IL-2 for at least about 2 days, more preferably for at least about ten days. Similar results have been obtained from cells cultured in the presence of IL-2 for as long as 30 days, with subculturing occurring approximately every 48 hours. As stated above, the cells are preferably stimulated with an antibody to a lymphocyte surface receptor during the first 48 hours of culture. Stimulation of cells with

antibody, with or without IL-2, can be for as short a time as 30 minutes, overnight, preferably 2-4 days.

The antibody to a lymphocyte surface receptor can be any of a variety of monoclonal antibodies against a surface antigen receptor complex. Useable antibodies include an anti-CD2, anti-CD4, anti-CD5, anti-CD28, anti-CD11b, etc., monoclonal antibody (MoAb). The antibody used in the present invention is preferably an anti-CD3 monoclonal antibody. The antibodies can be used alone or in various combinations with other antibodies. For example, anti-CD3 can be used in combination with anti-CD2, anti-CD4, anti-CD5, anti-CD28, or anti-CD11b, for effective results. Anti-CD3 or anti-CD2 can each be used individually as the antibody in the cultures. The anti-CD3 MoAb can be, but is not limited to, OKT3, WT32, Leu-4, SPV-T3c, RIV9, 64.1, etc. More preferably, the anti-CD3 MoAb is OKT3, which is available from Ortho, a division of Johnson & Johnson.

The interleukin-2 (IL-2) is a commercially available T cell growth factor. It can be a naturally occurring IL-2 or it can be recombinant IL-2. It is believed that other cytokines, including lymphokines, can also be used in the present invention to provide the lymphokine activated cells. These include IL-1, IL-4, IL-6, TNF, GM-CSF interferons, etc. It is envisioned that they can be used alone, in sequence, or in combination with IL-2 in the culturing media.

The immune cells, preferably T lymphocytes, and more preferably peripheral blood mononuclear lymphocytes can be depleted of specific T cell subsets by any method. Preferably, the PBLs are depleted of specific subsets by negative depletion using magnetic beads. Typically, this involves the labelling of the PBLs with an antibody to a lymphocyte surface receptor for the T cells that are to be removed from the total PBL population. This mixture of labelled and unlabelled cells are then mixed with goat anti-mouse IgG-coated magnetic beads. A complex of the beads and the labelled T cells, i.e., those cells

complexed with the surface receptor antibody, is formed. The beads/labelled T cell complexes are then separated from the mixture using a magnetic separator. In this way, a specific T cell subset, or portion thereof, can be removed from the PBL mixture.

The specific immune cell subset removed can be any that down-regulates the immunotherapeutic activity, preferably the cytotoxic activity, of the total immune cell population. This can include: CD4⁺, or any of its subsets such as 2H4 or 4B4; CD8⁺, or any of its subsets; NK cells, or any of its subsets; macrophages; B cells; and the like. Preferably, the immune cell subsets removed are T cell subsets, and more preferably they are CD4⁺ or CD8⁺ cells.

In general, a typical sample of PBLs from a sample of human whole blood contains about 20-30% CD8⁺ cells and about 30-50% CD4⁺ cells. In order to increase the immunotherapeutic activity, e.g, antitumor activity, of an immune cell population according to the present invention, the cells that inhibit or down-regulate the immunotherapeutic activity of the population need only be removed until an increase in the immunotherapeutic activity, as represented by cytotoxicity, by a factor of about 1.2 is observed in the remaining cell population. Preferably, in order to increase the immunotherapeutic activity of immune cells according to the present invention, the number of CD4⁺ or CD8⁺ cells are reduced in the depleted immune cell populations by at least about 75%, more preferably by at least about 90%. Most preferably, however, a "substantially completely depleted" immune cell populations, e.g., PBL populations, contain less than about 5% of the cell subset removed. For example, a "substantially completely CD4⁺-depleted immune cell population" contains less than about 5% CD4⁺ cells. Thus, the method of the present invention includes separating preferably at least about 75%, and more preferably at least about 90% of the CD4⁺ or CD8⁺

cells from PBLs to increase the immunotherapeutic activity, e.g., antitumor activity, of the remaining "depleted" cell population.

5 The increased immunotherapeutic activity of the immune cell populations is determined in vitro by the percent cytotoxicity, which is a measure of the ability of immune cells to destroy a radioactively labeled tumor target. That is, the antitumor activity is determined by a comparison of the level of radioactivity released in
10 tissue culture media from the effector/target combination to the level of radioactivity in the culture media released from the target alone. Thus, increased immunotherapeutic activity, as defined herein, of immune cells is typically demonstrated by an increase in the
15 percent cytotoxicity of the effector cells on human tumor cells. The human tumor cell lines can be any of a variety of cell lines commercially available, including leukemia cells and fresh tumor targets. Preferably, they are leukemia cells.

20 According to specific embodiments of the present invention, increased antitumor activity is demonstrated by an increase in the percent cytotoxicity level by a factor of at least about 1.2. Preferably, antitumor activity is demonstrated by an increase in the percent
25 cytotoxicity by a factor of at least about 2.0.

As can be seen from Figure 1, the amount of cytolytic activity of either the CD4⁺-depleted or the CD8⁺-depleted cultures is greater than that of unseparated, i.e., total or undepleted, PBLs regardless
30 of the effector to target ratio. The LAK activity was measured at several time points between 10 and 30 days of culture, with similar results regardless of the day of assay. Similar results are obtained with CD4⁺-depleted and CD8⁺-depleted populations in short-term, i.e., about
35 three to five day, cultures. It is envisioned that shorter time periods for culturing will produce similar results.

These observations could be explained in several manners. For example, the enhanced LAK activity could be the result of a relative enrichment in CD3⁺CD16⁺ and/or CD56⁺ NK cells, or CD3⁺CD4⁺CD8⁻ ($\gamma\delta$) T cells, both of which have been previously shown to mediate LAK activity. It has been determined, however, that the enhanced LAK, as well as NK activity, is preferably the result of the activation of the cell subpopulations, i.e., CD4⁺ or CD8⁺ T cells, that remain in the depleted cultures.

This was determined by labelling the cells from the depleted PBL populations with anti-CD8 MoAb (in the CD4⁺-depleted cultures) or anti-CD4 MoAb (in the CD8⁺-depleted cultures), and positively sorting them using a fluorescence-activated cell sorter (FACS). When cells from CD4⁺-depleted cultures are separated into CD8⁺ and CD4⁻CD8⁻ populations, and tested for LAK and NK activity immediately after sorting, both CD8⁺ cells and CD4⁻CD8⁻ cells mediate significant levels of LAK and NK activity.

As shown in Figure 2, the CD4⁻CD8⁻ cells demonstrate about 65% cytotoxicity at an effector to target ratio of about 30:1. The CD8⁺ cells demonstrate about 40% cytotoxicity at an effector to target ratio of about 30:1. Similar results are observed when CD8⁺-depleted populations are labelled with anti-CD4 MoAb and sorted into CD4⁺ and CD4⁻CD8⁻ populations (Figure 3). Additional phenotyping of the CD4⁻CD8⁻ cells in both types of cultures show them to be primarily CD16⁺Leu19⁺ or CD3⁺CD4⁻CD8⁻.

In comparison, however, when cultured with IL-2 and an antibody to a lymphocyte surface receptor in an undepleted population of PBLs, isolated CD4⁺ or CD8⁺ cells do not develop such significant levels of NK or LAK activity. As can be seen by the results shown in Figure 4, at an effector to target ratio of about 10:1, the cytotoxicity of CD4⁺ or CD8⁺ cell subsets is no more than about 10-15%, and at an effector to target ratio of about

30:1, the cytotoxicity of CD4⁺ or CD8⁺ cell subsets is no more than about 15-20%.

Results similar to those shown in Figure 4 were obtained at various times between 10 and 30 days of culturing undepleted PBLs with IL-2 and the anti-CD3 MoAb OKT3. The CD4⁺ and CD8⁺ cells were isolated by negative depletion, however it is also possible to isolate them by positive FACS selection. Control cultures of CD4⁺-depleted or CD8⁺-depleted cells were sorted to obtain CD8⁺ and CD4⁺ cells, respectively. As shown in Figure 4, CD8⁺ and CD4⁺ cells from the depleted cultures developed significant levels of LAK activity, i.e., at least about 40% cytotoxic activity at an effector to target ratio of about 30:1. Thus, although CD4⁺ and CD8⁺ cells do not show significant LAK or NK activity when tested immediately after isolation from CD3-LAK cultures, CD4⁺ and CD8⁺ cells can develop high LAK activity if one of these subsets is depleted from the PBL population prior to the initiation of culture.

Although not intending to be limiting in any manner, these results suggest that the development of LAK activity by T cells subpopulations is inhibited in the PBL cultures. It is believed that this inhibitory effect is the result of T cells, and possibly other immune cells, such as macrophages or B cells, that prevent the development of LAK activity by the other T cell subsets. Furthermore, it is believed that the inhibitory T cells generally exert their effect only if present throughout the entire culture period.

Positive Selection of Cell Subsets

Cell subpopulations, such as CD4⁺ or CD8⁺ cells, or specific cell subsets of these populations, separated from CD3-LAK cells, i.e., T-AK cells, show negligible LAK activity. These T-AK cells are typically cultured in the presence of IL-2 and an antibody to a lymphocyte surface receptor for about five days. It has been determined,

however, that if the CD4⁺ and CD8⁺ cells are subsequently cultured separately in the presence of IL-2 alone, each individual population rapidly develops LAK activity.

5 The initial culturing process of the undepleted PBL populations preferably occurs over a period of at least about three days, and more preferably at least about five days. The subsequent culturing process in IL-2 of each cell subset preferably occurs over a period of at least about three days, and more preferably at least
10 about ten days. The subsequent culturing process can be carried out for up to about 30 days.

The subsequent culture of the separate CD4⁺ and CD8⁺ cell populations is done in the presence of preferably about 10-1000 units/ml IL-2, more preferably
15 about 100-1000 units/ml IL-2. Upon being tested for LAK activity at various times throughout the culturing process of the separate cell subsets, both populations rapidly develop and maintain high levels of NK and LAK activity.

20 Specific immune cells, preferably T lymphocytes, can be separated from an unseparated, i.e., total population of immune cells, preferably PBLs by any method. Preferably, the specific cell subpopulations are separated from the total populations by positive
25 selection using fluorescence-labelled monoclonal antibodies. Typically, this involves adding a fluorescein isothiocyanate-conjugated MoAb or a phycoerythrin-conjugated MoAb to a cultured immune cell population, incubating the cells with the conjugate for
30 30 minutes at 4°C, washing the cells, and sorting or selecting out the labelled cells using a fluorescence-activated cell sorter. For positively selecting CD8⁺ cells the monoclonal antibody OKT8 can be used, and for positively selecting CD4⁺ cells the monoclonal antibody
35 OKT4 can be used, both of which are available from the Ortho Division of Johnson & Johnson.

As can be seen in Figure 5, both the CD4⁺ and CD8⁺ cell subsets show enhanced antitumor activity after

ten days of culturing in IL-2. Although not shown, enhanced antitumor activity is seen after only three days of culturing in IL-2. Thus, using this methodology both CD4⁺ and CD8⁺ T cells can develop high levels of LAK activity.

Although this is in no way limiting, the results of the depletion and positive selection experiments suggest that the inhibition involves some ongoing regulatory interaction between CD4⁺ and CD8⁺ cells that is effectively abolished once they are separated, and that it is not due to an event in the initial activation process. That is, the observation that either CD4⁺ or CD8⁺ T cells can rapidly acquire LAK activity once isolated from PBL cultures suggests that the absence of expression of LAK activity by these cells in the unseparated populations is not the result of an irreversible process. Rather, the inhibition requires the continued interactions by the reciprocal T cell subsets. Mixing experiments designed to test this hypothesis demonstrate that the maintenance of a suppressor effect requires the continued interaction of viable metabolically active T cell subsets.

The inhibition could be mediated through direct cell contact or via soluble factors. Regulatory networks have been described in which both T cell subsets must be present in order to obtain suppression of function [N.K. Damle et al., *J. Exp. Med.*, 158, 159 (1983)]. These data suggest that there are inhibitory signals which prevent the development of LAK activity by CD4⁺ or CD8⁺ T cells in unseparated, i.e., total or undepleted, PBL populations. That is, there is a negative regulation of T cell function in PBL populations that appear to be mediated by the T cells themselves.

There are several soluble factors which might be involved in regulating the development of LAK activity by T cells, including interleukin-4 (IL-4) and transforming growth factor- β (TGF- β). It is, of course,

not necessary that both T cell subsets are regulated by the same factor.

IL-4 has been shown to inhibit both the growth and development of effector functions by LAK cells, although those effects appear to be principally on NK cells rather than T cells. See, for example, M.B. Widmer et al., J. Exp. Med., 166, 1477 (1987); H. Spits et al., J. Immunol., 141, 29 (1988); A. Nagler et al., J. Immunol., 141, 2349 (1988); Y. Kawakami et al., J. Exp. Med., 168, 2183 (1988). IL-4 is made primarily by CD4⁺ T cells, which suggest that it could play a role in regulating the development of LAK activity in CD8⁺ cells; however, this is not intended to be limiting in any way. See D.B. Lewis et al., Proc. Natl. Acad. Sci. USA, 85, 9743 (1988).

TGF- β also has been shown to inhibit both NK and LAK activity. See, for example, A. Kasid et al., J. Immunol., 141, 690 (1988); and J.J. Mule et al., Cancer Immunol. Immunother., 26, 95 (1988). Furthermore, this inhibition has, in some cases, been shown to be based on the balance between the levels of IL-2 and TGF- β . See, for example, J.H. Kehrl et al., J. Exp. Med., 163, 1037 (1986).

The addition of human TGF- β_1 (TGF- β), which is available from R & D Systems, Minneapolis, MN, to either CD4⁺-depleted or CD8⁺-depleted populations upon the initiation of the culturing process results in the inhibition of the lytic function of the depleted populations. Specifically, the addition of TGF- β in concentrations ranging from 0.1-30 ng/ml upon initiating the culture, and during each of the subculturing steps, demonstrates a dose-dependent decrease in the lytic activity, i.e., LAK activity, of the T cells. This effect is reversible upon the removal of TGF- β from the culture medium.

TGF- β is produced by both CD4⁺ and CD8⁺ cells under certain culture conditions. Given that both T cell subsets produce TGF- β , it is possible that it is only

when both CD4⁺ and CD8⁺ cells are present that the level of TGF- β produced reaches the point where it has an inhibitory effect in the presence of high levels of IL-2. After depletion of either T cell subset, the levels of TGF- β would be too low to have an inhibitory effect on the T cells; however, this is not intended to be limiting in any way.

The observations that either CD4⁺ or CD8⁺ cells can, under the appropriate conditions, develop LAK activity and that the generation of that LAK activity is regulated by the presence of the reciprocal T cell subset, have implications for protocols for adoptive immunotherapy. For example, under certain conditions it is preferable to have LAK activity mediated by T cells. Thus, the present invention opens the possibility of including T cells with LAK activity in future clinical trials. Such cells could possibly achieve complementary or different antitumor effects than have been observed in conventional protocols.

The following examples are set forth as representative of specific and preferred embodiments of the present invention. These examples are not to be construed as limiting the scope of the invention in any manner. It should be understood that many variations and modifications can be made while remaining within the spirit and scope of the invention.

Example 1

Isolation and Culture of Cells with LAK Activity

Peripheral blood lymphocytes (PBLs) were isolated from heparinized venous blood (human whole blood) by centrifugation over Ficoll-Hypaque according to the method of A. Boyum, Scand. J. of Clin. Lab. Invest., 99, 77 (1968), which is incorporated herein by reference. Isolated mononuclear cells were washed three times with phosphate buffered saline (PBS, pH 7.4) (GIBCO Laboratories, Grand Island, NY) and counted. CD4⁺ and

CD8⁺ enriched cultures were obtained by negative depletion using magnetic beads (obtained from Baxter Healthcare Corporation, Deerfield, IL; also available from Advanced Magnetics, Massachusetts; or Dynal Corp., Norway). Briefly, PBLs were labelled by incubation with either of the monoclonal antibodies OKT4 or OKT8 (Ortho, Raritan, NJ) for 30 minutes on ice. The cells were then washed twice with cold PBS and mixed with goat anti-mouse IgG-coated magnetic beads (obtained from Baxter Healthcare; also available from Dynal Corp., Norway) at a bead:cell ratio of 10:1. The bead/cell mixture was incubated for 30 minutes at 4°C while rotating at 5-6 rpm. At the end of the incubation, the bead/cell suspension was diluted two-fold with cold PBS. Using a magnetic separator (Baxter Healthcare Corporation, Deerfield, IL), the beads were allowed to collect against the side of the test tube for five minutes. The supernatant containing unbound cells was then collected and transferred to a new tube. This process was repeated three times to completely remove the beads and bead-bound cells. The cells that remained in suspension (CD4⁺-depleted or CD8⁺-depleted PBLs) were washed and counted. This resulted in depleted PBL populations with less than about 5% contamination by the T cell subset removed. The cells remained essentially unchanged during subsequent culture.

Peripheral blood lymphocytes or depleted populations (5×10^6 cells) were cultured in 25 cm² flasks (Corning, Corning, NY) in 10 ml tissue culture medium (TCM). The TCM consisted of Rosewell Park Memorial Institute (RPMI) 1640 medium (available from GIBCO, Grand Island, NY) supplemented with 25 mM Hepes [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (penicillin/streptomycin mix available from GIBCO, Grand Island, NY), and 5% pooled heat-inactivated human serum. The cultures were supplemented with 1000 units/ml of highly purified recombinant human IL-2 from

E. coli (Hoffman-LaRoche, Nutley, NJ). [See, A. Wang et al., Science, 224, 1431 (1984); and S.A. Rosenberg et al., Science, 223, 1412 (1984), which are incorporated herein by reference]. The cultures were supplemented
5 with 10 ng/ml of the anti-CD3 MoAb OKT3 (Ortho Division, Johnson & Johnson, Raritan, NJ). The OKT3 was present in the culture during the first 48 hours. Thereafter, the OKT3 was diluted due to the addition of fresh TCM and IL-2. No additional OKT3 was added during the culturing
10 process. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂. After the first 48 hours of culture, the cells were counted and subcultured at 0.5 x 10⁶ cells/ml in TCM containing IL-2. Subsequently, the cells were counted and subcultured every 48 hours in
15 fresh TCM with IL-2 at a concentration of 0.5 x 10⁶ cells/ml.

Example 2

Cell Sorting by Immunofluorescence

PBLs, CD4⁺-depleted PBLs, or CD8⁺-depleted PBLs
20 were each cultured in OKT3 + IL-2 as described above. At various times during the culture period, the cells were sorted on a fluorescence-activated cell sorter (FACS). PBL populations were labelled with fluorescein isothiocyanate- (FITC) conjugated or phycoerythrin- (PE)
25 conjugated MoAb: OKT4 and OKT8 (Ortho). CD4⁺-depleted populations were labelled with OKT8, and CD8⁺-depleted populations were labelled with OKT4. The cells were incubated for 30 minutes at 4°C and were then washed twice with cold PBS containing 2% fetal bovine serum.
30 Cells were sorted on a FACS IV (Becton Dickinson, Mountain View, CA). Sorted cells were centrifuged and an aliquot retained to test the purity of the populations. All of the positively sorted populations used for determining LAK activity were more than 97% positive for
35 the desired surface marker.

Example 3Cell-Mediated Lympholysis (CML)

CML assays were done as described in S.-L. Wee et al., Hum. Immunol., 3, 45 (1981), which is incorporated herein by reference. Human tumor lines K562 (chronic myelogenous leukemia, obtained from American Tissue Type Culture Collection (ATTCC)) and HL60 (promyelocytic leukemia, ATTCC) were maintained in culture in RPMI 1640 with 10% fetal bovine serum (GIBCO, Grand Island, NY). Cells were subcultured at 0.5×10^6 /ml in fresh medium twice a week.

Cells of the line HL60 were not lysed by unstimulated PBLs and were therefore considered NK-resistant. LAK activity was measured as cytolytic against the NK-resistant targets HL60. NK activity was measured as cytolytic activity against the K562 targets.

Tumor cell line targets were labelled with 250-750 μ Ci of $\text{Na}^{51}\text{CrO}_4$ (5000 μ Ci/ml, New England Nuclear, Boston, MA) for one hour at 37°C. These cells were washed three times in TCM, resuspended in culture media that did not contain IL-2, counted, and aliquoted at 500 targets/well in a 96-well V bottom plate (Costar, Cambridge, MA) into which the effector cells, i.e., the peripheral blood lymphocytes or depleted populations cultured as described above in Example 1, had been previously aliquoted at set concentrations. The effector:target cell ratios ranged from 30:1 to 1:1. Plates were centrifuged at 65 g for five minutes and incubated in 5% CO_2 at 37°C for four hours, after which 100 μ l of media was harvested from each well into a scintillation vial with 2.5 ml of scintillation fluid (Biofluor, New England Nuclear, Boston, MA). Radioactivity was counted on a liquid scintillation counter (LKB, Turku, Finland).

Percent cytotoxicity was determined by the following equation (cpm = counts per minute):

$$\frac{(\text{experimental mean cpm}) - (\text{spontaneous release mean cpm})}{(\text{maximal release mean cpm}) - (\text{spontaneous release mean cpm})} \times 100$$

wherein "spontaneous release mean" is defined as the amount of ^{51}Cr released from target cells alone (background); "maximal release" is the total ^{51}Cr in the targets following lysis with a detergent such as Triton X-100; and "experiment mean" is the ^{51}Cr released in wells with targets and effectors.

Representative samples of the results of all experiments are displayed in Figures 1-5. Each data point in each figure represents an analysis of three separate samples analyzed after the same period of culture, i.e., between 10 and 30 days, and the same period of contact between the effectors and targets, i.e., about four hours.

Example 4

Immunophenotyping

Cells (1×10^6) were washed 3 times with HBSS after which they were incubated at 4°C with $20 \mu\text{l}$ of the corresponding monoclonal antibody (OKT4 or OKT8 from Ortho, Rareton, New Jersey). They were again washed 3 times with cold Hanks Balanced Saline Solution (HBSS) including 2% fetal calf serum and resuspended in 0.2% paraformaldehyde. Two color fluorescence measurements were performed on a Coulter Profile (Coulter Cytometry, Hialeah, FL) or a FACS IV (Becton Dickinson, Mountain View, CA).

Example 5

Mixing Experiments

CD4^+ or CD8^+ cells isolated from unseparated PBL T-AK cultures on day 5 were added at a 1:1 ratio into CD4^+ -depleted, or CD8^+ -depleted, populations, respectively, on day 0 of autologous cultures. Some of the cultures received irradiated cells. Lytic function was tested 4 days later as % cytotoxicity. The results as shown below in Table 1 demonstrated that the addition of nonviable irradiated cells did not prevent the development of lytic activity. However, the addition of

non-irradiated, i.e., metabolically active, CD4⁺ or CD8⁺ cells completely suppressed the development of LAK activity by the CD4⁺-depleted or CD8⁺-depleted populations, respectively.

5

Table 1

**CD4⁺ and CD8⁺ Cells From PBL Cultures Inhibits
the Development of Lytic Function by CD4⁺-
Depleted and CD8⁺-Depleted Cultures^a**

	<u>% Cytotoxicity</u>
	<u>30:110:13:1</u>
10	
CD4 ⁺ -Depleted	38 26 15
CD4 ⁺ -Depleted + CD4 ⁺ from PBL	-4 -1 1
CD4 ⁺ -Depleted + irrad. CD4 ⁺ ^b	42 26 11
15	
CD8 ⁺ -Depleted	25 14 9
CD8 ⁺ -Depleted + CD8 ⁺ from PBL	-1 2 -4
CD8 ⁺ -Depleted + irrad. CD8 ⁺ ^b	46 30 15

^a CD4⁺ and CD8⁺ cells were positively sorted out of PBL stimulated with OKT3 + IL-2 and cultured for 5 days. The cells were added at a 1:1 ratio to autologous cultures which had been depleted of CD4⁺ or CD8⁺ cells, respectively. Lytic function was assayed on cell line HL60.

20

^b Isolated CD4⁺ and CD8⁺ cells received 2500 rads.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention. The relevant portions of the references cited herein are incorporated by reference.

25

30

WHAT IS CLAIMED IS:

1. A method of enhancing the immunotherapeutic activity of immune cells comprising the steps of:

5 (a) separating at least one cell subpopulation from an immune cell population; wherein the cell subpopulation removed is capable of down-regulating the immunotherapeutic activity of the immunopopulation; and

10 (b) culturing the depleted immune cell population in the presence of an antibody to a lymphocyte surface receptor; wherein the depleted immune cell population exhibits increased immunotherapeutic activity by a factor of at least about 1.2.

15 2. The method according to claim 1, wherein the depleted immune cell population is cultured in the presence of an antibody to a lymphocyte surface receptor and a cytokine.

20 3. The method according to claim 1, wherein the cytokine is IL-2.

4. The method of claim 3, wherein:

(a) the step of separating cells comprises separating at least one cell subpopulation of T lymphocytes; and

25 (b) the step of culturing the depleted immune cell population comprises culturing the cells in a first medium comprising IL-2 and an antibody to a lymphocyte surface receptor, in combination, until the depleted population exhibits an increased immunotherapeutic activity as
30 represented by an increased cytotoxicity by a factor of at least about 1.2.

5. The method of any one of claims 1-4, wherein the step of separating cells comprises separating CD4⁺ lymphocytes from the immune cell population to form a CD4⁺-depleted immune cell population.

5 6. The method of claim 5, wherein the CD4⁺-depleted immune cell population includes CD8⁺ lymphocytes; wherein the CD8⁺ lymphocytes exhibit increased cytotoxicity.

7. The method of any one of claims 1-4, wherein the step of separating cells comprises separating CD8⁺ lymphocytes from the immune cell population to form a CD8⁺-depleted immune cell population.

8. The method of claim 7, wherein the CD8⁺-depleted immune cell population includes CD4⁺ lymphocytes; wherein the CD4⁺ lymphocytes exhibit increased cytotoxicity.

15 9. The method of claim 4, wherein the step of culturing comprises:

- (a) stimulating the depleted immune cell population in the first medium with an antibody to a lymphocyte surface receptor and IL-2 during the first 48 hours of culturing;
- 20 (b) removing the depleted immune cell population from the first medium; and
- (c) subculturing the depleted immune cell population in a second medium comprising IL-2 without any additional amount of an antibody to a lymphocyte surface receptor.
- 25

10. The method of claim 1, wherein the step of separating cells comprises separating at least about 75% of the cell subpopulation from the immune cells.

11. The method of claim 1, wherein the antibody to a lymphocyte surface receptor is an anti-CD3 monoclonal antibody.

5 12. The method of claim 5, wherein the increased immunotherapeutic activity is represented by an increased cytotoxicity by a factor of at least about 2.0.

13. A method of enhancing the immunotherapeutic activity of immune cells, comprising:

- 10 (a) culturing an immune cell population in the presence of IL-2 and an antibody to a lymphocyte surface receptor to form a cultured immune cell population;
- (b) separating at least one cell subpopulation, which is capable of developing immunotherapeutic activity, from the cultured immune cell population; and
- 15 (c) subculturing the separated cell subpopulation in a second medium comprising IL-2; wherein the separated cell subpopulation exhibits increased immunotherapeutic activity by a factor of at least about 1.2.
- 20

14. The method of claim 13, wherein:

- 25 (a) the step of culturing an immune cell population includes culturing the cells in a first medium comprising both IL-2 and an antibody to a lymphocyte surface receptor;
- (a) the step of separating cells comprises separating at least one T lymphocyte subpopulation, which is capable of developing cytotoxicity; and
- 30 (b) the step of subculturing the separated T lymphocyte subpopulation comprises subculturing until the subpopulation exhibits an increased

immunotherapeutic activity as represented by an increased cytotoxicity by a factor of at least about 1.2.

5 15. The method of claim 14, wherein the step of separating cells comprises separating CD4⁺ lymphocytes from the immune cell population to form a separated CD4⁺ lymphocyte subpopulation.

10 16. The method of claim 14, wherein the step of separating cells comprises separating CD8⁺ lymphocytes from immune cells to form a separated CD8⁺ lymphocyte subpopulation.

17. The method of claim 14, wherein the step of culturing a immune cell population in a first medium comprises:

- 15 (a) stimulating the immune cell population with an antibody to a lymphocyte surface receptor and IL-2 during the first 48 hours of culturing;
- (b) removing the immune cell population from the first medium; and
- 20 (c) subculturing the immune cell population in a second medium comprising IL-2 without any additional amount of an antibody to a lymphocyte surface receptor.

25 18. The method of claim 14, wherein the cytotoxicity of the separated lymphocyte subpopulation is increased by a factor of at least about 2.0.

19. A method of enhancing the immunotherapeutic activity of immune cells, comprising:

- 30 (a) culturing an immune cell population in the presence of IL-2 and an anti-CD3 monoclonal antibody to form a T-AK cell population;
- (b) separating CD4⁺ lymphocytes from the T-AK cell population;

5 (c) subculturing the CD4⁺ lymphocytes in a second medium comprising IL-2; wherein the CD4⁺ lymphocytes exhibit increased immunotherapeutic activity as represented by an increased cytotoxicity by a factor of at least about 1.2.

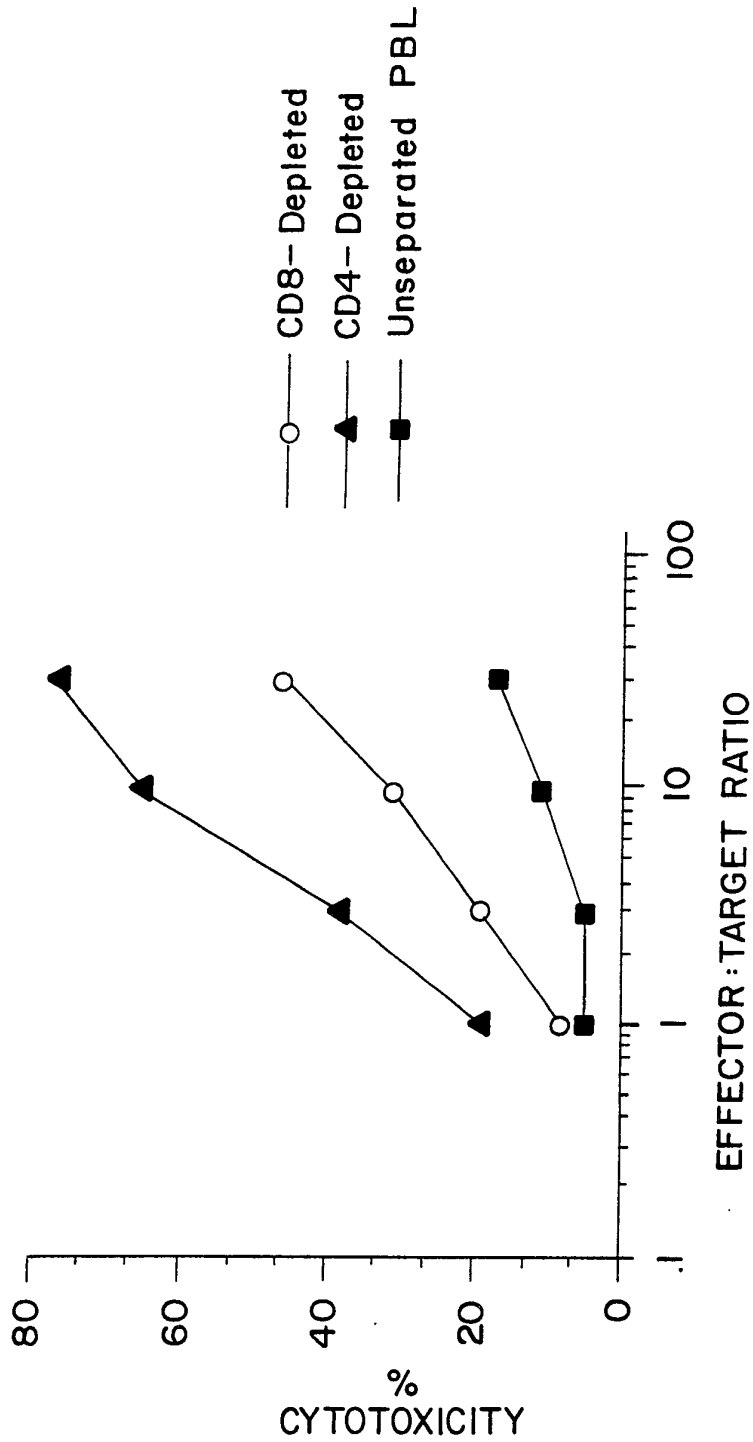
20. A method of enhancing the immunotherapeutic activity of immune cells, comprising:

- 10 (a) culturing an immune cell population in the presence of IL-2 and an anti-CD3 monoclonal antibody to form a T-AK cell population;
- (b) separating CD8⁺ lymphocytes from the T-AK cell population;
- 15 (c) subculturing the CD8⁺ lymphocytes in a second medium comprising IL-2; wherein the CD8⁺ lymphocytes exhibit increased immunotherapeutic activity as represented by an increased cytotoxicity by a factor of at least about 1.2.

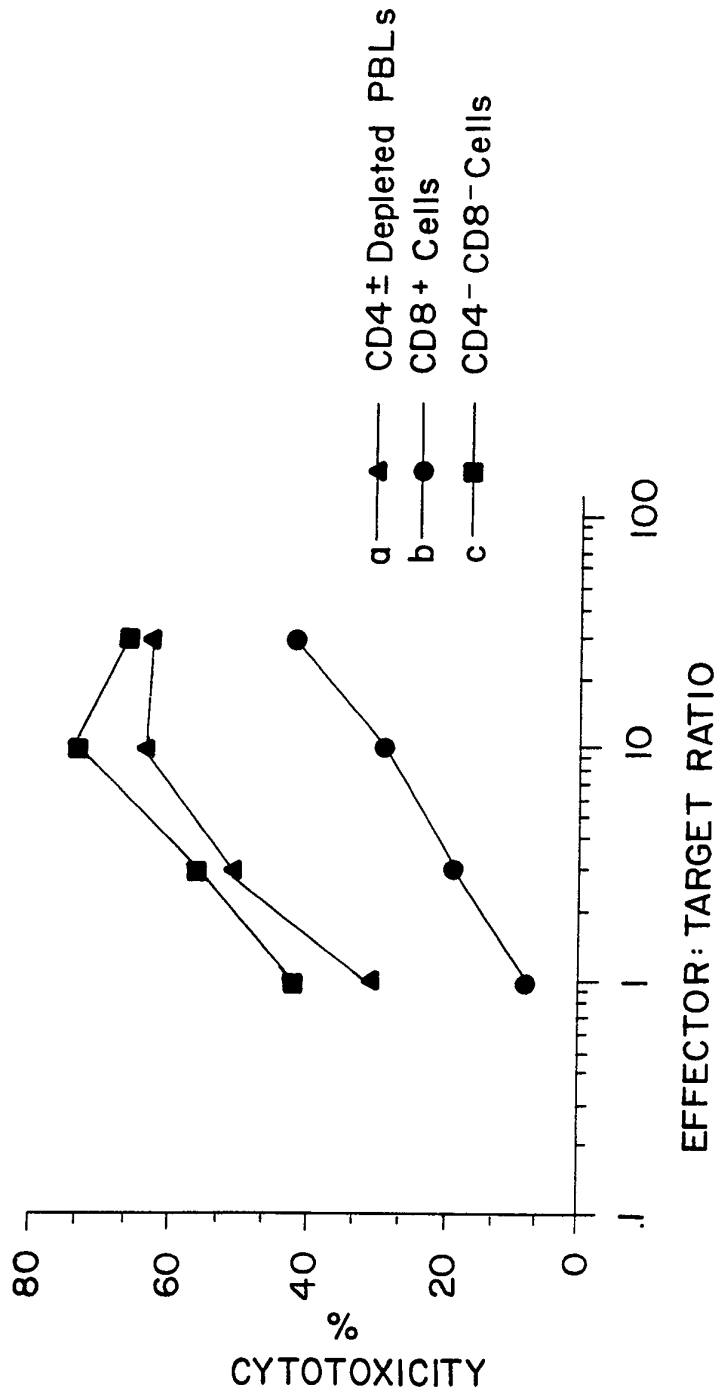
20 21. In a method of treating patients having a disease responsive to adoptive immunotherapy, wherein said patient is treated with stimulated immune cells, the improvement wherein the immunotherapeutic activity of said immune cells is enhanced by a method according to any one of claims 1-20.

FIG. 1

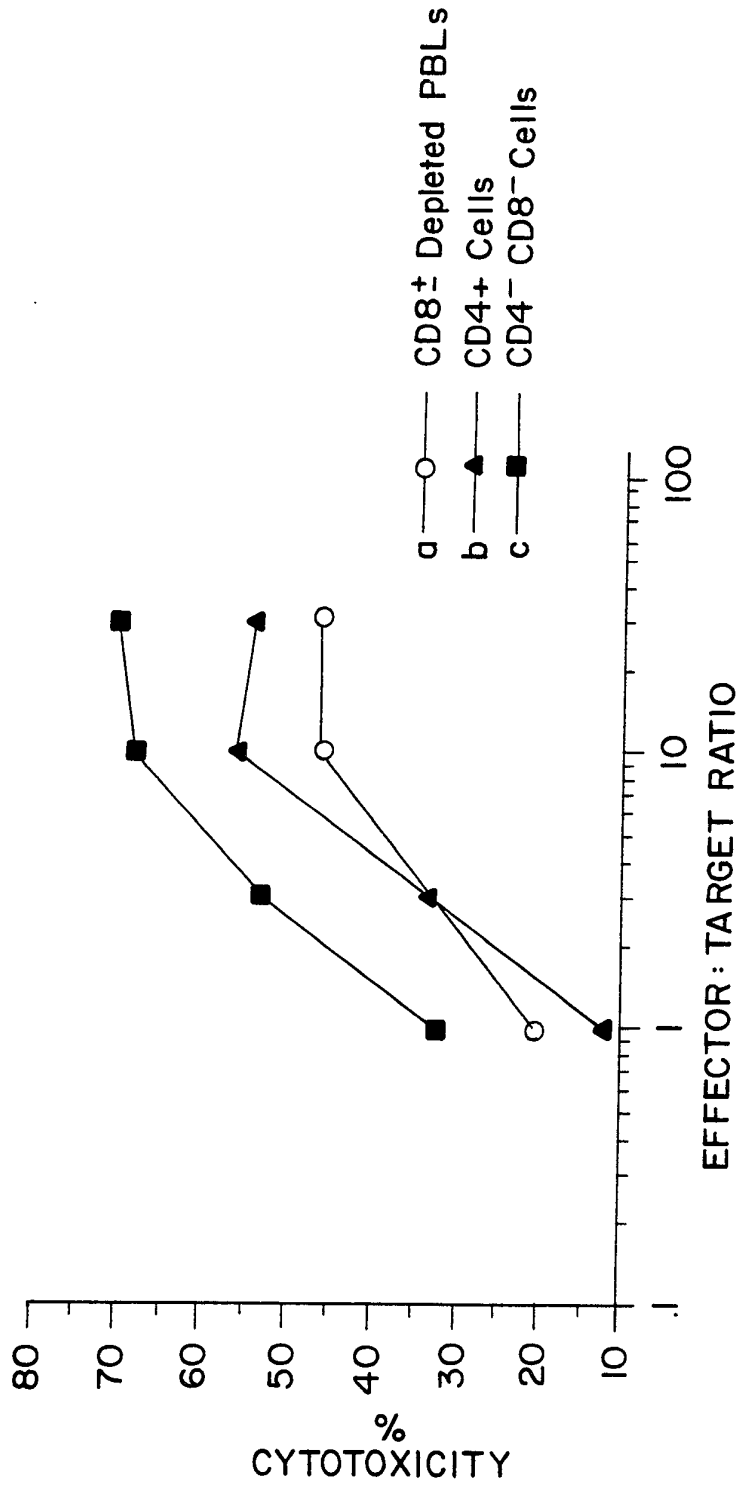
LAK ACTIVITY OF anti-CD3 +IL2
STIMULATED CULTURES



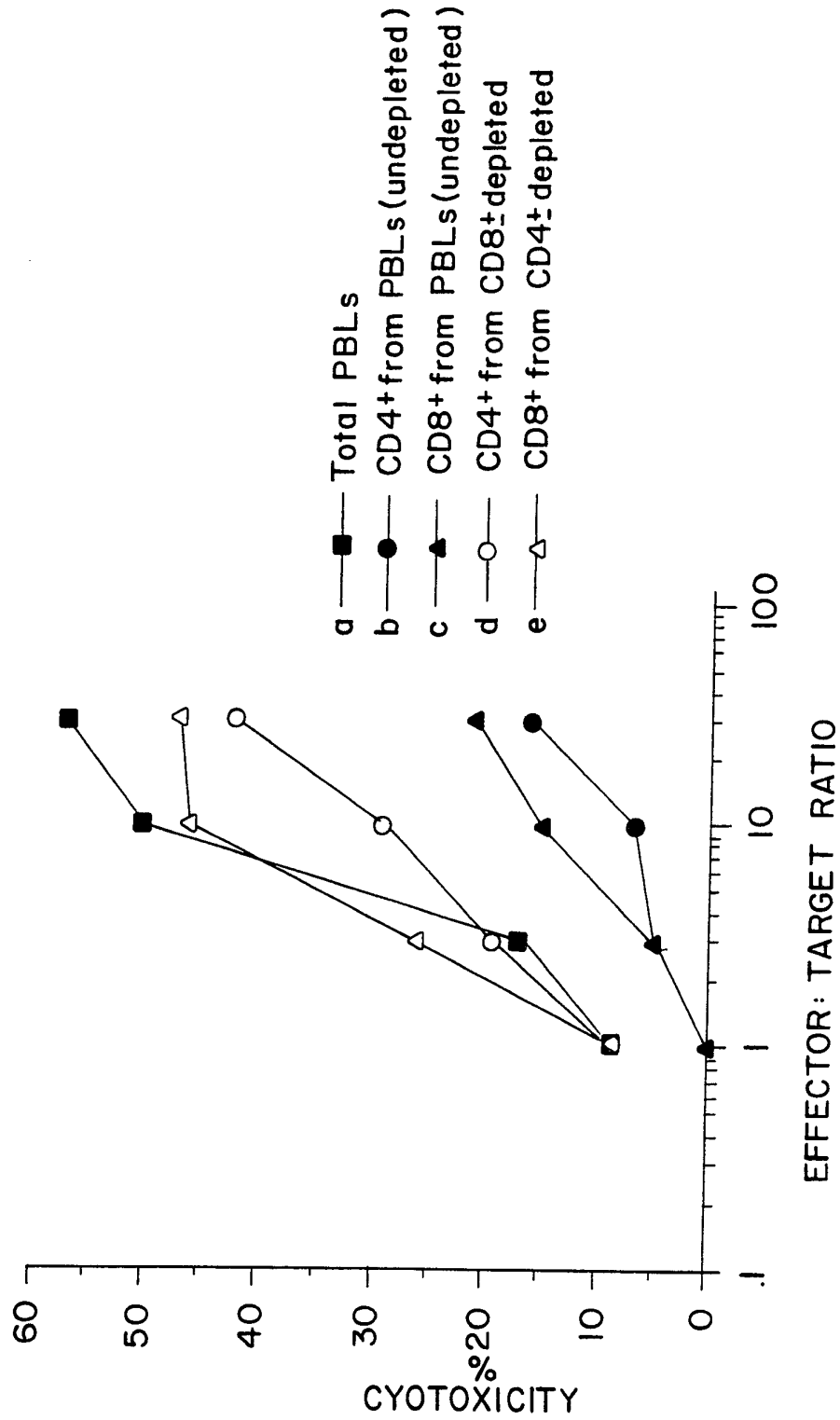
LAK ACTIVITY OF CD4±DEPLETED CULTURES
STIMULATED WITH anti-CD3 + IL2 **FIG.2**



LAK ACTIVITY OF CD8[±] DEPLETED CULTURES
STIMULATED WITH anti-CD3 + IL2 **FIG. 3**

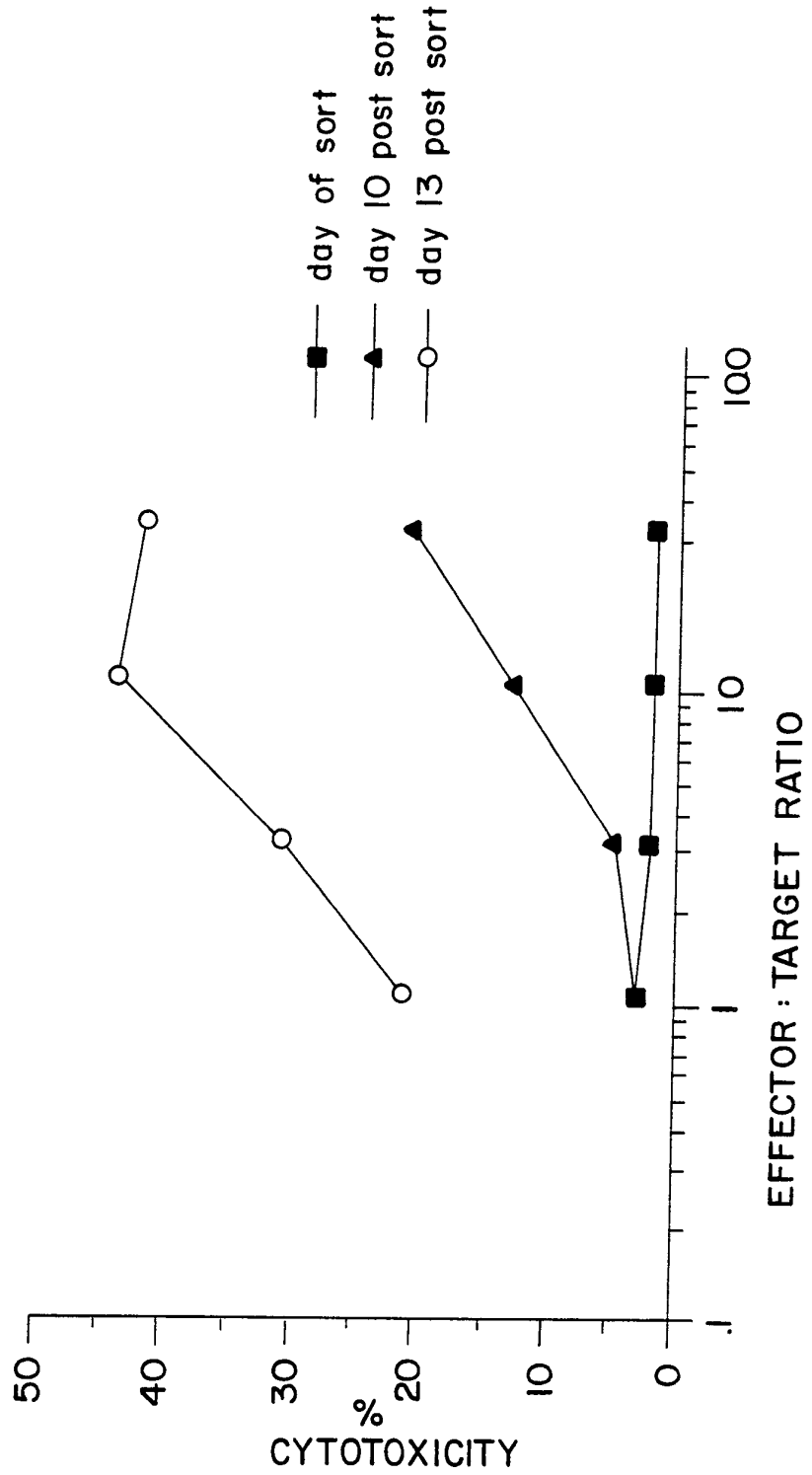


LAK ACTIVITY OF CD4+ AND CD8+ CELLS FROM UNDEPLETED PBL AND DEPLETED CULTURES **FIG.4**

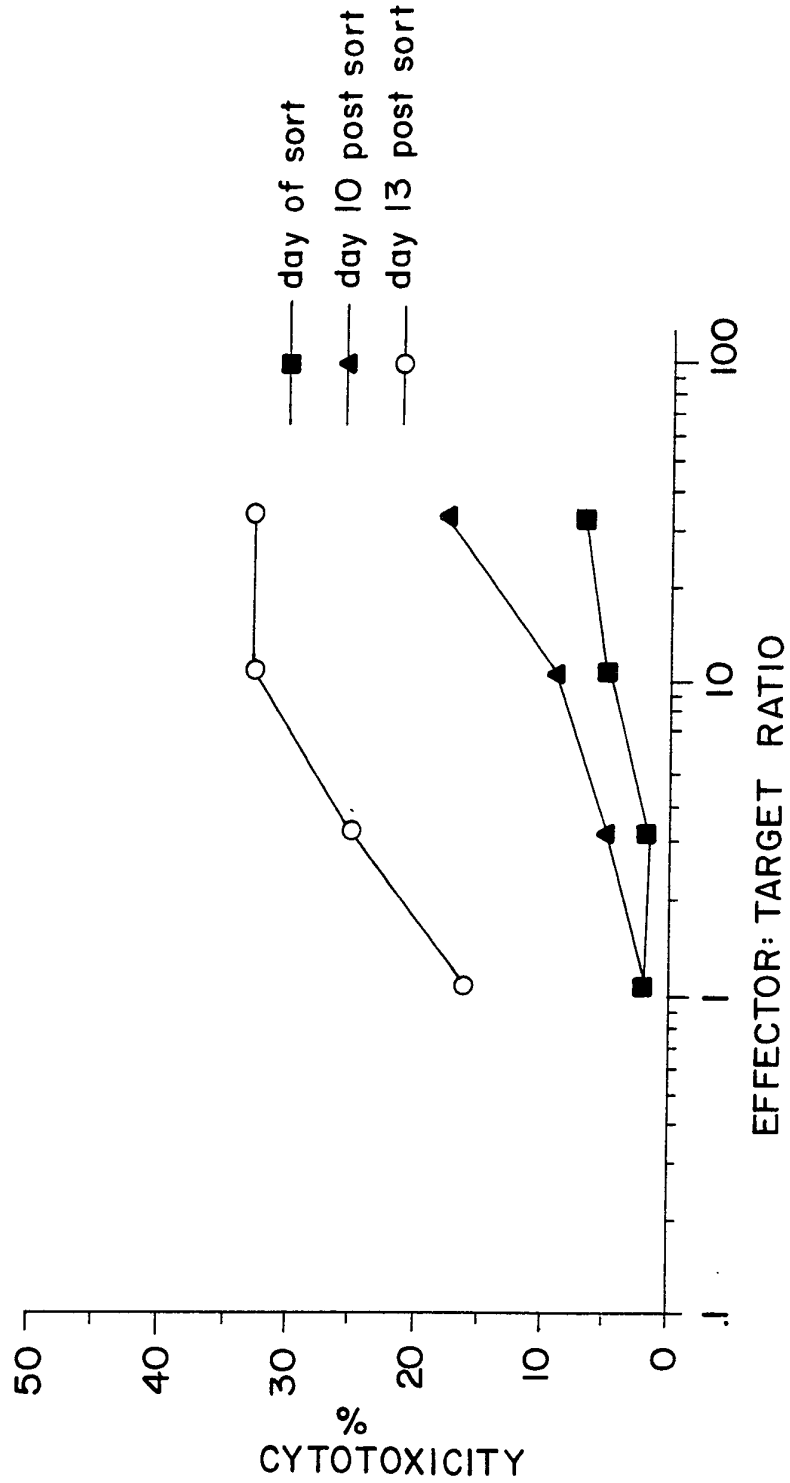


SUBSTITUTE SHEET

LAK ACTIVITY OF CD4+ CELLS ISOLATED FROM PBL CULTURED WITH OKT3+IL2
FIG. 5A



LAK ACTIVITY OF CD8+ CELLS ISOLATED FROM PBL CULTURED WITH OKT3+ IL2 **FIG. 5B**



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/02873**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :C12N 5/02, 5/08; A61K 35/14

US CL :435/240.2, 240.25; 424/93V

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 240.25; 424/93V

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
aps, dialog**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of Immunology, Volume 138, Number 8, issued 15 April 1987. Ochoa, et. al. "Long-term growth of lymphokine-activated killer (LAK) cells: Role of anti-CD3, β -IL 1, interferon-lambda and - β ", pages 2728-2733 (see especially, the paragraph bridging pages 2728 and 2729).	1-20
Y	FASEB Journal, Volume 3, Number 3 (Part I), issued 1989. Ochoa, et. al. "T cells can develop high LAK activity: Possible regulatory circuits". Page A826 (see entire abstract).	1-20
X,P	The Journal of Immunology, Volume 146, Number 10, issued 15 May, 1991. Geller, et. al. "Generation of lymphokine-activated killer activity in T cells: Possible regulatory circuits". Pages 3280-3288 (see entire document).	1-20

 Further documents are listed in the continuation of Box C.
 See patent family annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be part of particular relevance
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Date of the actual completion of the international search

25 June 1992

Date of mailing of the international search report

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