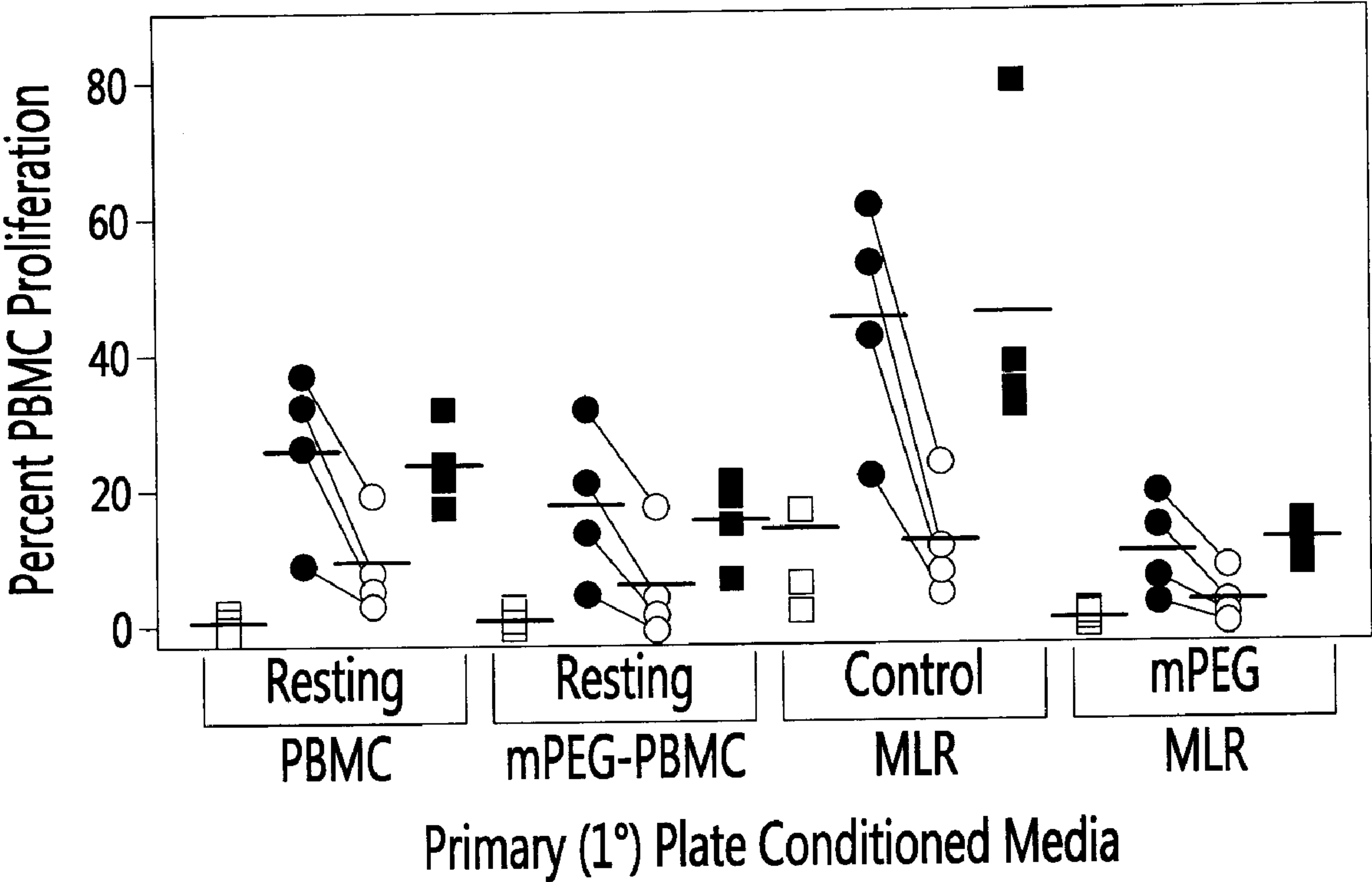




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(72) **Inventeurs/Inventors:**
SCOTT, MARK D., CA;
WANG, DUNCHENG, CA;
TOYOFUKU, WENDY M., CA
(73) **Propriétaire/Owner:**
CANADIAN BLOOD SERVICES, CA
(74) **Agent:** NORTON ROSE FULBRIGHT CANADA
LLP/S.E.N.C.R.L., S.R.L.

(54) **Titre :** METHODE PERMETTANT DE CONFERER UNE TOLERANCE IMMUNITAIRE AU MOYEN DE LEUCOCYTES
ANTIGENIQUES MODIFIES AUX POLYMERES
(54) **Title:** METHOD FOR INDUCING IMMUNE TOLERANCE USING POLYMER-MODIFIED ANTIGENIC LEUKOCYTES



(57) **Abrégé/Abstract:**
This application relates to cellular based-therapies for increasing the level of Regulatory T cells and/or decreasing the level of pro-inflammatory T cells to induce anergy or immune tolerance. To provide these therapeutic effects, a polymer- modified leukocyte is contacted with a second immunogenic leukocyte. Cellular preparations and processes for achieving cellular therapy are also provided.

ABSTRACT

This application relates to cellular based-therapies for increasing the level of Regulatory T cells and/or decreasing the level of pro-inflammatory T cells to induce anergy or immune tolerance. To provide these therapeutic effects, a polymer-modified
5 leukocyte is contacted with a second immunogenic leukocyte. Cellular preparations and processes for achieving cellular therapy are also provided.

METHOD FOR INDUCING IMMUNE TOLERANCE USING POLYMER-MODIFIED ANTIGENIC LEUKOCYTES

CROSS-REFERENCE TO RELATED APPLICATIONS

This is the first application filed for the present invention.

5 TECHNOLOGICAL FIELD

This application relates to the use of antigenic leukocytes covalently modified with a polymer to augment the level of regulatory T (Treg) cells and/or decrease the level of pro-inflammatory T cells for the induction of a state of immune tolerance or anergy. These antigenic leukocytes are useful for the treatment of various conditions
10 associated with excessive immune response auto-immunity and/or inflammation.

BACKGROUND

Acute and chronic rejection of donor tissues and organs remains a significant clinical problem in transplantation medicine. To minimize or prevent rejection, the administration of immunosuppressive agents is typically required. Acute and chronic
15 rejection are primarily T lymphocyte-mediated events that require allogeneic recognition of the foreign tissue and the subsequent proliferation of alloresponsive T cells. Indeed, because of the central role of the T cell in rejection, it is the primary target of current immunosuppressive drugs (e.g., cyclosporine A, FK506). In general, these pharmacologic agents target either the T cell activation (e.g., cyclosporine A that
20 inhibits IL-2 responsiveness) or the proliferation (e.g., methotrexate) of the alloresponsive T cells. However all of today's clinically approved anti-rejection drugs are beset by chronic toxicity; consequently, significant research is underway to identify alternative means of preventing acute and chronic rejection.

A biomaterials approach to the prevention of allorecognition is the direct
25 "immunocamouflage" of donor cells (e.g., erythrocytes, lymphocytes, and pancreatic islets) to prevent allorecognition (Scott et al., 1997; Murad et al., 1999A; Murad et al., 1999B; Bradley et al., 2001; Chen et al., 2001; Chen et al., 2003; McCoy et al., 2005; Chen et al., 2006; Bradley et al., 2006; Sutton et al., 2010; Le et al., 2010). The immunocamouflage of cells is induced by the direct grafting of low immunogenicity
30 polymers to the cell membrane. Previous studies have demonstrated that the immunocamouflage of erythrocytes and lymphocytes resulted in the loss of allorecognition both *in vitro* and *in vivo*. Moreover, in contrast to pharmacologic agents, the grafted polymer exhibited both extremely low toxicity and immunogenicity.

It would be highly desirable to be provided with a cellular preparation capable of inducing a state of anergy or immunotolerance by increasing the ratio of the level of regulatory T cells (such as Treg) to pro-inflammatory T cells (such as Th1 and Th17). The cellular preparation could induce anergy or tolerance either by increasing Treg
5 levels, decrease pro-inflammatory T cell levels or both. This preparation could be useful for treating, preventing and/or alleviating the symptoms associated to an abnormal/excessive immune condition, such as an auto-immune disease, a response to a vaccine or a tissue/cell transplantation.

BRIEF SUMMARY

10 One aim of the present invention is to provide a cellular preparation capable of inducing a state of anergy or immunotolerance by increasing the ratio of the level of regulatory T cells (such as Treg) to the level of pro-inflammatory T cells (such as Th1 and Th17). The cellular preparation could induce anergy or tolerance either by increasing Treg levels, decrease pro-inflammatory T cell levels or both. This preparation is useful for
15 treating, preventing and/or alleviating the symptoms associated to an abnormal/excessive immune condition, such as an auto-immune disease, a response to a vaccine or a tissue/cell transplantation.

In accordance with the present invention there is provided a method of increasing a ratio of the level of regulatory T (Treg) cells to the level of pro-inflammatory T cells in a
20 subject in need thereof. Broadly, the method comprises administering : (i) a cellular preparation comprising a first immunogenic leukocyte having a cytoplasmic membrane associated to a low-immunogenic biocompatible polymer; and/or (ii) a supernatant of a cell culture of the cellular preparation and a second immunogenic leukocyte. The method is to provide an increase in the ratio of the level of Treg cells to the level of pro-
25 inflammatory T cells in the subject. In an embodiment, the cytoplasmic membrane of the first immunogenic cell has a membrane-associated protein covalently bound to the low-immunogenic biocompatible polymer. In another embodiment, the first immunogenic leukocyte is allogeneic to the second immunogenic leukocyte. In yet another embodiment, the leukocyte is a T cell. In still another embodiment, in (ii), the
30 first immunogenic leukocyte or second immunogenic leukocyte is from the subject. In yet another embodiment, the low-immunogenic biocompatible polymer is a polyethylene glycol (such as for example mPEG) and/or 2-alkyloxazoline (POZ). In still another embodiment, the increased ratio between the level of Treg cells and the level of pro-inflammatory T cells is for treating, preventing and/or alleviating the symptoms

associated to an auto-immune disease of the subject (such as, for example, type I diabetes, rheumatoid arthritis, multiple sclerosis, psoriasis, lupus, immune thrombocytopenia, experimental autoimmune encephalomyelitis, autoimmune uveitis, inflammatory bowel disease and/or Crohn's disease). In still another embodiment, the increased ratio between the level of Treg cells and the level of pro-inflammatory T cells is for preventing the onset of an excessive immune reaction in the subject (such as, for example, an excessive immune reaction to a vaccine). In a further embodiment, the increased ratio between the level of Treg cells and the level of pro-inflammatory T cells is for preventing the rejection of transplanted cells or tissue of the subject.

10 In accordance with the present invention there is provided a cellular preparation for increasing a ratio of regulatory T (Treg) cells to pro-inflammatory T cells in a subject. The cellular preparation comprises (i) a first immunogenic leukocyte having a cytoplasmic membrane associated to a low-immunogenic biocompatible polymer; and/or (ii) a supernatant of a cell culture of the first immunogenic leukocyte and a
15 second immunogenic leukocyte. In an embodiment, the cytoplasmic membrane of the first immunogenic leukocyte has a membrane-associated protein covalently bound to the low-immunogenic biocompatible polymer. In another embodiment, the first immunogenic leukocyte is allogeneic to the second immunogenic leukocyte. In yet another embodiment, the leukocyte is a T cell. In still another embodiment, in (ii), the
20 first immunogenic leukocyte or second immunogenic leukocyte is from the subject. In yet another embodiment, the low-immunogenic biocompatible polymer is a polyethylene glycol (such as for example mPEG) and/or 2-alkyloxazoline (POZ). In still another embodiment, the increased ratio between the level of Treg cells and the level of pro-inflammatory T cells is for treating, preventing and/or alleviating the symptoms
25 associated to an auto-immune disease of the subject (such as, for example, type I diabetes, rheumatoid arthritis, multiple sclerosis, psoriasis, lupus, immune thrombocytopenia, experimental autoimmune encephalomyelitis, autoimmune uveitis, inflammatory bowel disease and/or Crohn's disease). In still another embodiment, the increased ratio between the level of Treg cells and the level of pro-inflammatory T cells
30 is for preventing the onset of an excessive immune reaction in the subject (such as, for example, an excessive immune reaction to a vaccine). In a further embodiment, the increased ratio between the level of Treg cells and the level of pro-inflammatory T cells is for preventing the rejection of transplanted cells or tissue of the subject.

In accordance with the present invention there is provided a process for increasing
35 and/or for providing the ability of a cellular preparation to increase a ratio of Regulatory

T (Treg) cells to pro-inflammatory T cells in a subject. Broadly the process comprises (i) associating a low-immunogenic biocompatible polymer to a cytoplasmic membrane of a first immunogenic leukocyte to obtain a modified first immunogenic leukocyte; and (ii) formulating the preparation obtained in step (i) for administration to the subject. In an embodiment, the process further comprises, in step (i), covalently binding the low-immunogenic biocompatible polymer to a membrane-associated protein of the cytoplasmic membrane of the first immunogenic leukocyte. In another embodiment, the process further comprises, in step (i), placing the modified first immunogenic leukocyte in contact, in a cell culture medium, with a second immunogenic leukocyte. In still another embodiment, the process further comprises, in step (i) after the modified first immunogenic leukocyte has contacted the second immunogenic leukocyte, isolating the supernatant of the cell culture medium. In still another embodiment, the first immunogenic leukocyte is allogeneic to the second immunogenic leukocyte. In another embodiment, the leukocyte is a T cell. In still another embodiment, the first immunogenic leukocyte or second immunogenic leukocyte is from the subject. In yet another embodiment, the low-immunogenic biocompatible polymer is a polyethylene glycol (such as for example mPEG) and/or 2-alkyloxazoline (POZ). In embodiments where the low-immunogenic biocompatible polymer is mPEG, it can be covalently bound to the membrane protein by contacting the first immunogenic leukocyte with methoxypoly(-ethylene glycol) succinimidyl valerate. In another embodiment, the POZ is covalently bound to the membrane protein by using a linker molecule. The linker molecule may comprise, *inter alia*, cyanuric chloride, imidazolyl formate, succinimidyl succinate, succinimidyl carbonate, succinimidyl glutarate, N-hydroxysuccinimide, 4-nitrophenol, and 2,4,5-trichlorophenol. The linker molecules listed above are exemplary only, and the invention is not intended to be limited to those particular examples. Any linker molecule capable of covalently attaching to the polymer (as exemplified by PEG, POZ, HPG) and mediating the linkage of the polymer to the cell membrane may be similarly used. In another embodiment, the process further comprises formulating the formulated cellular preparation in a vaccine.

Throughout this application, various terms are used according to their plain definition in the art. However, for purposes of clarity, some specific terms are defined below.

Allogeneic cell. A cell is considered "allogeneic" with respect to another cell if both cells are derived from the same animal species but presents sequence variation in at least one genetic locus. A cell is considered "allogeneic" with respect to a subject if the cell is derived from the same animal species as the subject but presents sequence

variation in at least one genetic locus when compared to the subject's respective genetic locus. Allogeneic cells induce an immune reaction (such as a rejection) when they are introduced into an immunocompetent host.

5 *Anergy and Tolerance.* In the present context, the term "anergy" refers to a non-specific state of immune unresponsiveness to an antigen to which the host was previously sensitized to or unsensitized to. It can be characterized by a decrease or even an absence of lymphokine secretion by viable T cells when the T cell receptor is engaged by an antigen. In the present context, the term "tolerance" refers to an acquired specific failure of the immunological mechanism to respond to a given antigen, induced by
10 exposure to the antigen. Tolerance refers to a specific nonreactivity of the immune system to a particular antigen, which is capable, under other conditions, of inducing an immune response. However, in the present context, the terms "anergy" and "tolerance" are used interchangeably since the compositions and methods presented herewith can be used to achieve both anergy and tolerance.

15 *Autologous cell.* A cell is considered "autologous" with respect to another cell if both cells are derived from the same individual or from genetically identical twins. A cell is considered "autologous" to a subject, if the cell is derived from the subject or a genetically identical twin. Autologous cells do not induce an immune reaction (such as a rejection) when they are introduced into an immuno-competent host.

20 *Immunogenic cell.* A first cell is considered immunogenic with respect to a second cell when it is able to induce an immune response in the latter cell. The second cell can be located in an immunocompetent subject. Preferably, the immune response is a cell-based immune response in which cellular mediator can be produced. In the context of this application, the immunogenic cells are immune cells, such as white blood cells or
25 leukocytes.

Low-immunogenic biocompatible polymer. As used herein, a "low-immunogenic polymer" refers to a polymer which is not or is unlikely to elicit an immune response in an individual. This low-immunogenic polymer is also capable of masking an antigenic determinant of a cell and lowering or even preventing an immune response to the
30 antigenic determinant when the antigenic determinant is introduced into a subject. A "biocompatible polymer" refers to a polymer which is non-toxic when introduced into a subject. Exemplary low-immunogenic biocompatible polymers includes, but are not limited to, polyethylene glycol (for example methoxypoly(ethylene glycol)), hyperbranched polyglycerol (HPG) and 2-alkyloxazoline (POZ).

Peripheral blood mononuclear cells (PBMC). This term refers to the cell population recuperated/derived from the peripheral blood of a subject (usually a mammal such as a human). PBMC usually contains T cells, B cells and antigen presenting cells.

Pharmaceutically effective amount or therapeutically effective amount. These expressions refer to an amount (dose) of a cellular preparation effective in mediating a therapeutic benefit to a patient (for example prevention, treatment and/or alleviation of symptoms of an immune-associated disorder in which the ratio of Tregs to pro-inflammatory T cells is low when compared to a healthy subject). It is also to be understood herein that a "pharmaceutically effective amount" may be interpreted as an amount giving a desired therapeutic effect, either taken in one dose or in any dosage or route, taken alone or in combination with other therapeutic agents.

Prevention, treatment and alleviation of symptoms. These expressions refer to the ability of a method or cellular preparation to limit the development, progression and/or symptomology of a immune-associated disorder associated to an abnormal/excessive immune response. Broadly, the prevention, treatment and/or alleviation of symptoms encompasses increasing the levels of Treg cells and/or decreasing the levels of pro-inflammatory T cells. A method or cellular preparation is considered effective or successful for treating and/or alleviating the symptoms associated with the disorder when a reduction in the pro-inflammatory state (when compared to an untreated and afflicted individual) in the treated individual (previously known to be afflicted with the disorder) is observed. A method or cellular preparation is considered effective or successful for preventing the disorder when a reduction in the pro-inflammatory state (when compared to an untreated and afflicted individual) in the treated individual is observed upon an immunological challenge (such as, for example, an antigenic challenge).

Pro-inflammatory T cells. In the present context, pro-inflammatory T cells are a population of T cells capable of mediating an inflammatory reaction. Pro-inflammatory T cells generally include T helper 1 (Th1 or Type 1) and T helper 17 (Th17) subsets of T cells. Th1 cells partner mainly with macrophage and can produce interferon- γ , tumor necrosis factor- β , IL-2 and IL-10. Th1 cells promote the cellular immune response by maximizing the killing efficacy of the macrophages and the proliferation of cytotoxic CD8+ T cells. Th1 cells can also promote the production of opsonizing antibodies. T helper 17 cells (Th17) are a subset of T helper cells capable of producing interleukin 17 (IL-17) and are thought to play a key role in autoimmune diseases and in microbial infections. Th17 cells primarily produce two main members of the IL-17 family, IL-17A

and IL-17F, which are involved in the recruitment, activation and migration of neutrophils. Th17 cells also secrete IL-21 and IL-22.

Regulatory T cells. Regulatory T cells are also referred to as Treg and were formerly known as suppressor T cell. Regulatory T cells are a component of the immune system that suppress immune responses of other cells. Regulatory T cells usually express CD3, CD4, CD8, CD25, and Foxp3. Additional regulatory T cell populations include Tr1, Th3, CD8+CD28-, and Qa-1 restricted T cells. Regulatory T cells actively suppress activation of the immune system and prevent pathological self-reactivity, *i.e.* autoimmune disease. The critical role regulatory T cells play within the immune system is evidenced by the severe autoimmune syndrome that results from a genetic deficiency in regulatory T cells. The immunosuppressive cytokines TGF- β and Interleukin 10 (IL-10) have also been implicated in regulatory T cell function. Similar to other T cells, a subset of regulatory T cells can develop in the thymus and this subset is usually referred to as natural Treg (or nTreg). Another type of regulatory T cell (induced Treg or iTreg) can develop in the periphery from naïve CD4+ T cells. The large majority of Foxp3-expressing regulatory T cells are found within the major histocompatibility complex (MHC) class II restricted CD4-expressing (CD4+) helper T cell population and express high levels of the interleukin-2 receptor alpha chain (CD25). In addition to the Foxp3-expressing CD4+ CD25+, there also appears to be a minor population of MHC class I restricted CD8+ Foxp3-expressing regulatory T cells. Unlike conventional T cells, regulatory T cells do not produce IL-2 and are therefore anergic at baseline. An alternative way of identifying regulatory T cells is to determine the DNA methylation pattern of a portion of the foxp3 gene (TSDR, Treg-specific-demethylated region) which is found demethylated in Tregs.

Splenocytes. This term refers to the cell population obtained from the spleen of a subject (usually a mammal such as a rodent). Splenocytes usually comprise T cell, B cell as well as antigen presenting cells.

Syngenic cell. A cell is considered “syngenic” with respect to a subject (or a cell derived therefrom) if it is sufficiently identical to the subject so as to prevent an immune rejection upon transplantation. Syngenic cells are derived from the same animal species.

Viable. In the present context, the term “viable” refers to the ability of a cell to complete at least one cell cycle and, ultimately proliferate. By opposition, the term “non-viable” refers to a cell which is no longer capable of completing at least one cell cycle. By comparison, the term “cycle arrest” refers to a cell which has been treated to halt its cell

cycle progression (usually with a pharmacological agent) but which is still capable of re-entering the cell cycle (usually when the pharmacological agent is removed).

White blood cells. White blood cells are also referred to as leukocytes (or leucocytes) and are cells of the immune system. Five different and diverse types of leukocytes exist, but they are all produced and derived from a multipotent cell in the bone marrow known as a hematopoietic stem cell. White blood cells include neutrophils (neutrophil granulocytes or polymorphonuclear (PMN) leukocytes), eosinophil (eosinophil granulocytes), basophils (basophil granulocytes), lymphocytes (including B cells as well as T cells), natural killer (NK) and monocytes.

Xenogenic cell. A cell is considered "xenogenic" with respect to a subject (or a cell derived from the subject) when it is derived from a different animal species than the subject. A xenogenic cell is expected to be rejected when transplanted in an immuno-competent host.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the nature of the invention, reference will now be made to the accompanying drawings, showing by way of illustration, a preferred embodiment thereof, and in which:

Figure 1 shows diagrammatically the conditioned media protocol. A primary (1°) two-way mixed lymphocyte reaction (MLR) was initiated using two HLA-disparate populations consisting of unmodified or polymer-grafted (1 mM SVAmPEG; 5 kDa) PBMC. Within the mPEG-MLR, only one donor population was PEGylated. At 72 h, the conditioned media from the wells were collected. Secondary (2°) mixed lymphocyte reactions using control and PEGylated PBMC from the same donors were initiated. A mitogen (PHA) stimulation control was added to assure that the media collected would support proliferation. 1° MLR Conditioned media: 1. Resting unmodified PBMC; 2. Resting mPEG-PBMC; 3. Control MLR; and 4. mPEG-MLR. 1° MLR/2° MLR Cell Types or Stimulation: A. Resting PBMC; B. Resting mPEG PBMC; C. MLR; D. mPEG MLR; P: PHA stimulation.

Figure 2 shows 1° mixed lymphocyte reaction (MLR) results. Primary (1°) media cytokine levels at 72 h. IL-2 (A), IFN- γ (B) IL-17A (C), TNF- α (D) and IL-6 (E), levels are significantly reduced in the PEGylated two-way MLR utilizing modified and unmodified PBMC populations from HLA disparate individuals. The cytokine profile was analyzed using the BD cytometric bead array. Values shown are the mean $\pm$ SD of a

minimum of 4 independent experiments. Percent non-viable cells within the control and PEGylated (SVAmPEG; 5 kDa) resting PBMC was assessed by propidium iodine exclusion (F).

Figure 3 shows 2° mixed lymphocyte reaction (MLR) results. Shown is the proliferation index (percent PBMC proliferation) of the secondary MLR (□ resting PBMC, ● control MLR, ○ mPEG MLR, ■ PHA stimulation) that were conducted in the indicated (x-axis) conditioned media. As shown, relative to all other conditioned media, the media from the 1° plate control MLR demonstrated a significant ($p < 0.01$) pro-proliferative effect in the 2° MLR. This effect was noted on even resting PBMC and PHA-stimulated cells. In contrast, the 1° conditioned media from the mPEG-MLR demonstrated a significant anti-proliferative effect in the 2° MLR. As noted by the lines connecting paired experiments, PEGylation of one donor population resulted in reduced proliferation in all conditioned media experiments. No significant differences were noted between fresh media in a parallel secondary plate and the resting PBMC conditioned media. Shown are the individual results of 4 independent experiments and the mean (line). PEGylated cells were modified with 1 mM SVAmPEG (5 kDa).

Figure 4 illustrates the effects of the various conditioned media on the levels of Treg and Th17. PEGylation of human lymphocytes resulted in a significant *in vitro* immunomodulatory effects as noted by changes in the percentage of Treg (A) and Th17 (B) T cell populations. Results are provided for Treg levels (upper panel and columns), for Th17 levels (lower panel and columns) as well as percent PBMC proliferation (line) for 2° plates (black column→resting PBMC; grey column→control MLR; white column→mPEG MLR; hatched column→PHA stimulation) having received 1° conditioned media (defined in x-axis). As shown, the 1° media from the mPEG-MLR favored production of Treg cells and a decreased population of Th17 lymphocytes. In contrast, the 1° media from the control MLR enhanced Th17 cell production and greatly inhibited Treg levels. The relative ratio of Th17:Treg was highly correlated with lymphocyte proliferation as denoted by the right y-axis and the embedded line graph. The high levels of Tregs in both the resting mPEG-PBMC and in mPEG-MLR correlated with low levels of proliferation. In contrast, an increased level of Th17 cells was associated with the 1° media from the control MLR and PHA stimulation. PEGylated cells were modified with 1 mM SVAmPEG (5 kDa).

Figure 5 illustrates Treg levels in the spleen (A), in the brachial lymph nodes (B) or in the blood (C) in function of time (hours post injection) following administration of donor splenocytes or control (△ naïve; ▲ soluble mPEG; □ syngeneic cells; ■ mPEG

syngeneic cells; • allogeneic cells; ○ mPEG alloegeneic cells). PEGylation of allogeneic donor murine splenocytes resulted in a significant *in vivo* immunomodulatory effect giving rise to significantly elevated Treg lymphocytes. As noted, in all three tissues, a significant ($p < 0.001$ at 120 h) increase in Treg lymphocytes over that
 5 observed in naïve mice was noted in mice receiving mPEG-modified allogeneic donor cells. In stark contrast, a significant decrease in Tregs (relative to naïve mice) is noted in mice transfused with unmodified allogeneic splenocytes. In comparing the absolute difference between the control PEGylated splenocytes (dotted area) the differential impact of donor cell PEGylation can be fully appreciated. Importantly, as noted at 120
 10 h, transfusion of soluble mPEG, syngeneic cells or mPEG-syngeneic cells had no significant effect on the Treg lymphocyte population. The range observed in naïve mice is denoted by the grey bars. PEGylated murine splenocytes were modified with 1 mM SVAmPEG (20 kDa).

Figure 6 illustrates Th17 levels in the spleen (A), in the brachial lymph nodes (B) or in
 15 the blood (C) in function of time (hours post injection) following administration of donor splenocytes or control (Δ naïve; $\blacktriangle$ soluble mPEG; $\square$ syngeneic cells; $\blacksquare$ mPEG syngeneic cells; • allogeneic cells; ○ mPEG alloegeneic cells). PEGylation of allogeneic donor murine splenocytes resulted in a significant *in vivo* immunomodulatory effect as evidenced by baseline levels of Th17 lymphocytes. As shown, unmodified
 20 allogeneic splenocytes resulted in a dramatic increase ($p < 0.001$ at all time points >24 h) in Th17 lymphocytes. However, PEGylation of the allogeneic donor cells completely abrogates this increase and the Th17 levels stay in the range seen in naïve mice (grey zone). In comparing the absolute difference between the control and PEGylated splenocytes (dotted area) the differential impact of donor cell PEGylation can be fully
 25 appreciated. Importantly, as noted at 120 h, transfusion of soluble mPEG, syngeneic cells or mPEG-syngeneic cells had no significant effect on the Th17 lymphocyte population. The range observed in naïve mice is denoted by the grey bars. PEGylated murine splenocytes were modified with 1 mM SVAmPEG (20 kDa).

Figure 7 shows the ratio of Treg/Th17 levels five days following administration of donor
 30 splenocytes. PEGylation of allogeneic donor murine splenocytes resulted in a significant *in vivo* immunomodulatory effect. The panels in (A) show the ratio of Treg/Th17 levels in spleen (top panel), “brachial” lymph node (middle panel), and peripheral blood (lower panel). The graph in (B) compares the ratio when non-modified allogeneic cells (right side) or PEGylated allogeneic cells (left side) are administered (○
 35 for spleen, $\square$ for lymph nodes, • for blood). * denotes statistical significance ($p < 0.001$).

Figure 8 shows the long-term immunomodulatory effects of PEGylation of donor cells. The immunomodulatory effects of the PEGylated splenocytes is long lived and prevents changes in Treg and Th17 levels consequent to rechallenge with unmodified allogeneic cells. Results are shown for percentage of Tregs (upper panel) and Th17 cells (lower panel) in the spleen (right panel), brachial lymph nodes (middle panel) and peripheral blood (left panel) for mice transfused with allogeneic splenocytes (■), mPEG allogeneic splenocytes (□), conditioned serum of mice transfused with mPEG allogeneic splenocytes (○) or conditioned medium from 1° mPEG allogeneic splenocytes on 2° allogeneic splenocytes (●). Shaded area (gray) on the graph indicate Treg and Th17 levels in naïve mice. As shown, 30 days post transfusion of the polymer modified splenocytes, Treg levels remain significantly elevated while Th17 levels remain similar to or decreased from that of naïve mice. Furthermore, transfusion of unmodified allogeneic splenocytes to mice previously treated with PEGylated allogeneic cells does not result in decreased Treg or increased Th17 lymphocyte populations. Indeed, Treg levels remain significantly elevated above that seen in naïve mice and very similar to those levels observed at 5 days post PEGylated splenocytes transfusion. The range observed in naïve mice is denoted by the grey bars. PEGylated murine splenocytes were modified with 1 mM SVAmPEG (20 kDa).

Figure 9 shows that immunomodulation is not haplotype-specific. Initial one-way MLR (open symbol) consisting of C57Bl/6 (H-2b) splenocytes challenged with unmodified or PEGylated irradiated Balb/c (H2-d) splenocytes. Following 48 h of challenge, duplicate samples were challenged with unmodified-non-irradiated C3H (H-2k) splenocytes (two-way MLR). The addition of the fresh responder cells from a third, H2-disparate mouse strain (C3H), at 48 h did not reverse the attenuation of proliferation in responder cells co-incubated with irradiated, cmPEG-modified Balb/c splenocytes. In contrast, the proliferation in the control (0 mM) MLR was significantly ($p < 0.001$) enhanced by the addition of the C3H splenocytes. The data shown represented the co-culturing of 5.12×10^6 C57Bl/6 splenocytes with 5.12×10^6 irradiated, mPEG-derivitized Balb/c splenocytes. After 48 h of incubation, fresh C3H responder cells were added to duplicate wells. The results were expressed as the average mean $\pm$ standard deviation of triplicate samples from a representative experiment. PEGylated murine splenocytes were modified with the indicated concentrations (mM) of activated mPEG (5 kDa).

Figure 10 provides an hypothetical representation of immune modulation. Immune modulation via pharmacologic and immunocamouflage therapy. Panel A: Current immunomodulation therapy almost exclusively targets the recipient's immune system

and does not address the inherent antigenicity and immunogenicity of allogeneic tissues. Response to non-self is in large part mediated by cell-cell interactions between Antigen Presenting Cells (APC; e.g., dendritic cells) and naïve T lymphocytes (Thp). This cell-cell interaction is characterized by adhesion, allorecognition and co-

5 stimulation events. Consequent to allorecognition, cytokine/chemokine burst occurs followed by proliferation of pro-inflammatory T cells (e.g., CTL, Th17, Th1 populations), immunoglobulin production and decreased evidence of regulatory T cells (Treg). Current therapeutic agents are primarily cytotoxic agents preventing T cell activation (e.g., cyclosporine and rapamycin) or T cell proliferation (e.g., methotrexate,

10 corticosteroids, azathiaprine). Additionally some blocking antibodies have been investigated. Panel **B**: In contrast, PEGylation of donor PBMC results in loss of appropriate cell-cell interaction leading to loss of the cytokine burst, decreased/absent proliferation, evidence of apoptosis of alloresponsive T cells and increased levels of Regulatory T (Treg) cells that, in aggregate, provides a tolerogenic/anergic state both

15 *in vitro* (Figs. 2, 3, and 7) and *in vivo* (Figs. 4 to 6). Shown with the schematic is a DNA laddering gel of an unmodified MLR (A) and a PEGylated MLR (B) showing enhanced apoptosis consequent to PEGylation. Size of T cell population denotes increase or decrease in number. Size of B cell indicates antibody response.

Figure 11 illustrates significant changes in the levels of Th17 and Treg lymphocytes are

20 noted in the spleen, brachial lymph node and pancreatic lymph nodes upon conversion of NOD mice from non-diabetic to diabetic. These changes are characterized by dramatically increased Th17 (in the spleen, from 0.05 to 3.84%; in the brachial lymph node from 0.01% to 0.67%; in the pancreatic lymph node from 0.05% to 1.05%) and significantly decreased Treg (in the spleen, from 16.5% to 2.0%; in the brachial lymph

25 node from 11.8% to 1.8% and in the pancreatic lymph node, from 12.7% to 4.1%) lymphocytes. Tregs: *, $p < 0.001$ from non-diabetic NOD mice. Th17: ** $p < 0.001$ from non-diabetic NOD mice.

Figure 12 illustrates cellular proliferation in a 2-way MLR of PEGylated or POZylated cells at day 10. Results are shown for resting cells (○), mPEG-MLR (■) and POZ-MRL

30 (□) as percentage in proliferation (with respect to resting cells) in function of grafting density.

DETAILED DESCRIPTION

In accordance with the present invention, there is provided a preparation for increasing the levels of regulatory T cells and/or decreasing the levels of pro-inflammatory T cells

for inducing a state of anergy, tolerance, loss of pro-inflammatory, and/or immuno-
quiescence in a subject in need thereof. The preparation is obtained by contacting a
first immunogenic cell (which has been modified to bear on its surface a low-
immunogenic biocompatible polymer) and a second immunogenic cell. This contact can
5 be made directly *in vivo*. Alternatively, the conditioned media of the culture of the first
and second immunogenic cell can also be used.

As it will be shown below, polymer-based bioengineering of donor cells provides a
significant opportunity to modulate the responsiveness (*i.e.*, immunoquiescent versus
pro-inflammatory) of the recipients immune system. It is hypothesized that polymer-
10 modified white blood cells (*e.g.* leukocytes), and preparations arising from such cells,
can be used to induce Tregs and/or attenuate Th17/1 and NK (natural killer) cell
upregulation, to prevent the pro-inflammatory immune response to allogeneic donor
cells. Moreover, it is proposed that polymer-modified allogeneic white blood cells (*e.g.*
leukocytes) can be used therapeutically in various diseases (such as auto-immunity or
15 an excessive immune response) to increase the levels Treg cells and/or decrease
proinflammatory effector cells, to ultimately increase the ratio of regulatory T cells to
pro-inflammatory T cells thereby attenuating the incidence and/or severity of the
disease pathology.

The present application provides experimental evidence that the PEGylation of
20 allogeneic human peripheral blood mononuclear cells (PBMC) or murine splenocytes
can give rise to immunoquiescence (tolerance and/or anergy). This anergy was shown
in vitro by conducting and analyzing mixed lymphocyte reactions (MLR) and
conditioned media experiments for lymphocyte proliferation, differentiation and cytokine
production. For lymphocyte differentiation, both *in vitro* and *in vivo*, the
25 expansion/contraction of the regulatory T (Treg; favoring tolerance or anergy) and Th17
(pro-inflammatory and allorejection) populations were quantitated. To more fully assess
the systemic biologic effect of polymer-mediated immune tolerance, *in vivo* murine
studies were also conducted to determine both the Treg and Th17 population
modulations as well as whether differential effects were noted in the spleen, lymph
30 nodes and blood of control and treated animals.

Methods for modulating the Treg/pro-inflammatory T cells ratio

The present application provides methods and cellular preparations for increasing the
ratio of the level of regulatory T cells with respect to the level of pro-inflammatory T
cells. In the present application, the ratio can be increased either by augmenting the
35 level of regulatory T cells in the subject or decreasing the level of pro-inflammatory T

cells in the subject. Alternatively, the ratio can be increased by augmenting the level of regulatory T cells in the subject and decreasing the level of pro-inflammatory T cells in the subject. When the ratio Treg/pro-inflammatory T cells is increased in a subject, it is considered that a state of anergy and/or of increased tolerance is present. The
5 induction of a state of anergy or immunotolerance in individuals experiencing an abnormally elevated immune reaction can be therapeutically beneficial for limiting the symptoms or pathology associated with the abnormally elevated immune reaction. In some embodiments, it is not necessary to induce a state of complete anergy or tolerance, a partial induction of anergy or tolerance can be beneficial to prevent, treat
10 and/or alleviate the symptoms of a disorder associated with a pro-inflammatory state (such as, for example, an auto-immune disease or an excessive immune response).

In order to increase the Treg/pro-inflammatory T cells ratio, a cellular preparation can be administered to the subject in a therapeutically effective amount. In such instance, the cellular preparation comprises a first immunogenic cell that has been modified with
15 a low-immunogenic biocompatible polymer. The first cell is considered immunogenic with respect to the subject because it is able to induce an immune response (e.g. a cell-mediated immune response) in the subject. It is possible to determine if two cells are considered immunogenic with respect to one another by conducting conventional *in vitro* assays, such as a mixed lymphocyte reaction. It is also expected that MHC-
20 disparate cells would be considered immunogenic with respect to one another. In an embodiment, the first immunogenic cell can be allogeneic to the subject. In another embodiment, the first immunogenic cell can be xenogenic to the subject. However, the first immunogenic cell cannot be autologous or syngenic to the subject.

An alternative way of increasing the ratio, it is also possible to administer the
25 supernatant of a conditioned cell culture of a first immunogenic cell that has been modified with a low-immunogenic biocompatible polymer and a second immunogenic cell. In some embodiment, this cell culture supernatant can comprise a first immunogenic cell or a derivative thereof (a part of the cytoplasmic membrane from the first immunogenic cell for example). In some embodiments, the second
30 immunogenic cell is not modified to bear a low-immunogenic biocompatible polymer on its surface. Alternatively, the second immunogenic cell can be modified to bear a low-immunogenic biocompatible polymer on its surface. The second cell is considered immunogenic with respect to the first cell because if the first cell was not modified and placed into contact with the second cell, an immune response (e.g. a cell-mediated
35 immune response) would occur. It is possible to determine if two cells are considered

immunogenic with respect to one another by conducting conventional *in vitro* assays, such as the mixed lymphocyte reaction. It is also expected that MHC-disparate cells would be considered immunogenic with respect to one another. In an embodiment, the first immunogenic cell can be allogeneic to the second immunogenic cell. In another
5 embodiment, the first immunogenic cell can be xenogenic to the second immunogenic cell. However, the first immunogenic cell cannot be autologous or syngenic to the second immunogenic cell.

An alternative way of increasing the ratio, it is also possible to administer the supernatant of a conditioned serum of an animal (such as a rodent) that has been
10 transfused with a first immunogenic cell that has been modified with a low-immunogenic biocompatible polymer. In some embodiment, this conditioned serum can comprise a first immunogenic cell or a derivative therefrom (a part of the cytoplasmic membrane from the first immunogenic cell for example). The first cell is considered immunogenic with respect to the animal if the first cell was not modified and transfused
15 into the animal, an immune response (e.g. a cell-mediated immune response) would occur. In another embodiment, the first immunogenic cell can be allogeneic or xenogenic with respect to the animal. However, the first immunogenic cell cannot be autologous or syngenic to the animal.

The first and/or second immunogenic cells can be derived from any animals, but are
20 preferably derived from mammals (such as, for example, humans and mice).

In the methods and cellular preparations provided herewith, the surface of the first immunogenic cell is modified with a low-immunogenic biocompatible polymer. For some specific application, it may be preferable to modify the first immunogenic cell with a single type of low-immunogenic biocompatible polymer. However, for other
25 applications, it is possible to modify the first immunogenic cell with at least two different types of low-immunogenic biocompatible polymers.

In order to achieve this modification, the low-immunogenic biocompatible polymer can be covalently bound to a membrane-associated protein of the first immunogenic cell. The membrane-associated protein must have at least a portion which is accessible on
30 the external surface of the cytoplasmic membrane of the first immunogenic cell for being covalently attached to the polymer. For example, the membrane-associated protein can be partially embedded in the cytoplasmic membrane or can be associated with the external surface of the membrane without being embedded in the cytoplasmic membrane. The low-immunogenic biocompatible polymer can be covalently bound to a
35 plurality of membrane-associated proteins. In some embodiment, the low-immunogenic

biocompatible polymer can be polyethylene glycol (methoxy polyethylene glycol for example). The polyethylene glycol can be directly and covalently bound to a membrane-associated protein or, alternatively, a linker attaching the low-immunogenic biocompatible polymer can be used for attaching the polymer to the protein.

5 Exemplary linkers are provided in U.S. Patent Serial No. 8,007,784.

In an alternative or complementary embodiment, the low-immunogenic biocompatible polymer can be inserted in the cytoplasmic membrane by using a lipid-modified polymer.

10 In the methods and cellular preparations provided herewith, the first immunogenic cell and the second immunogenic cell are leukocytes or stem cells. Leukocytes can be obtained from isolating peripheral blood mononuclear cells (PBMC) from the subject. Alternatively, the leukocytes can be obtained from the spleen (e.g. splenocytes). Leukocytes usually include T cells, B cells and antigen presenting cells. In the methods and cellular preparations provided herewith, the first and/or second immunogenic cells
15 are not erythrocytes since the polymer-modified erythrocytes are not capable of increasing the ratio Treg/pro-inflammatory T cells when they were administered in a subject. However, traces of erythrocytes in the first and/or second immunogenic cells are tolerated (for example, less than about 10%, less than about 5% or less than about 1% of the total number of cells in the preparation).

20 Even though it is not necessary to further purify the leukocytes to conduct the method or obtain the cellular preparation, it is possible to use a pure cell population or a relatively homogenous population of cells as the first immunogenic cells and/or as the second immunogenic cells. This pure cell population and relative homogenous population of cells can, for example, essentially consist essentially a single cell type of
25 T cells, B cells, antigen presenting cells (APC) or stem cells. Alternatively, the population of cells can consist essentially of more than one cell type. The population of cells can be obtained through conventional methods (for example cell sorting or magnetic beads). In an embodiment, when the population of cells consist of a single cell type, the percentage of the cell type with respect to the total population of cells is at
30 least 90%, at least 95% or at least 99%. The relatively homogenous population of cells are expected to contain some contaminating cells, for example less than 10%, less than 5% or less than 1% of the total population of cells.

The cell culture supernatant used in the method or in the cellular preparation can be obtained by co-culturing a first immunogenic leukocyte preparation with a second
35 immunogenic leukocyte preparation. It is also possible to co-culture a first immunogenic

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homogenous cell population (such as, for example, a T pure cell population or a substantially pure T cell population) with a second immunogenic leukocyte preparation. It is also contemplated to culture a first immunogenic leukocyte preparation with a second immunogenic population of cells (such as, for example, a pure T cell population or a substantially pure T cell population).

In addition, when the subject's own cells are used in the cell culture (to provide the culture supernatant), they can be modified to be covalently bound to the low-immunogenic biocompatible polymer and cultured with a (second) immunogenic cell. Alternatively, they can remained unmodified (e.g. not covalently bound to the low-immunogenic biocompatible polymer) and cultured with a (first) immunogenic cell which has been modified to be covalently bound to the low-immunogenic biocompatible polymer.

In the methods and preparations presented herewith, it is possible to inhibit/limit the proliferation of the immunogenic cell. For example, when a first immunogenic cell is administered to the subject, it can be treated/modified prior to its administration into the subject in order to inhibit/limit the immunogenic cell from proliferating in the subject. For example, the immunogenic cell can be irradiated (e.g. γ -irradiation) prior to its introduction in the subject or its introduction into a culture system. Upon irradiation, the immunogenic cell is not considered viable (e.g. capable of proliferation). Alternatively, the first immunogenic cell can be treated with a pharmacological agent which halts cell cycle progression. Upon the administration of such pharmacological agent, the first immunogenic cell can be considered viable since it can resume cellular proliferation when the agent is removed from the cell-containing medium.

When the cell culture supernatant is used in the method or in the cellular preparations, it is possible to inhibit/limit the proliferation of one of the two immunogenic cell population. As indicated above, the inhibition of cellular proliferation can be achieved by various means, including irradiation and the use of pharmacological agent. In this particular embodiment, it is important that only one of the two cell populations be inhibited/limited from proliferating and that the other cell population be able to proliferate.

The conditioned serum used in the method or in the cellular preparation can be obtained by transfusing a first immunogenic leukocyte preparation to the subject. It is also possible to transfuse a first immunogenic homogenous cell population (such as, for example, a T pure cell population or a substantially pure T cell population) to the subject. The serum is recuperated from the subject after a time sufficient to induce in

the transfused subject a state of anergy or tolerance. As indicated above, optionally, the first immunogenic cellular preparation can be inhibited/limited from proliferating prior to the transfusion.

As shown herein, the contact between a first (modified) immunogenic cell and a second immunogenic cell (*in vivo* or *in vitro*) induces a state of anergy or immune tolerance in the treated subject. This state of anergy or immune tolerance is induced by either directly administering the first modified immunogenic cell or the cell culture supernatant of the first and second immunogenic cell. In addition, the state of anergy can persist long after the administration of the modified first immunogenic cells or the cellular preparations (as shown below, at least 30 days in mice). Consequently, the methods and cellular preparations described herein are useful for the treatment, prevention and/or alleviation of symptoms associated with abnormal/excessive immune responses and conditions associated thereto.

Autoimmunity arises consequent to an animal/individual's immune system recognizing their own tissues as "non-self". Autoimmunity is largely a cell-mediated disease with T lymphocytes playing a central role in "self" recognition and are, in many cases, also the effector cells.

The Non-Obese Diabetic (NOD) mouse is an inbred strain that exhibits the spontaneous development of a variety of autoimmune diseases including insulin dependent diabetes. The murine autoimmune diabetes develops around 16 to 20 weeks of age and has been extensively used to study the mechanisms underlying autoimmune-mediated diabetes, therapeutic interventions and the effect of viral enhancers on disease pathogenesis. Diabetes develops in NOD mice as a result of insulinitis, a leukocytic infiltrate of the pancreatic islets. This can be exacerbated if mice are exposed to killed mycobacterium or other agents (Coxsackie virus). Multiple studies have established that the pathogenesis of diabetes in the NOD mouse is very similar to that observed in human type I diabetes (T1D) in that it is characterized by the breakdown of multiple tolerance pathways and development of severe insulinitis of the islets prior to β -cell destruction. Moreover, T cells (including Th1, Th17 and Tregs) have been identified as key mediators of the autoimmune disease process though other cells (NK cells, B-cells, DC and macrophages) are also observed. Indeed, the NOD mouse model has translated into successful clinical human trials utilizing T-cell targeting therapies for treatment of many autoimmune diseases, including T1D.

Treatment of most autoimmune diseases is problematic and normally focused on addressing disease symptoms, not causation. Typically, treatment for chronic

autoimmune disease is via systemic steroid (e.g., dexamethasone) administration to induce a general immunosuppression and to act as an anti-inflammatory agent. It is believed that one mechanism of this immunosuppression may be the induction of Treg cells. In addition to steroids, the administration of IVIg (pooled, polyvalent, IgG purified from the plasma of >1 000 blood donors) can also effectively treat some autoimmune diseases including immunothrombocytopenia (ITP). Interestingly, the onset of diabetes in NOD mice can also be delayed, but not fully blocked by administration of IVIg and this may correlate with induction of Tregs (and/or IL-10). Hence, novel approaches to increase Treg cells (and/or IL-10) while decreasing inflammatory T cell responses (e.g., Th17, NK cells) could be beneficial in treating autoimmune diabetes.

A state of anergy or immune tolerance can be considered therapeutically beneficial in subjects experiencing (or at risk of experiencing) an abnormal immune response, such as for example an auto-immune disease. Some auto-immune diseases are associated with either low levels of Tregs and/or elevated levels of pro-inflammatory T cells (such as Th17 and/or Th1). Such auto-immune diseases include, but are not limited to, type I diabetes, rheumatoid arthritis, multiple sclerosis, lupus, immune thrombocytopenia, experimental autoimmune encephalomyelitis, auto-immune uveitis, psoriasis inflammatory bowel disease and Crohn's disease. Because it is shown herein that the modified first immunogenic cell and/or the cellular preparations are beneficial for increasing the ratio Tregs/pro-inflammatory T cells, it is expected that administration of the cellular preparations to afflicted subject will alleviate symptoms associated with the auto-immune disease.

A state of anergy or tolerance can also be considered therapeutically beneficial in subjects at risk of developing an abnormally elevated/excessive immune response. Such abnormally elevated immune response can be observed in subjects receiving a vaccine. For example, it has been shown that subjects receiving a respiratory syncytial virus (RSV) vaccine develop an excessive immune response. Such abnormally elevated immune response can also be observed in subjects receiving a transplant (cells or tissues). In these conditions, the methods and cellular preparations can be applied to prevent or limit the elevated/excessive immune response. The modified first immunogenic cell and/or cellular preparations can be co-administered with the vaccine or the transplant. Alternatively, the modified first immunogenic cell and/or cellular preparations can be administered prior to the administration of the vaccine or the introduction of the transplant to induce a state of anergy or tolerance in the subject.

In the methods and cellular preparations described herein, it is contemplated that the cell culture supernatant be optionally administered with other therapeutic agents known to be useful for the treatment, prevention and/or alleviation of symptoms of conditions associated to an excessive/abnormal immune response, such as, for example,
5 cortisone, IL-10, IL-11 and/or IL-12.

Processes for obtaining cellular preparations

The cellular preparations used in the methods described can be obtained by first covalently binding a low-immunogenic biocompatible polymer to a first immunogenic cell. In this first step, it is important low-immunogenic biocompatible polymer be grafted
10 at a density sufficient for preventing allo-recognition of the first immunogenic cell by the second immunogenic cell or the treated subject's cells. In order to determine if the second immunogenic cell is capable of allo-recognizing the first immunogenic cell (and ultimately determine if the selected polymer and grafting is appropriate), various techniques are known to those skilled in the art and include, but are not limited to, a
15 standard mixed lymphocyte reaction (MLR), high molecular weight mitogen stimulation (e.g. PHA stimulation) as well as flow cytometry (to determine the immunocamouflage of surface markers, see Chen and Scott, 2006).

It is also important that the polymer exhibit low-immunogenicity and be biocompatible to the treated subject. It is shown below that polyethylene glycol (particularly
20 methoxypoly(ethylene glycol)) and POZ have been successfully used to modified a first immunogenic cell and provide to this cell the ability to increase a ratio of Treg cells to pro-inflammatory T cells in a subject. These experimental results show that other low-immunogenic biocompatible polymers can also be used to modify the first immunogenic cell because they have also been shown to prevent allorecognition of the first
25 immunogenic cell and are biocompatible. These other low-immunogenic biocompatible polymers include, but are not limited to an hyperbranched polyglycerol (HPG). In some embodiments, it is preferable to use a single type of polymers. In other embodiments, it is possible to use a plurality of different types of polymers.

In an embodiment, the low-immunogenic biocompatible polymer can be covalently
30 associated with the membrane-associated protein(s) by creating a reactive site on the polymer (by deprotecting a chemical group) and contacting the polymer with the first immunogenic cell. For example, for covalently binding a methoxypoly(ethylene glycol) to a first immunogenic cell, it is possible to incubate a methoxypoly(-ethylene glycol) succinimidyl valerate (reactive polymer) in the presence of the first immunogenic cell.
35 The contact between the reactive polymer and the first immunogenic cell is performed

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under conditions sufficient for providing a grafting density which will prevent allorecognition of the first immunogenic cell by the second immunogenic cell or the immune cells of the subject. A linker can optionally be used. Examples of such polymers and linkers are described in U.S. Patents 5,908,624; 8,007,784 and
5 8,067,151.

In another embodiment, the low-immunogenic biocompatible polymer can be integrated within the lipid bilayer of the cytoplasmic membrane by using a lipid-modified polymer.

The first immunogenic cell can be optionally inhibited/limited from proliferating prior to its introduction in the subject or its contact with the second immunogenic cell. For
10 example, the first immunogenic cell can be irradiated to halt its proliferation prior to its administration to the subject or its contact with the second immunogenic cell. Alternatively, the first immunogenic cell can first be modified with the low-immunogenic biocompatible polymer and then inhibited/limited from proliferating.

In an optional embodiment, the modified first immunogenic cell can be placed in a cell
15 culture with the second immunogenic cell and the supernatant of this cell culture can be administered to the subject in need thereof. The supernatant can be modified (e.g. filtered) to remove the first and/or second immunogenic cell. When the first and second immunogenic cells are cultured in the same medium (or in the same culture system), one of the two immunogenic cell populations can be inhibited/limited from proliferating.
20 For example, the modified first immunogenic cell can be inhibited/limited from proliferating prior to its co-culture with the second immunogenic cell. Alternatively, the second immunogenic cell can be inhibited/limited from proliferating prior to its co-culture with the modified first immunogenic cell. In the co-culture systems, it is important that at least one of the two immunogenic cell populations be able to
25 proliferate and be considered viable.

In the process described herein, the first immunogenic leukocyte can be allogeneic to the subject's cells or to second immunogenic leukocyte. Alternatively, the first immunogenic cell can be xenogeneic to the subject's cells or to the second immunogenic cell.

30 When a co-culture system is used, it is possible to culture a first immunogenic leukocyte preparation (such as, for example a PBMC) with a second immunogenic leukocyte preparation (such as, for example a PBMC). It is also possible to culture a first immunogenic relatively homogenous cell population (such as, for example, a T cell population) with a second immunogenic leukocyte preparation (such as, for example a

PBMC). It is also contemplated to culture a first immunogenic leukocyte preparation (such as, for example a PBMC) with a second immunogenic relatively homogenous population of cells (such as, for example, a T cell population).

5 In other embodiments, a conditioned serum can be used. The conditioned serum used in the method or in the cellular preparation can be obtained by transfusing a first immunogenic leukocyte preparation to the subject. It is also possible to transfuse a first immunogenic homogenous cell population (such as, for example, a T pure cell population or a substantially pure T cell population) to the subject. The serum is recuperated from the subject after a time sufficient to induce in the transfused subject a
10 state of anergy or tolerance. As indicated above, optionally, the first immunogenic cellular preparation can be inhibited/limited from proliferating prior to the transfusion.

Once the first immunogenic cell has been modified it or the conditioned supernatant/serum has been obtained it can be formulated for administration to the subject. The formulation step can comprise admixing the cellular preparation obtained
15 (at a therapeutically effective dose) with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, and/or carriers. The formulations are preferably in a liquid injectable form and can include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces. The formulations can comprise pharmaceutically
20 acceptable solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol).

In addition, if the modified first immunogenic cell or the conditioned serum/supernatant are destined to be used to prevent an excessive immune reaction triggered by a
25 vaccine, they can be formulated for administration with the vaccine. The modified first immunogenic cell or the conditioned serum/supernatant can be formulated for simultaneous administration with the vaccine by admixing the vaccine with the modified first immunogenic cell or the conditioned serum/supernatant. Alternatively, the modified first immunogenic cell or the conditioned serum/supernatant can be formulated for
30 administration prior to or after the vaccine, for example in a formulation that is physically distinct from the vaccine.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I – MATERIAL AND METHODS

Human PBMC and dendritic cell preparation. Human whole blood was collected in heparinized vacutainer blood collection tubes (BD, Franklin Lakes, NJ) from healthy volunteer donors following informed consent. PBMC were isolated from diluted whole blood using FicollPaque PREMIUM™ (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) as per the product instructions. The PBMC layer was washed twice with 1x Hank's Balanced Salt Solution (HBSS; without CaCl₂ and MgSO₄; Invitrogen by Life Technologies, Carlsbad, CA) and resuspended in the appropriate media as needed for mixed lymphocyte reactions and flow cytometric analysis of Treg and Th17 phenotypes. Dendritic cells (DC) were prepared from isolated PBMC as described by O'Neill and Bhardwaj (O'Neill et al., 2005). Briefly, freshly isolated PBMC were overlaid on Petri dishes for 3 h of in AIM V serum free culture medium (Invitrogen, Carlsbad, CA). Non-adherent cells were gently washed off the plate. The adherent cells (monocyte rich cells) were treated with IL-4 and GM-CSF (50 and 100 ng/mL respectively; R&D Systems, Minneapolis, MN) in AIM V medium. Cells were again treated with IL-4 and GM-CSF on days 2 and 5. On day 6, cells were centrifuged and resuspended in fresh media supplemented with DC maturation factors (TNF- α , IL-1b, IL-6; R&D Systems, Minneapolis, MN) and prostaglandin E2 (SigmaAldrich, St. Louis, MO). The mature DC-like cells were harvested on day 7 and CD80, CD83, CD86 and HLA-DR expressions were determined to confirm DC maturation via flow cytometry (FACSCalibur™ Flow Cytometer, BD Biosciences, San Jose, CA).

Murine splenocyte and tissue harvesting. All murine studies were done in accordance with the Canadian Council of Animal Care and the University of British Columbia Animal Care Committee guidelines and were conducted within the Centre for Disease Modeling at the University of British Columbia. Murine donor cells used for the *in vivo* donation and *in vitro* studies were euthanized by CO₂. Three allogeneic strains of mice were utilized for syngeneic and allogeneic *in vitro* and *in vivo* challenge: Balb/c, H-2^d; C57Bl/6, H-2^b; and C3H, H-2^k. Murine spleens, brachial lymph nodes, and peripheral blood were collected at the indicated days. Mouse spleens and brachial lymph nodes were dissected and placed into cold phosphate buffered saline (PBS; 1.9 mM NaH₂PO₄, 8.1mM Na₂HPO₄, and 154 mM NaCl, pH 7.3) containing 0.2% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO.) and kept on ice until ready to process. Whole blood was collected in heparinized tubes via cardiac puncture. Murine donor splenocytes were prepared from freshly harvested syngeneic or allogeneic spleens via homogenization into a cell suspension in PBS (0.2% BSA) using the frosted end of two

- microscope slides. The resultant cell suspension was spun down at 500 x g. The splenocyte pellet was resuspended in 1 mL of 1 x BD Pharm Lyse™ lysing buffer (BD Biosciences, San Diego, CA) and incubated for 1 min at room temperature. Lymph node cells were harvested via tissue homogenization as described above, washed
5 twice and resuspended in PBS (0.2% BSA) for flow cytometric analysis of Th17, Treg and murine haplotype. Recipient peripheral blood lymphocytes were prepared via lysis of the red cells (BD Pharm Lyse lysing buffer; BD Biosciences, San Diego, CA) at 1x concentration, followed by washing (1x) and resuspension in PBS (0.2% BSA) for flow analysis of Th17, Treg and murine haplotype.
- 10 *mPEG modification (PEGylation) of PBMCs and splenocytes.* Human PBMC and murine splenocytes were derivitized using methoxypoly(-ethylene glycol) succinimidyl valerate (mPEG-SVA; Laysan Bio Inc. Arab, AL) with a molecular weight of 5 or 20 kDa as previously described (Scott et al., 1997; Murad et al, 1999A; Chen et al., 2003; Chen et al., 2006). Grafting concentrations ranged from 0 to 5.0 mM per 4×10^6
15 cells/mL. Cells were incubated with the activated mPEG for 60 min at room temperature in isotonic alkaline phosphate buffer (50 mM K_2HPO_4 and 105 mM NaCl; pH 8.0), then washed twice with 25 mM HEPES/RPMI 1640 containing 0.01% human albumin. Human PBMC were resuspended in AIM V media at a final cell density of 2.0×10^6 cells/mL for use in the MLR. Murine splenocytes used for *in vivo* studies were
20 resuspended in sterile saline at a final cell density of 2.0×10^8 cells/ml for i.v. injection. To determine if the simple presence of the mPEG polymer itself altered the immune response either *in vitro* and *in vivo*, additional studies were done with unactivated polymer incapable of covalent grafting to the cell surface. For these studies, allogeneic human (*in vitro* studies) or syngeneic and allogeneic murine splenocytes (*in vivo*
25 studies) were treated with non-covalently bound mPEG (soluble mPEG) under the same reaction conditions described for the covalent grafting studies. For clarity, “soluble mPEG” refers to cells treated with non-covalently grafted polymer while “mPEG-modified” refers to treatment with activated polymer resulting in the covalent grafting of the mPEG to the cell membrane.
- 30 *In vitro and in vivo cell proliferation.* Cell proliferation (both *in vitro* and *in vivo*) was assessed via flow cytometry using the CellTrace™ CFSE (Carboxyfluorescein diacetate, succinimidyl ester) Cell Proliferation Kit (Invitrogen by Life Technologies e Molecular probes, Carlsbad, CA). Human and murine cells labeling was done according to the product insert at a final concentration of 2.5 mM CFSE per 2×10^6
35 cells total. Donor and recipient cell proliferation was differentially determined by

haplotype analysis. In some experiments, cell proliferation was measured by ^3H -thymidine incorporation. In these experiments, donor splenocytes (5.12×10^6 cells per well) were co-incubated in triplicate in 96-well plates at 37°C , 5% CO_2 for 3 days. On day 3, all wells were pulsed with ^3H -thymidine and incubated for 24 h at 37°C , 5% CO_2 .

5 Cellular DNA was collected on filter mats using a Skatron cell harvester (Suffolk, U.K.) and cellular proliferation was measured by ^3H -thymidine incorporation.

Mixed lymphocyte reaction (MLR) - control and conditioned media. The effects of polymer grafting (5 kDa SVAmPEG) on allorecognition *in vitro* were assessed using two-way MLR (Murad et al, 1999A; Chen et al., 2003; Chen et al., 2006). PBMC from
10 two MHC-disparate human donors were label with CFSE as described. Each MLR reaction well contained a total of 1×10^6 cells (single donor for resting or mitogen stimulation or equal numbers for disparate donors for MLR). Cells were plated in multiwell flat-bottom 24-well tissue culture plates (BD Biosciences, Discovery Labware, Bedford, MA). PBMC proliferation, cytokine secretion, as well as Treg and Th17
15 phenotyping was done at days 10 and 14. For flow cytometric analysis, the harvested cells were resuspended in PBS (0.1% BSA). While time course studies (Days 4, 7, 10 and 14) were done, the presented studies show days 10 and 14. These extended studies are, in fact, the most stringent test of the immunomodulatory effects of the grafted polymer as membrane remodeling over this time could have resulted in a latter
20 onset of proliferation. To investigate *in vitro* whether polymer grafting to allogeneic PBMC gave rise to tolerance or anergy, secondary (2°) MLR studies were conducted using conditioned media. Conditioned media from a primary (1°) 2 way-MLR was collected at 72 h for conducting a secondary (2°) MLR as schematically shown in Fig. 1. Conditioned media from the 1° MLR included: A1) Resting Unmodified PBMC; B2)
25 Resting PEGylated PBMC; C3) two-way MLR; and D4) two-way mPEG-MLR. The 2° MLR utilized freshly prepared MHC-disparate donors (either the same as or different from) the initial plate and plated as described above. As shown in Fig. 1, the 2° MLR samples included: A) Resting PBMC; B) two-way MLR; P) Mitogen Stimulation; D) two-way mPEG-MLR. For these studies, PBMC were derivatized using 1 mM 5 kDa SVAmPEG. Mitogen stimulation (PHA-P; SigmaAldrich, St. Louis, MO) of donor
30 PBMC in the secondary plates was done to assess the proliferation potential and viability of cells incubated in the conditioned media. Human PBMC were challenged with 2 mg/ml per 1×10^6 cells of PHA-P. All plates were incubated at 37°C (5% CO_2). Following 13 days of incubation (37°C , 5% CO_2), the cell culture supernatants were
35 collected and cells were harvested from the 2° MLR plates. Cell proliferation was measured via CFSE-dilution of $\text{CD}3^+\text{CD}4^+$ lymphocytes by flow cytometry.

Immunophenotyping by flow cytometry. The T lymphocytes populations (double positive for CD3⁺ and CD4⁺) in both the *in vitro* and *in vivo* studies were measured by flow cytometry using fluorescently labeled CD3 and CD4 monoclonal antibodies (BD Pharmingen, San Diego, CA). Human and mouse Regulatory T lymphocytes (Treg) were CD3⁺/CD4⁺ and FoxP3⁺ (transcription factor) while inflammatory Th17 lymphocytes cells were CD3⁺/CD4⁺ and IL-17⁺ (cytokine) as measured per the BD Treg/Th17 Phenotyping Kit (BD Pharmingen, San Diego, CA). After the staining, the cells (1 x 10⁶ cells total) were washed and resuspended in PBS (0.1% BSA) prior to flow acquisition. Isotype controls were also used to determine background fluorescence. All samples were acquired using the FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) and CellQuest Pro™ software for both acquisition and analysis.

Cytokine quantitation. Cell culture supernatants were collected from the 1° two-way MLR plate and stored at -80°C prior to analysis. Conditioned media aliquots from a minimum of four independent experiments were used for quantification of supernatant cytokine levels using the BD Cytometric Bead Array (CBA) system (BD Biosciences, San Diego, CA) for flow cytometry. The CBA system is a multiplexed bead based immunoassay used to quantitate multiple cytokine levels in a single sample simultaneously by fluorescence-based emission and flow cytometry. Cytokine measured included: IFN γ , TNF- α , IL-10, IL-5, IL-4, and IL-2 using the BD Human Th1/Th2 Cytokine Kit I™. The IL-6 and IL-17A levels were measured using the BD CBA Human Soluble Protein Flex Set™. Both assays were performed following the manufacturer's product instruction manual. Briefly, cell culture supernatants of resting unmodified PBMC, unmodified MLR, PEGylated (5 kDa SVAmPEG; one donor) resting PBMC, PEGylated MLR, and mitogen (PHA) stimulated PBMC were incubated at room temperature in the dark with a mixture of each cytokine antibody-conjugated capture bead and the PE-conjugated detection antibody. Following the incubation, the samples were washed and acquired using a FACSCalibur™ flow cytometer and analyzed using Cell-Quest Pro™ software. Cytokine protein levels were determined using the BD Cytometric Bead Array™ and FCAP Array™ analysis software (BD Biosciences, San Diego, CA and Soft Flow Inc, St. Louis Park, MN).

In vivo murine studies. To investigate whether mPEG grafting to leukocytes would have systemic *in vivo* effects, a murine adoptive transfer system was employed using three genetically different strains: Balb/c, H-2^d; C57Bl/6, H-2^b; and C3H, H-2^k (Chen et al., 2003; Chen et al., 2006). All mice (donors and recipients) were 9-11 weeks old. Donor splenocytes were prepared and CFSE labeled as described. Control and mPEG-

grafted (1mM, 20 kDa SVAmPEG) syngeneic or allogeneic cells (20×10^6 splenocytes) were transfused intravenously (i.v.) via the tail vein into recipient animals. BALB/c and C57BL/6 mice injected with sterile saline served as control animals. Animals were euthanized by CO₂ at predetermined intervals (24 h, 48 h, 72 h, 96 h and 120 h) at which time blood, brachial lymph nodes and spleen were collected and processed for Th17/Treg phenotyping analysis and splenocyte proliferation studies by flow cytometry. Donor cell engraftment and proliferation were assessed via flow cytometry using murine haplotype (H-2K^b vs. H-2K^d) analysis. To determine the persistence of the immunomodulation, mice were re-challenged (2° challenge) 30 days after the initial transfer of allogeneic or mPEGallogeneic splenocytes with unmodified allogeneic cells. At 5 days post 2° challenge, Treg and Th17 phenotyping of murine splenocytes isolated from the spleen, lymph node and peripheral blood was again assessed via flow cytometry.

Statistical analysis. Data analysis was conducted using SPSS™ (v12) statistical software (Statistical Products and Services Solutions, Chicago, IL, USA). For significance, a minimum p value of <0.05 was used. For comparison of three or more means, a one-way analysis of variance (ANOVA) was performed. When significant differences were found, a post-hoc Tukey test was used for pair-wise comparison of means. When only two means were compared, student-t tests were performed.

20 **EXAMPLE II – *IN VITRO* AND *IN VIVO* IMMUNOMODULATION**

The material and methods used in this example are provided in Example I.

To determine the effects of immunocamouflage on the immune response, initial *in vitro* experiments examined the cytokine burst characterizing control and polymer modified MLR. The polymer-mediated immunocamouflage of human PBMC resulted in significant changes in the cytokine profile of the conditioned media obtained from the 1° MLR plate (Figs. 1 and 2). As shown in Fig. 2, control MLRs yielded elevated concentrations of IL-2, IFN- γ , IL-17A, TNF- α and IL-6 relative to resting unmodified or PEGylated PBMC. In contrast to the control MLR, the mPEG-MLR (one donor population PEGylated with 1 mM 5 kDa SVA-mPEG) resulted in the virtually complete inhibition ($p < 0.001$) of secretion for the proinflammatory cytokines examined. However, IL-10 was preferentially elevated in the mPEG-MLR. In the conditioned media, IL-10 levels were 2.01 ± 1.26 , 8.90 ± 2.10 , 1.69 ± 0.64 and 1.33 ± 0.73 ng/ml for the resting PBMC, mPEG-MLR, resting mPEGPBMC and Control MLR, respectively. As noted, in the mPEG-MLR, IL-10 levels were significantly ($p < 0.01$) increased suggesting the induction of an immunosuppressive state. Importantly, this

cytokine quiescent state was not due to loss of cell viability as evidenced by the very low levels of non-viable cells detected following 72 h incubation (Fig. 2).

The conditioned media produced from the initial 72 h MLR exerted a significant effect on the 2° MLR as demonstrated in Fig. 3. While the 1° media from resting PBMC showed no significant effect on the 2° MLR, the media from the 1° Control MLR demonstrated a significant ($p < 0.01$) pro-proliferative effect in the 2° MLR. As shown, the mean proliferation index of the 2° MLR increased from 26.05 ± 12.47 to 44.72 ± 17.13 in the presence of conditioned media from the 1° Control MLR. The pro-inflammatory effect of the 1° MLR media was noted on even the resting PBMC and PHA-stimulated cells. In contrast, the 1° conditioned media from the mPEG-MLR demonstrated a significant ($p < 0.001$) anti-proliferative effect in not only the 2° MLR but also the PHA-stimulated cells. The differential proliferation response between the control and mPEG-MLR conditions for matching experiments is noted by the lines connecting paired experiments. While not shown, soluble mPEG (5 kDa) had no effect on cytokine levels in the 1° conditioned media nor on the proliferation of PBMC mediated by allorecognition (control MLR) or by mitogen (PHA) stimulation.

Furthermore, as shown in Fig. 4, the proliferation index was positively correlated with an increased population of Th17 T cells and inversely correlated with Treg lymphocytes numbers. As demonstrated, the 1° conditioned media from the control MLR yielded elevated levels of Th17 cells and decreased levels of Treg lymphocytes. In comparison, the 1° media from the mPEG-MLR resulted in significantly elevated ($p < 0.001$) levels of Treg cells and a virtually non-existent population of Th17 lymphocytes. The source of the conditioned media also impacted the efficacy of PHA stimulation. As shown, conditioned media from the mPEG-MLR significantly inhibited mitogen proliferation while the control MLR conditioned media significantly enhanced proliferation relative to both media from resting PBMC ($p < 0.01$) and resting mPEG-PBMC ($p < 0.001$).

Hence, the *in vitro* experiments demonstrated that covalent grafting mPEG to human PBMC resulted in an immunomodulatory effect governed in part by changes in the Th17 and Treg populations. Moreover, these conditioned media experiments demonstrated that this immunomodulatory effect arises from soluble factors that might be able to induce a systemic effect *in vivo*. To determine if similar effects would be observed *in vivo*, a murine splenocyte adoptive transfer model was utilized. As demonstrated in Fig. 5, PEGylated donor splenocytes resulted in a significant *in vivo* immunomodulatory effect giving rise to elevated levels of Treg lymphocytes within the

spleen, brachial lymph node, and peripheral blood. As noted, in all three tissues, a significant ($p < 0.001$ at 120 h) time-dependent increase in Treg lymphocytes over that observed in naïve mice was noted in mice receiving mPEG-modified allogeneic donor cells. In stark contrast, a significant ($p < 0.001$) decrease in Tregs (≥ 48 h post-injection relative to naïve mice) is noted in mice transfused with unmodified allogeneic splenocytes. The absolute difference between the unmodified (control) and PEGylated splenocytes, shown by the stippled area, demonstrates the true magnitude of the differential impact of donor cell PEGylation. Importantly, as noted at 120 h, transfusion of soluble mPEG, syngeneic cells or mPEG-syngeneic cells had no significant effect on the Treg lymphocyte population.

As foreshadowed by our *in vitro* human PBMC findings (Example II), murine Th17 lymphocyte levels were influenced by the PEGylation state of the allogeneic donor cells (Fig. 6). While unmodified allogeneic murine donor cells resulted in a significant ($p < 0.001$), time-dependent, increase in the Th17 cell population in the spleen, brachial lymph node and peripheral blood, the covalent grafting of mPEG to the membrane of the donor splenocytes resulted in the complete abrogation of the increase. Indeed, the Th17 population remained at resting levels. The absolute difference between the unmodified and mPEG-modified donor cells is denoted by the stippled area. As with the Treg population, transfusion of soluble mPEG, syngeneic cells or mPEG-syngeneic cells had no significant effect on the Th17 lymphocyte population at 120 h.

As also shown on Fig. 7, normal mice have significantly higher levels of Tregs (Spleen $\sim 10\%$ total CD4⁺ T cells) relative to Th17 T Cells (Spleen $\sim 0.05\%$ total CD4⁺ T cells). Further, treatment with unmodified allogeneic cells results in production of Th17 cells and loss of Tregs. In contrast, polymer modified allogeneic cells maintain (even increase) Tregs and prevents Th17 production.

As might be anticipated, the mPEG-allogeneic splenocyte mediated increase in Treg cells in the peripheral blood samples occurred later in the studied time course (96 h) compared to either of the lymphatic tissues (spleen and lymph nodes; 48 h). This clearly suggests that T cell proliferation initially occurred within the lymphatic tissues and secondarily migrated into the peripheral blood. A similar time dependency was noted with the Th17 proliferation induced by the unmodified splenocyte populations. Proliferation initially occurred within lymphatic tissue within ~ 48 h and only appeared within the peripheral blood after ~ 96 h.

Of importance was the observation that the immunomodulatory effects of the PEGylated splenocytes were long lived and prevented subsequent changes in Treg

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and Th17 levels consequent to rechallenge with unmodified allogeneic cells. As shown in Fig. 8, 30 days post transfusion with polymer modified splenocytes, Treg levels remain significantly elevated and are similar to levels recorded at 120 h post challenge. In contrast, Th17 levels remained similar to or decreased from that observed in naïve mice at day 30. Of even more interest, a secondary adoptive transfer of unmodified allogeneic splenocytes (30 days post 1^o challenge; measured at 120 h) to mice previously treated with PEGylated allogeneic showed no significant decrease in Treg cells, or increase in Th17 cells, relative to the day 30 levels. This was in direct contrast to that observed in naïve mice (Fig. 5) injected with unmodified allogeneic cells that demonstrated a dramatic decrease in Treg lymphocytes. Indeed, Treg levels remain significantly elevated above that seen in naïve mice and very similar to those levels observed at 5 days post PEGylated splenocytes transfusion.

To determine if the observed *in vivo* murine findings gave rise to a tolerance to a specific H-2 haplotype or a more generalized anergy to allogeneic tissues, *in vitro* two-way murine MLR studies of three allogeneic splenocyte populations (Balb/c, H-2^d; C57Bl/6, H-2^b; and C3H, H-2^k) were done. As demonstrated in Fig. 9, the immunomodulation arising following exposure to polymer-grafted H-2 disparate splenocytes is not specific to the haplotype of the mPEGmodified splenocytes thereby suggestive of an anergic state. As shown, PEGylation of stimulator (*i.e.*, irradiated and incapable of proliferation) splenocytes very effectively attenuated allorecognition and proliferation of the responder cell population within a one-way MLR. Moreover, for comparative purposes, the anti-proliferative dose-response effect of cyclosporine A (CSA; which induces a pharmacologically-induced anergy) in a one-way murine MLR under the same experimental condition is shown. Interestingly, the type of polymer-modified cell is important. Human lymphocytes and murine splenocytes express high levels of “self-antigens” (Human Leukocyte Antigens (HLA) and mouse H-2 proteins). If cells devoid of these highly immunogenic antigens are used in the murine model, no changes in either Tregs or Th17 cells are observed. In mice injected with unmodified allogeneic erythrocytes, Treg levels within the spleen, lymph node and peripheral blood were (respectively): 91.7%, 95.0% and 107.0% of control mouse values. Similarly unchanged, Th17 levels were (respectively): 71.2%, 112.1% and 79.2% of control mouse values. Thus, allogeneic murine RBC do not elicit any significant changes in the systemic levels of either Treg or Th17 lymphocytes. This finding was observed despite some antigenic differences between the RBC in H-2 disparate mice. In support of the low immunogenicity of these genetically different RBC, allogeneic RBC exhibit normal *in vivo* circulation nor do they elicit a significant immune response. Hence, polymer

coupled to a low-immunogenicity allogeneic cell can not induce the immunomodulation noted with the highly immunogenic splenocytes.

Bioengineering of donor cells and/or tissues may provide significant opportunities to attenuate both the recognition and rejection of allogeneic tissues. One polymer-mediated approach to this end is the immunocamouflage of donor cells via the covalent grafting of mPEG (or other low immunogenicity polymers) to the exterior face of cell membranes. Consequent to membrane derivatization, mPEG-modified allogeneic and xenogeneic cells demonstrated a global, multivalent, attenuation of antigenicity and immunogenicity. This effect arose in part from charge camouflage and significantly diminished cell:cell (e.g., T cell:APC or T cell:islet cell) and ligand:receptor (e.g., antibody:antigen or CD28:CD80) interactions. The efficacy of this immunocamouflage was dependent on polymer molecular weight (*i.e.*, size) and grafting density.

However, the inhibition of cell:cell and ligand:receptor interactions are a 'local' immunomodulatory event arising from the steric and charge camouflaging effects of the grafted polymer. For the induction of tolerance, a systemic and persistent immunomodulatory effect would be necessary. As demonstrated herein, covalent grafting of mPEG to allogeneic lymphocytes (human PBMC or murine splenocytes) dramatically reduced allorecognition at both the local (cell:cell; MLR) and systemic (*in vivo* murine models) levels. Importantly, as demonstrated in our *in vivo* studies, it is not the donor cells that differentiate into Treg or Th17 cells, rather it is the recipients immune system that responds to the control or PEGylated splenocytes and upregulates production of either Th17 (upon challenge with unmodified splenocytes) or Treg (upon challenge with mPEG-splenocytes) populations. This was noted by both the absence of CFSE-staining (only donor cells were stained) and H-2 phenotyping of the Th17 and Treg cell populations.

The observed immunosuppressive state induced by PEGylated lymphocytes is surprisingly long lasting *in vivo*. As noted in Fig. 8, the elevated levels of Treg lymphocytes noted at day 5 persist to day 30. Moreover, the presence of these Treg (as well as other probable immunological events) prevents a pro-inflammatory response to unmodified allogeneic splenocytes administered at day 25. Indeed, no increase in Th17 lymphocytes is noted in the immunomodulated mice. Moreover, for the systemic tolerance/anergy to occur, the polymer must be grafted to a highly immunogenic cell type (e.g., lymphocyte and/or antigen presenting cells) as less immunogenic cells, such as H-2 disparate erythrocytes, do not alter the immune (Treg/Th17) response. While allogeneic murine erythrocytes do express antigenic

differences at the membrane, these cells are only weakly immunogenic eliciting weak IgG responses and typically remaining in the vascular circulation with a near normal half-life. Also of critical importance, induction of both local and systemic immunomodulation requires the covalent grafting of the polymer to the cell, as soluble mPEG $\pm$ allogeneic cells has no effect on the population dynamics of either Treg or Th17 lymphocytes *in vitro* or *in vivo*.

The balance between Treg and Th17 cells has been identified as a key factor that orchestrates the tolerance/inflammation level of human immune system. Regulatory T cells provide suppressor effect and maintain tolerance, while Th17 cells mediate and are indicative of a pro-inflammatory state. Hence, the polymer-mediated modulation of this balance may be clinically useful. Recent findings have shown that cyclosporine, a clinically used immunosuppressive agent, has substantial effects on the Treg/Th17 cell response; though this may be mediated by Th17 cytotoxicity as Treg cells cultured in the presence of rapamycin, but not cyclosporine A, are found to suppress ongoing alloimmune responses. Additionally, mycophenolic acid, another immunosuppressive agent, was found to shift the lymphocyte polarization by inhibiting IL-17 expression in activated PBMC *in vitro*. Of clinical importance, all of these pharmacologic agents exert significant systemic toxicity and their ongoing use requires substantial monitoring.

As evidenced by these results, induction of tolerance or anergy in transfusion and transplantation medicine by the polymer-mediated immunocamouflage of allogeneic leukocytes may provide a less toxic approach than current conventional pharmacologic agents. Current efforts to prevent and/or regulate the consequences of allorecognition involve phenotype matching (ranging from blood group to HLA matching) and the use of immunosuppressive agents (Fig. 10A). While extensive tissue matching (e.g., blood groups, HLA) can dramatically enhance transfusion or transplantation success, the necessity of tissue matching dramatically reduces the potential pool of donor tissues. Even in a tissue as plentiful as blood, extensive non-ABO matching for chronically transfused patients (e.g., sickle cell disease), while considered desirable, is costly and often difficult to achieve due to the scarcity of appropriately matched donor cells. This difficulty is greatly exaggerated with less common tissues and organs (e.g., islets and kidneys).

Thus, pharmacological interventions have been employed to enhance the probability of successful donor tissue engraftment (Fig. 10B). The data presented here suggests that polymer encapsulation "of", or grafting "to", donor tissue may be further enhanced or replaced by a tolerogenic or anergic approach. Rather, the prechallenge of a potential

tissue recipient with PEGylated donor specific (or simply allogeneic; see Fig. 9) PBMC several (~5) days prior to tissue transplantation could be used to induce a tolerogenic state within the recipient as shown in Fig. 10B. Elevated levels of Tregs and the down-regulation of Th17 cells would diminish the risks of both hyper-acute and acute rejection of the donor tissue. There are several substantial advantages for this approach. Primary amongst these are the easy collection of donor specific (or simply allogeneic) PBMC, the ease of PEGylation of the PBMC as well as the ease of administration to the transplant recipient. While a potential risk of lymphocyte transfusions is transfusion associated graft versus host disease (TA-GVHD) in immunosuppressed patients, it was previously demonstrated that PEGylation effectively blocks TA-GVHD in a murine model (Chen et al., 2003; Chen et al., 2006). Moreover, this process could be used inconjunction with irradiated PBMC thus obviating any risk of TAGVHD. Irradiated cells retain their allostimulatory effects and PEGylation similarly inhibits this allorecognition and proliferation.

In summary, polymer modification of allogeneic donor lymphocytes prevents allorecognition at the cell:cell level and also gives rise to systemic immunomodulation. The systemic immunomodulation is evidenced by a significant up-regulation of Treg cells and a significant down-regulation of pro-inflammatory Th17 cells. This immunomodulation is persistent (~30 days) and prevents subsequent pro-inflammatory responses to unmodified allogeneic cells. The polymer effect is dependent upon its covalent grafting to allogeneic cells as soluble PEG itself has no immunomodulatory effects. The clinical use of PEGylated (or other covalently grafted polymers) allogeneic lymphocytes to pre-challenge tissue recipients 5 days or more to transplantation may be useful in inducing a tolerogenic state and preventing acute rejection and/or enhancing tissue engraftment.

EXAMPLE III – IN VIVO IMMUNOMODULATION IN NOD MICE

In the NOD mice, autoimmune destruction of the pancreatic islets occurs within approximately 16 weeks and was confirmed with elevated blood glucose measures. The lymphocytes from pre-diabetic and diabetic animals has been obtained from the spleen, the brachial lymph node and the pancreatic lymph node. These lymphocytes have been submitted to flow cytometry using anti-IL-17A (PE) and anti-FoxP3 (Alexa 697) antibodies. As shown in Figure 11, significant changes in the levels of Th17 and Treg lymphocytes are noted in the spleen, brachial lymph node and pancreatic lymph nodes upon conversion of NOD mice from non-diabetic to diabetic state. These changes are characterized by dramatically increased Th17 (top numbers in each

panels) and significantly decreased Treg (lower numbers in each panels) lymphocytes. Tregs: *, $p < 0.001$ from non-diabetic NOD mice. Th17: ** $p < 0.001$ from non-diabetic NOD mice.

The NOD mice (8 to 10 week-old) have been treated with allogeneic leukocytes (as described in Example I) and mPEG-allogeneic leukocyte (as described in Example I) and were compared to untreated control mice (naïve or NOD in Table 1). Th17 levels have been measured in various tissues (as described in Example I). Peripheral blood samples of the groups were pooled for analysis, all other samples were measured individually. Five male NOD mice per group were used. The results are shown in Table 1 provided below.

Table 1. Treatment of NOD mice with unmodified or mPEG-modified allogeneic cells. Unmodified cells results in a potent inflammatory state as shown by increased Th17 cells. In contrast, administration of mPEG-allogeneic cells does not induce inflammation. * $p < 0.001$ relative to unmodified allogeneic cell treated.

Tissue	Th17		
	NOD	Allogeneic	mPEG Allogeneic
Blood	0.38	0.67	0.17*
Spleen	0.10 ± 0.01	2.32 ± 0.38	$0.11 \pm 0.01^*$
Brachial L. Node	0.08 ± 0.01	1.25 ± 0.35	$0.06 \pm 0.01^*$
Pancreatic L. Node	0.05 ± 0.01	0.27 ± 0.08	$0.07 \pm 0.01^*$

EXAMPLE IV – POZ POLYMER FOR INDUCING TOLERANCE OR ANERGY

Human PBMC and dendritic cell preparation. Human whole blood was collected in heparinized vacutainer blood collection tubes (BD, Franklin Lakes, NJ) from healthy volunteer donors following informed consent. PBMC were isolated from diluted whole blood using FicollPaque PREMIUM™ (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) as per the product instructions. The PBMC layer was washed twice with 1x Hank's Balanced Salt Solution (HBSS; without CaCl_2 and MgSO_4 ; Invitrogen by Life Technologies, Carlsbad, CA) and resuspended in the appropriate media as needed for mixed lymphocyte reactions and flow cytometric analysis of Treg and Th17 phenotypes. Dendritic cells (DC) were prepared from isolated PBMC as described by O'Neill and Bhardwaj (O'Neill et al., 2005). Briefly, freshly isolated PBMC were overlaid on Petri dishes for 3 h of in AIM V serum free culture medium (Invitrogen, Carlsbad, CA). Non-adherent cells were gently washed off the plate. The adherent cells

(monocyte rich cells) were treated with IL-4 and GM-CSF (50 and 100 ng/mL respectively; R&D Systems, Minneapolis, MN) in AIM V medium. Cells were again treated with IL-4 and GM-CSF on days 2 and 5. On day 6, cells were centrifuged and resuspended in fresh media supplemented with DC maturation factors (TNF- α , IL-1 β , IL-6; R&D Systems, Minneapolis, MN) and prostaglandin E2 (SigmaAldrich, St. Louis, MO). The mature DC-like cells were harvested on day 7 and CD80, CD83, CD86 and HLA-DR expressions were determined to confirm DC maturation via flow cytometry (FACSCaliburTM Flow Cytometer, BD Biosciences, San Jose, CA).

mPEG modification (PEGylation) of PBMCs and splenocytes. Human PBMC and murine splenocytes were derivitized using methoxypoly(-ethylene glycol) succinimidyl valerate (mPEG-SVA; Laysan Bio Inc. Arab, AL) with a molecular weight of 20 kDa as described in Example I. Grafting concentrations ranged from 0 to 3.0 mM per 4×10^6 cells/mL.

POZ modification (POZylation) of PBMCs and splenocytes. N-hydroxysuccinimidyl ester of polyethyloxazoline propionic acid (SPA-PEOZ; Serina Therapeutics, Huntsville, AL) with a molecular weight of 20 kDa were grafted on the cells as described in Example I. Grafting concentrations ranged from 0 to 3.0 mM per 4×10^6 cells/mL.

In vitro and in vivo cell proliferation. Cell proliferation (both *in vitro* and *in vivo*) was assessed via flow cytometry using the CellTraceTM CFSE (Carboxyfluorescein diacetate, succinimidyl ester) Cell Proliferation Kit (Invitrogen by Life Technologies e Molecular probes, Carlsbad, CA) as described in Example I.

Mixed lymphocyte reaction (MLR) - control and conditioned media. The effects of polymer grafting (20 kDa SVAmPEG or 20 kDa POZ) on allorecognition *in vitro* were assessed using two-way MLR (Murad et al, 1999A; Chen et al., 2003; Chen et al., 2006) as described in Example I.

A 2-way MLR was conducted using either PEGylated or POZylated human cells. As shown on Figure 12, the grafting of equimolar concentrations of wither 20 kDa mPEG or PEOZ (POZ) on a human mixed lymphocyte reaction (MLR) had similar effects on cellular proliferation.

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While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention

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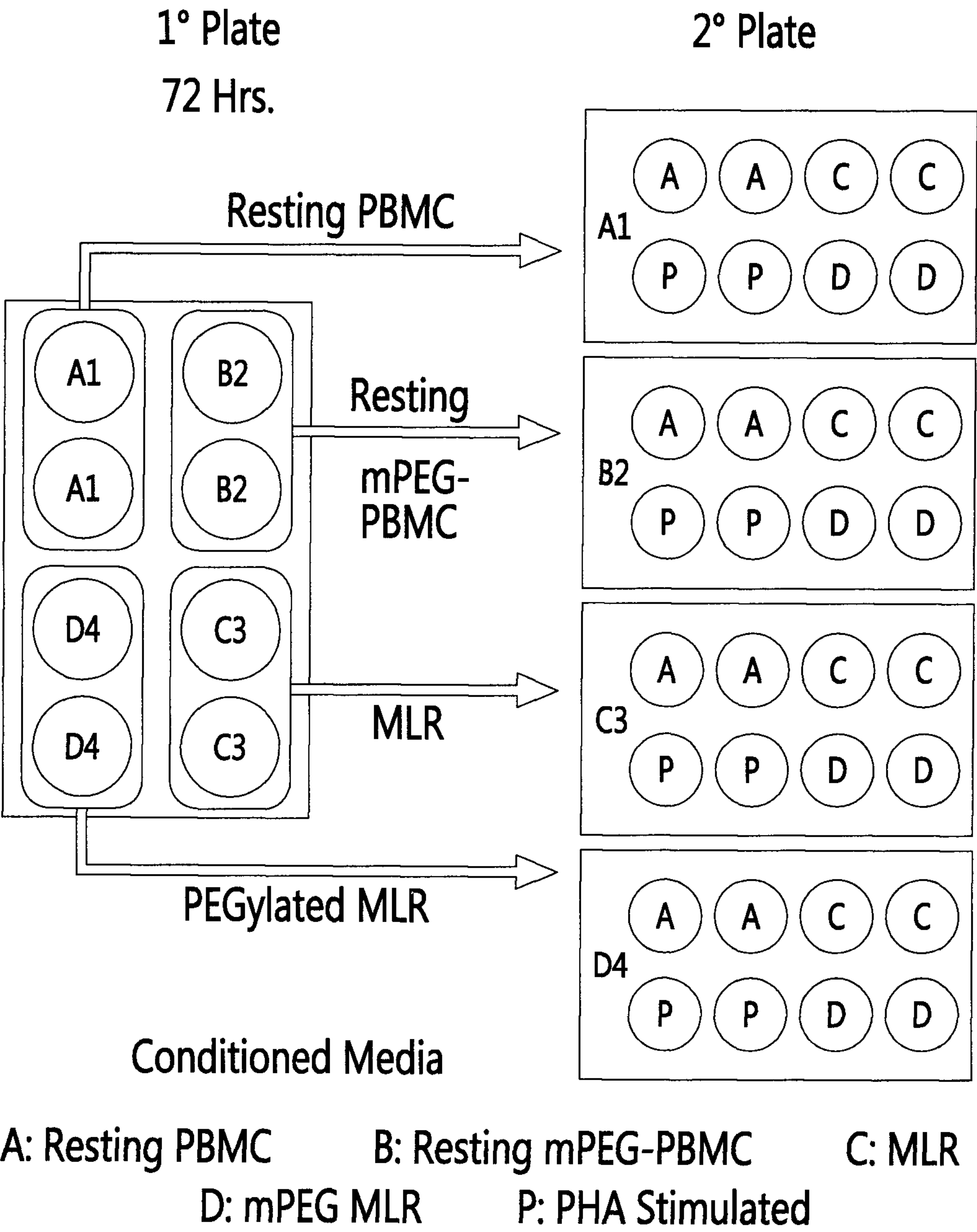
following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

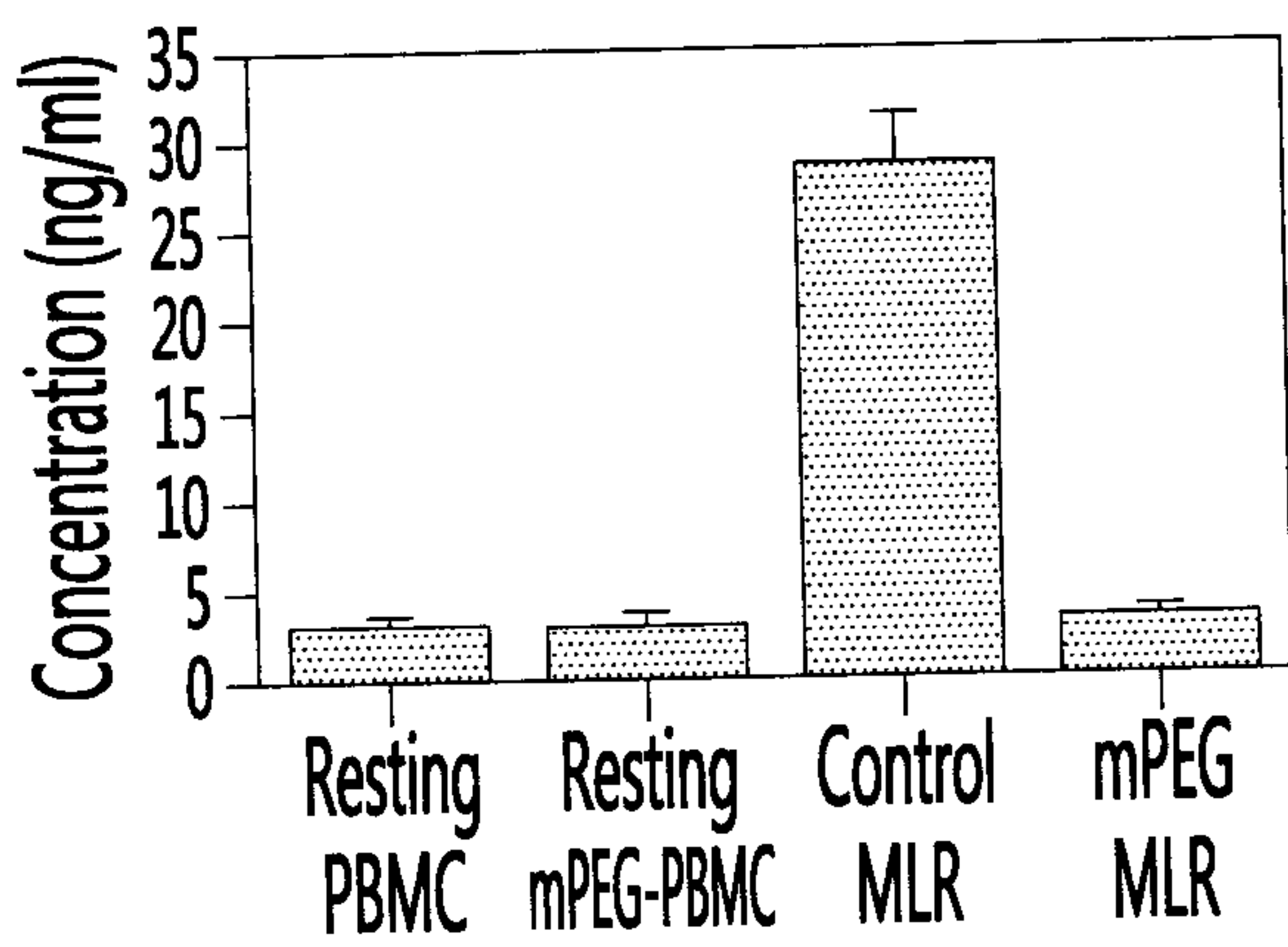
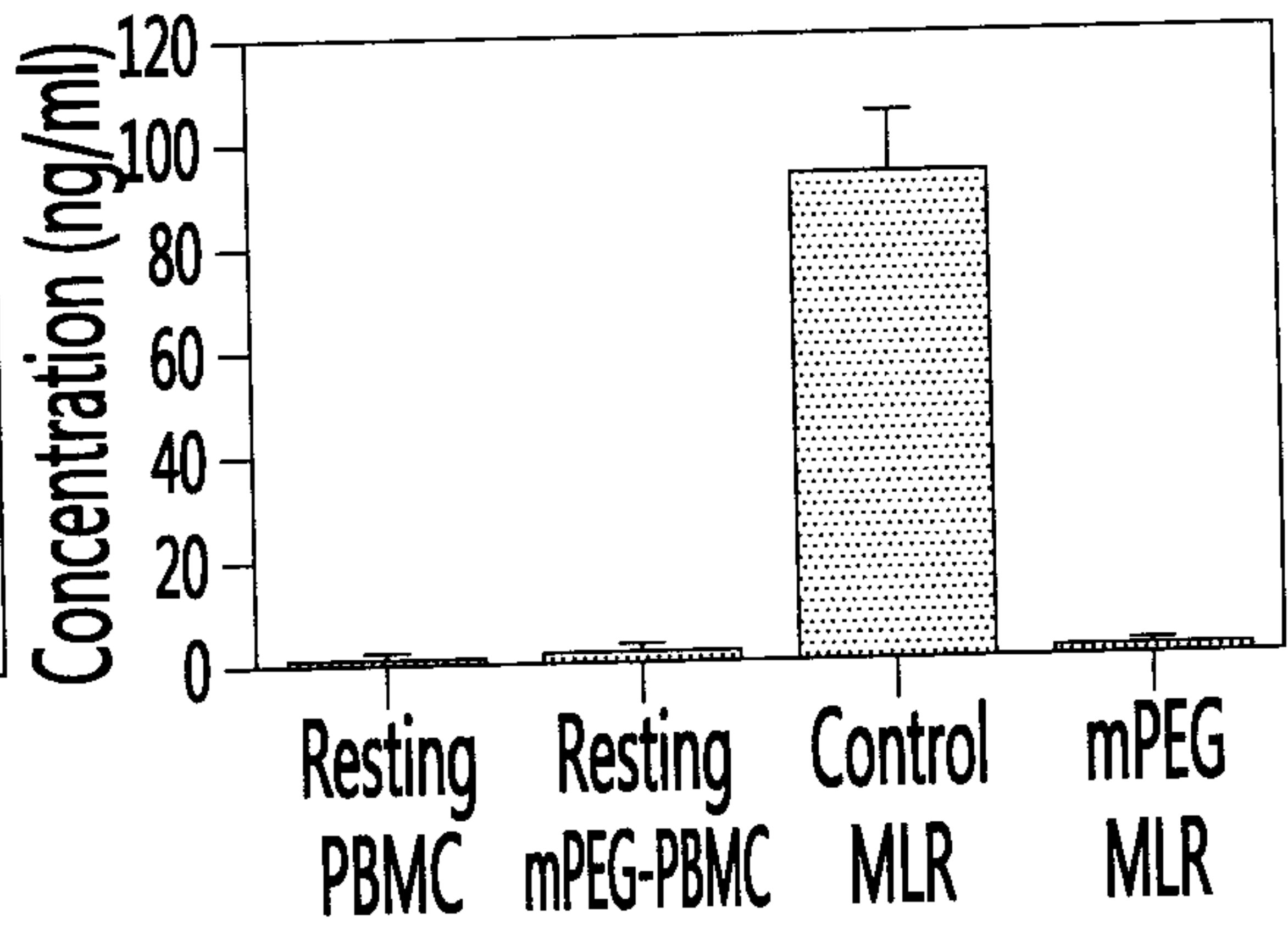
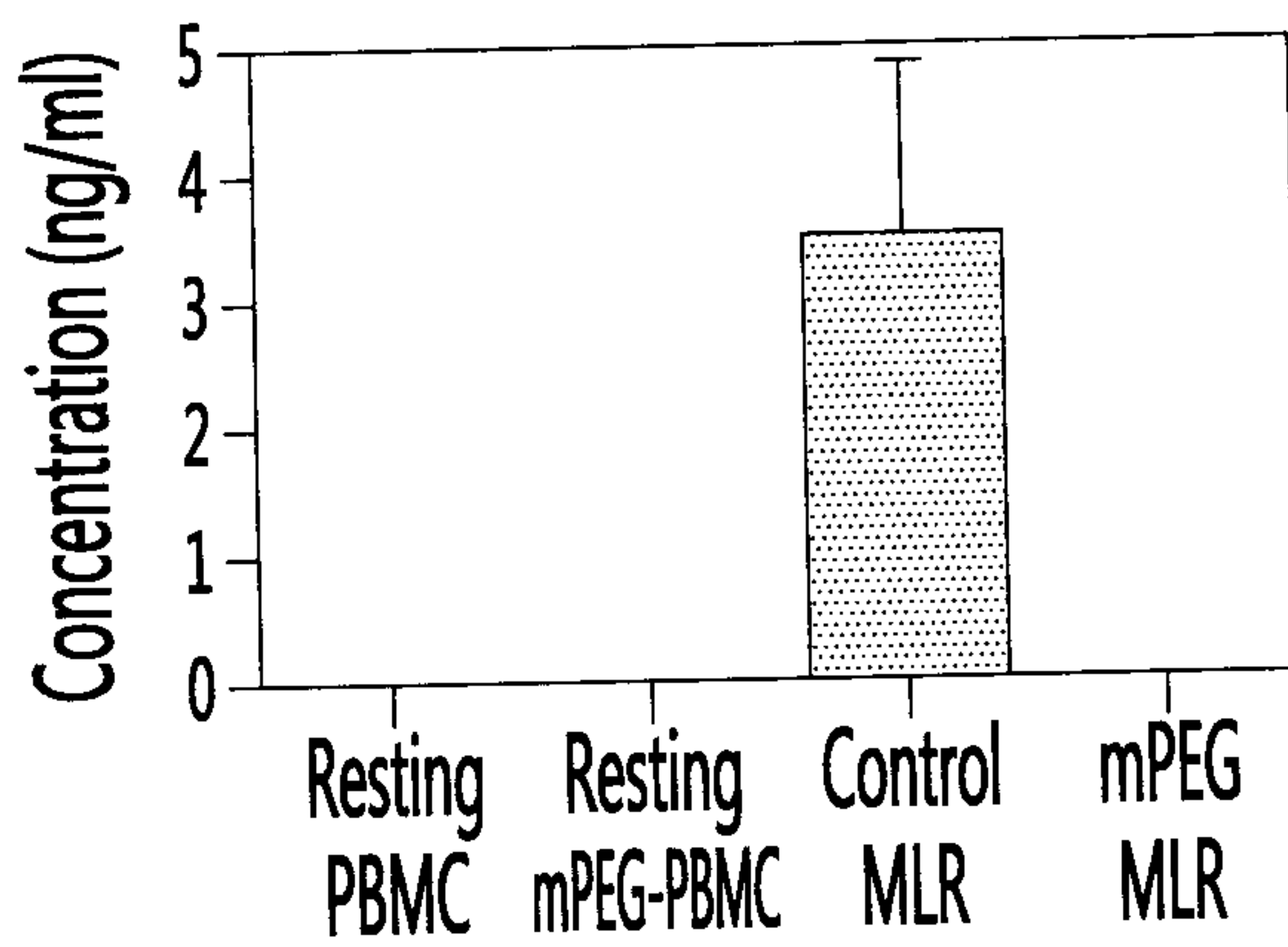
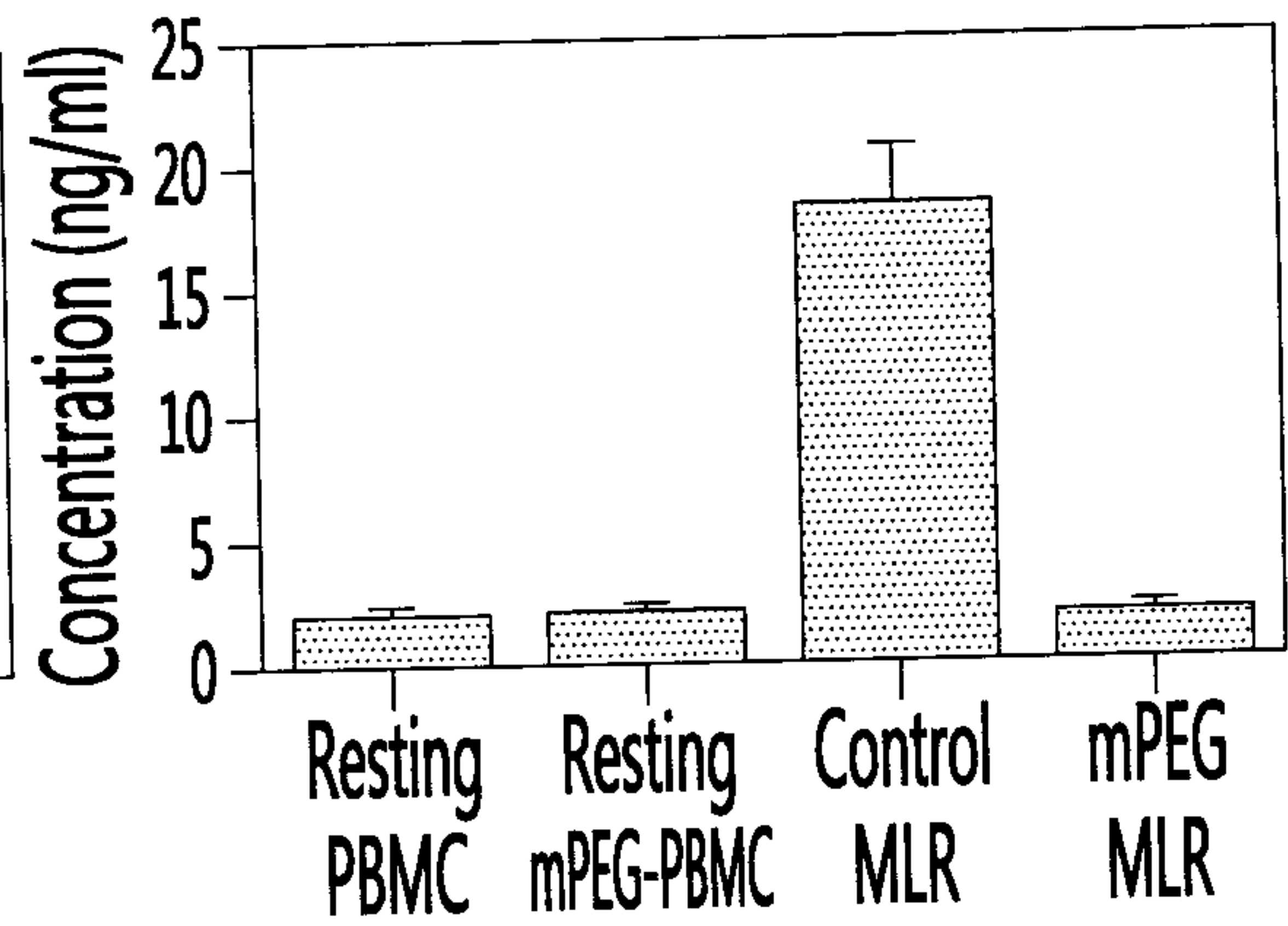
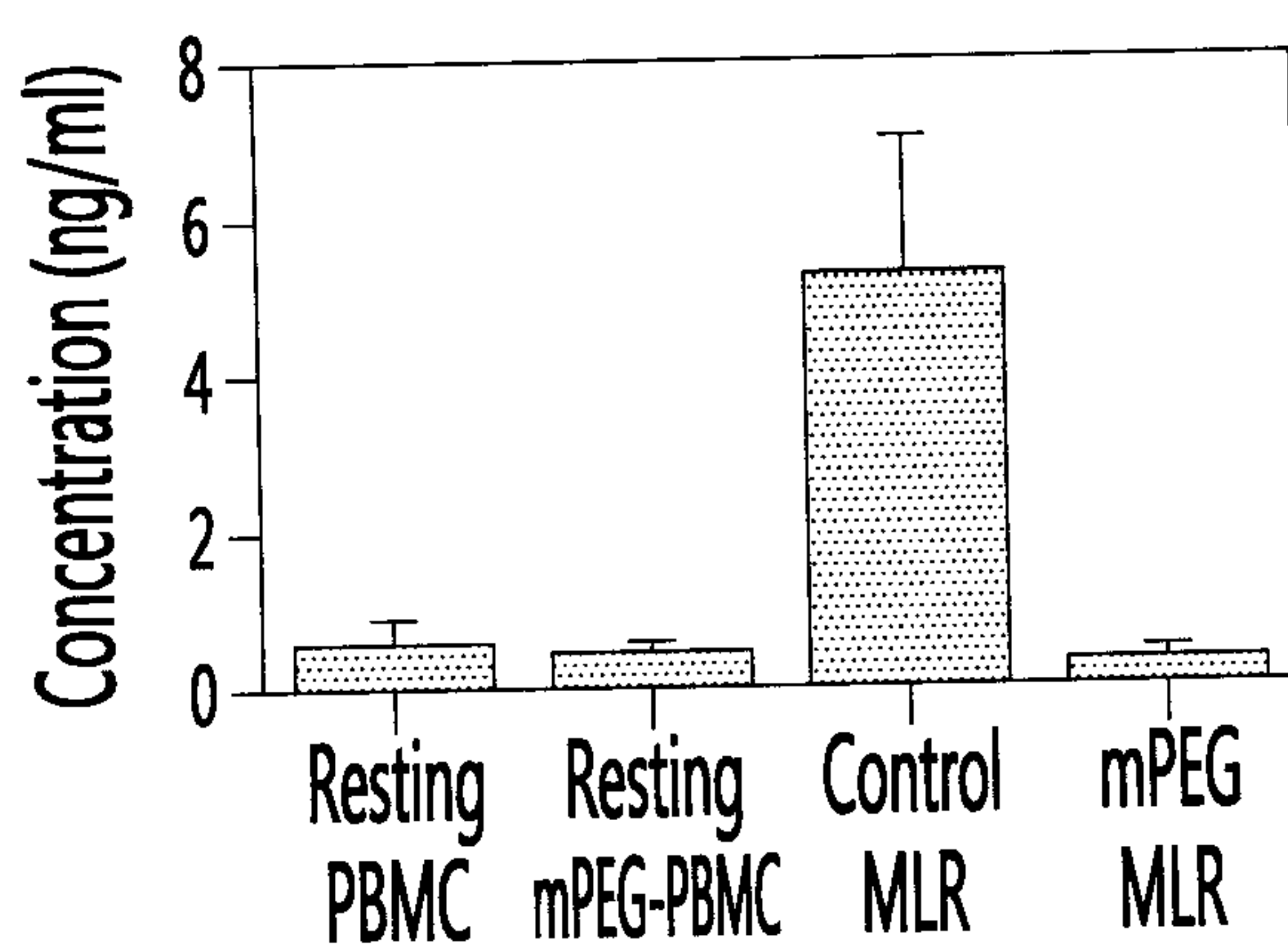
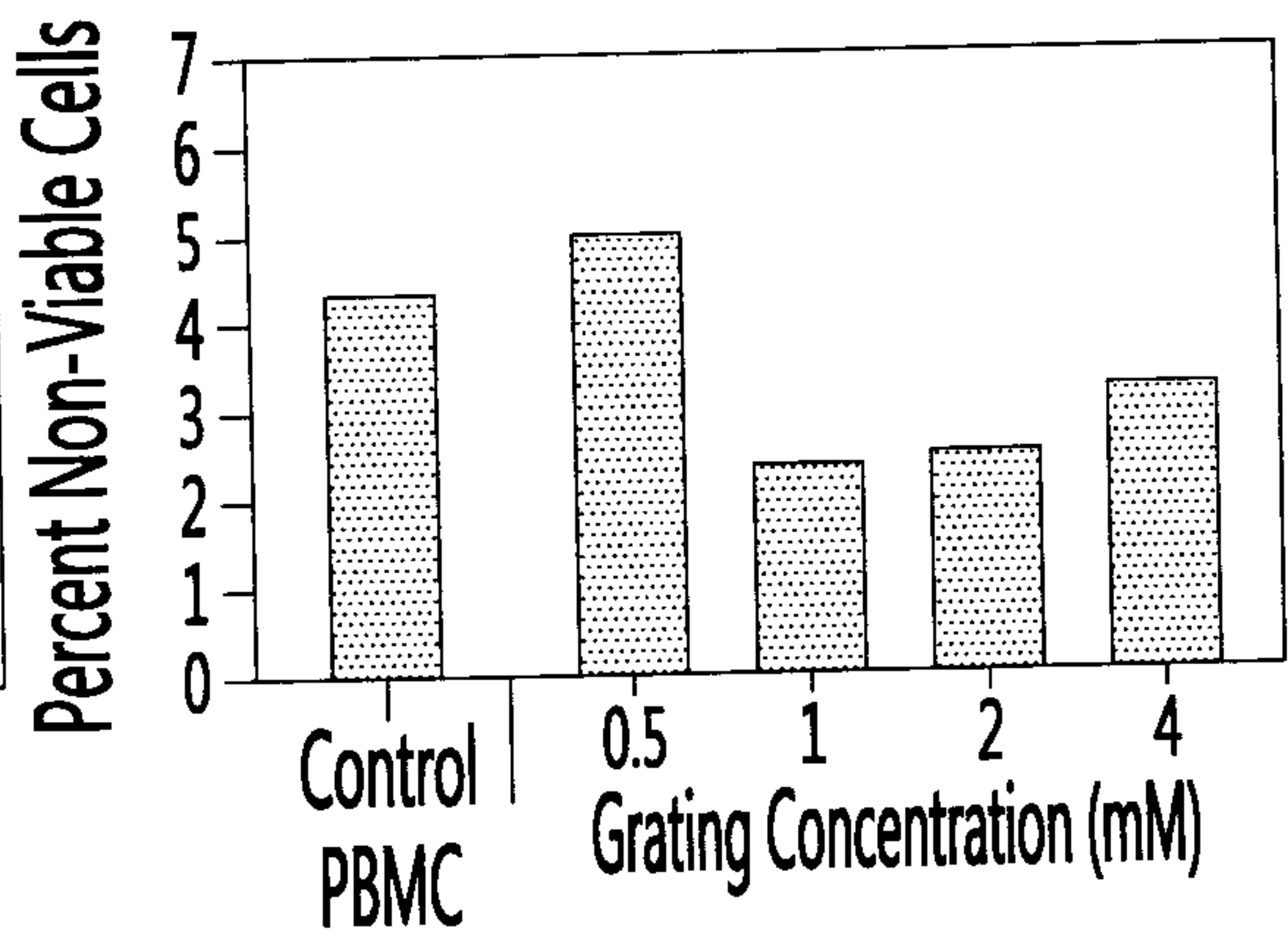
1. A cellular preparation for inducing increased expansion of endogenous regulatory T cells by increasing a ratio of regulatory T (Treg) cells to pro-inflammatory T cells in a subject, wherein the cellular preparation comprises a first immunogenic leukocyte having a cytoplasmic membrane associated to a low-immunogenic biocompatible polymer, wherein the low-immunogenic polymer is polyethylene glycol, 2-alkyloxazoline or hyperbranched glycerol.
2. The cellular preparation of claim 1, wherein the cytoplasmic membrane has a membrane-associated protein covalently bound to the low-immunogenic biocompatible polymer.
3. The cellular preparation of claim 1 or 2, wherein the first immunogenic leukocyte is a T cell.
4. The cellular preparation of any one of claims 1 to 3, wherein the low-immunogenic biocompatible polymer is a polyethylene glycol.
5. The cellular preparation of any one of claims 1 to 3, wherein the low-immunogenic biocompatible polymer is 2-alkyloxazoline (POZ).
6. The cellular preparation of any one of claims 1 to 5, wherein the increased ratio between the level of Treg cells and the level of pro-inflammatory T cells is for the treatment, prevention and/or alleviation of symptoms associated to an auto-immune disease in the subject.
7. The cellular preparation of claim 6, wherein the auto-immune disease is at least one of type I diabetes, rheumatoid arthritis, multiple sclerosis, psoriasis, lupus, immune thrombocytopenia, experimental autoimmune encephalomyelitis, autoimmune uveitis, inflammatory bowel disease and Crohn's disease.
8. The cellular preparation of any one of claims 1 to 5, wherein the increased ratio between the level of Treg cells and the level of pro-inflammatory T cells is for the prevention of an excessive immune reaction in the subject.

9. The cellular preparation of claim 8, wherein the excessive immune reaction is in response to a vaccine.
10. The cellular preparation of any one of claims 1 to 5, wherein the increased ratio between the level of T reg and the level of pro-inflammatory T cells is for the prevention of the symptoms associated to the rejection of transplanted cells or tissue in the subject.
11. A process for increasing and/or providing the ability of a cellular preparation to increase expansion of endogenous regulatory T cell by increasing a ratio of regulatory T (Treg) cells to pro-inflammatory T cells in a subject, said process comprising :
 - (i) associating a low-immunogenic biocompatible polymer to a cytoplasmic membrane of a first immunogenic leukocyte to obtain a modified first immunogenic leukocyte; and
 - (ii) formulating the preparation obtained in step (i) for administration to the subject,wherein the low-immunogenic polymer is polyethylene glycol, 2-alkyloxazoline or hyperbranched glycerol.
12. The process of claim 11, further comprising, in step (i), covalently binding the low-immunogenic biocompatible polymer to a membrane-associated protein of the cytoplasmic membrane of the first immunogenic leukocyte.
13. The process of claim 11 or 12, wherein the first immunogenic leukocyte is from the subject.
14. The process of claim 13, wherein the first immunogenic leukocyte is a T cell.
15. The process of any one of claims 11 to 14, wherein the low-immunogenic biocompatible polymer is a polyethylene glycol.
16. The process of claim 15, wherein the polyethylene glycol is a methoxy polyethylene glycol (mPEG).

17. The process of claim 16, comprising contacting the first immunogenic leukocyte with methoxypoly(-ethylene glycol) succinimidyl valerate.
18. The process of any one of claims 11 to 14, wherein the low-immunogenic biocompatible polymer is 2-alkyloxazoline (POZ).
19. The process of claim 12, wherein 2-alkyloxazoline (POZ) is covalently bound to the membrane protein with a linker.
20. The process of any one of claims 11 to 19, further comprising formulating the formulated preparation in a vaccine.



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Figure 2AFigure 2BFigure 2CFigure 2DFigure 2EFigure 2F

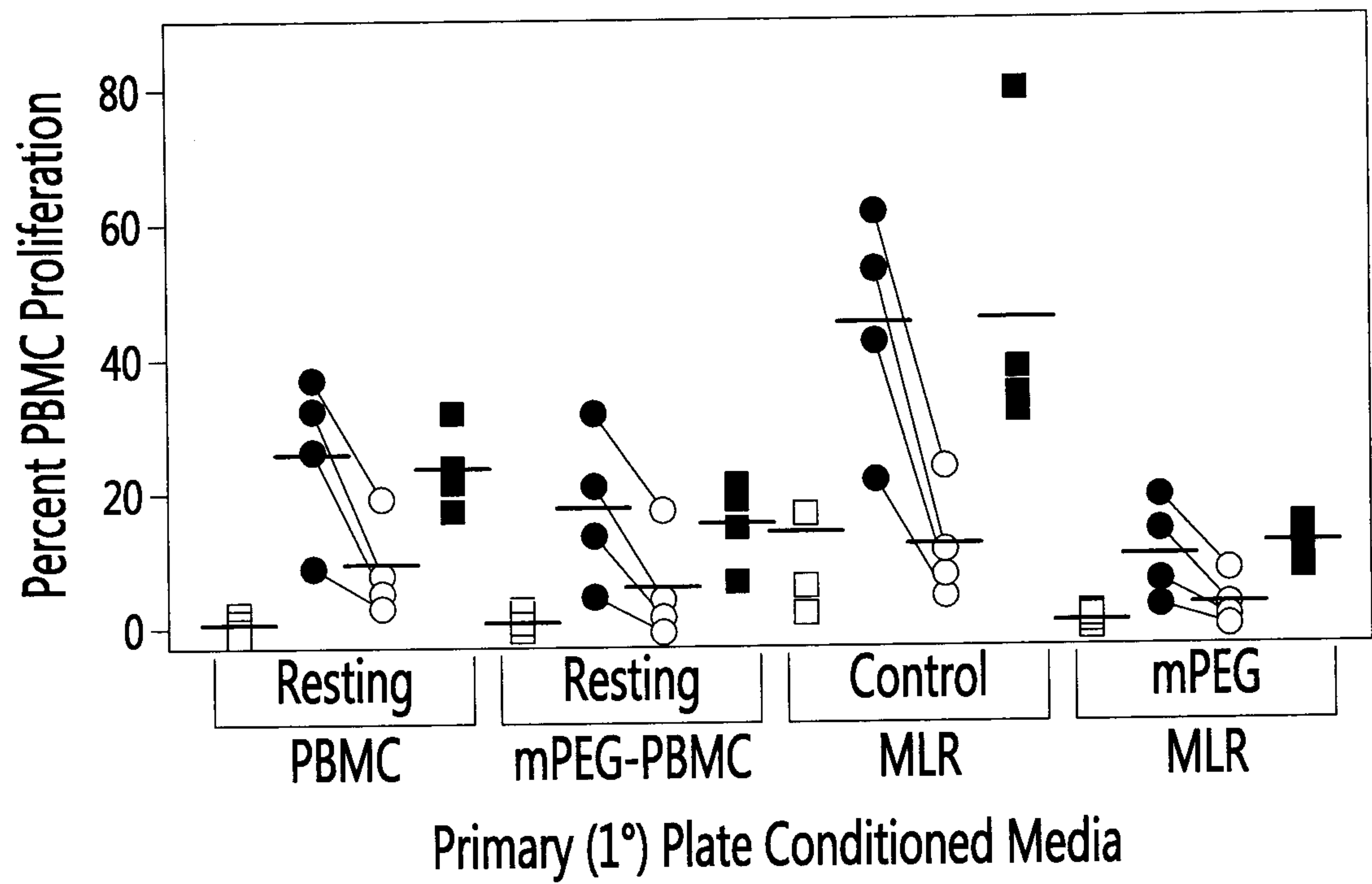
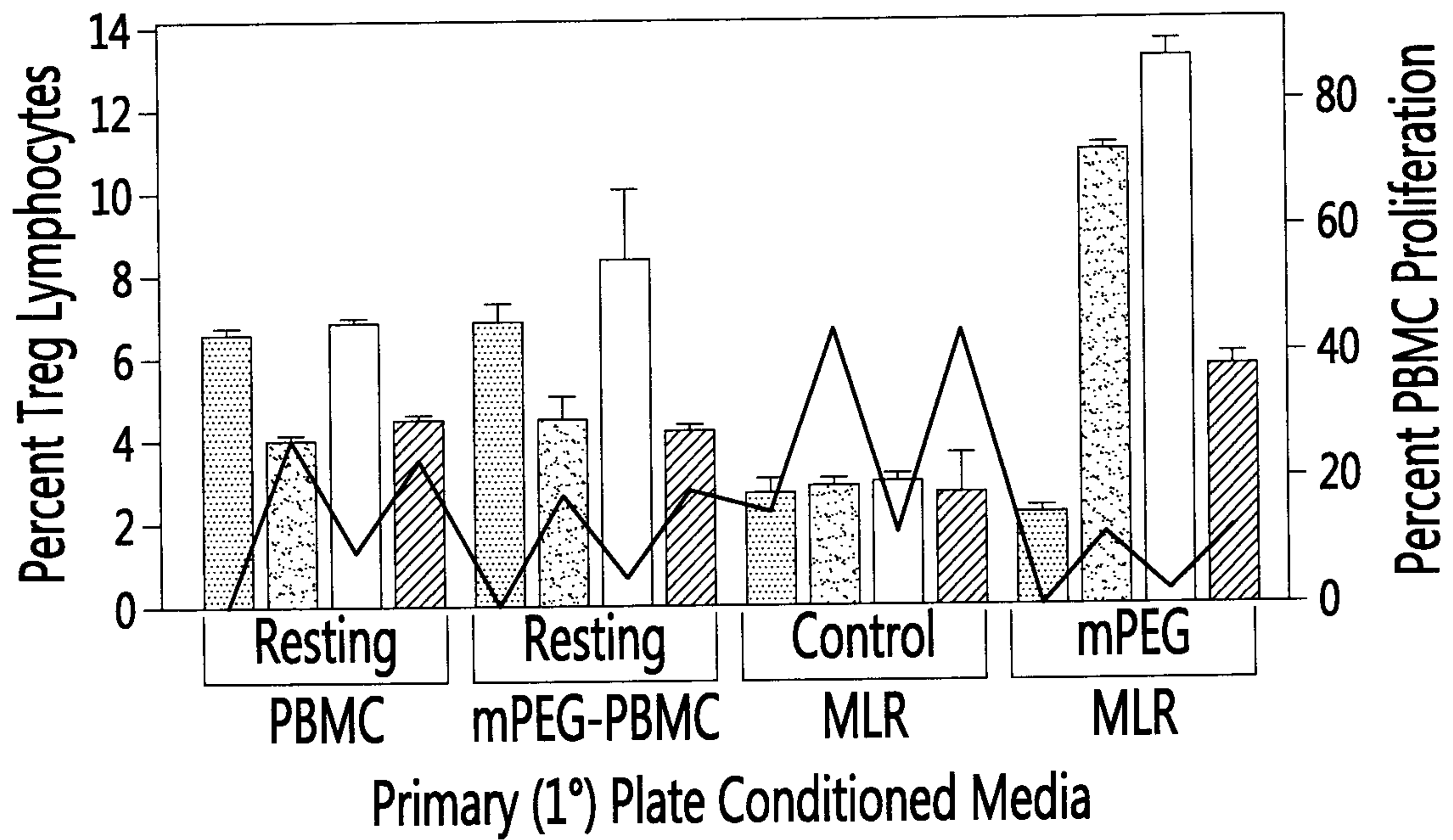
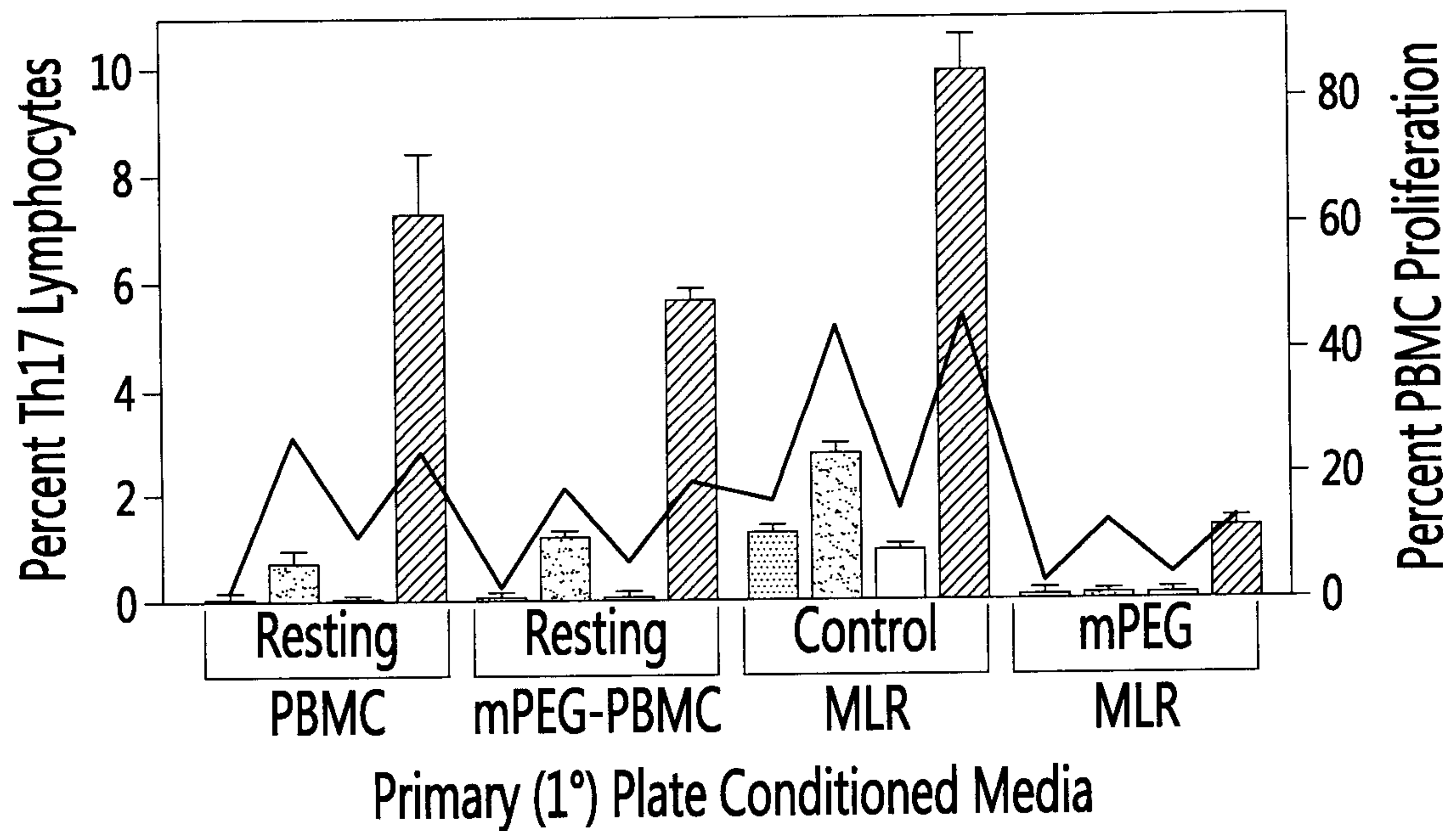
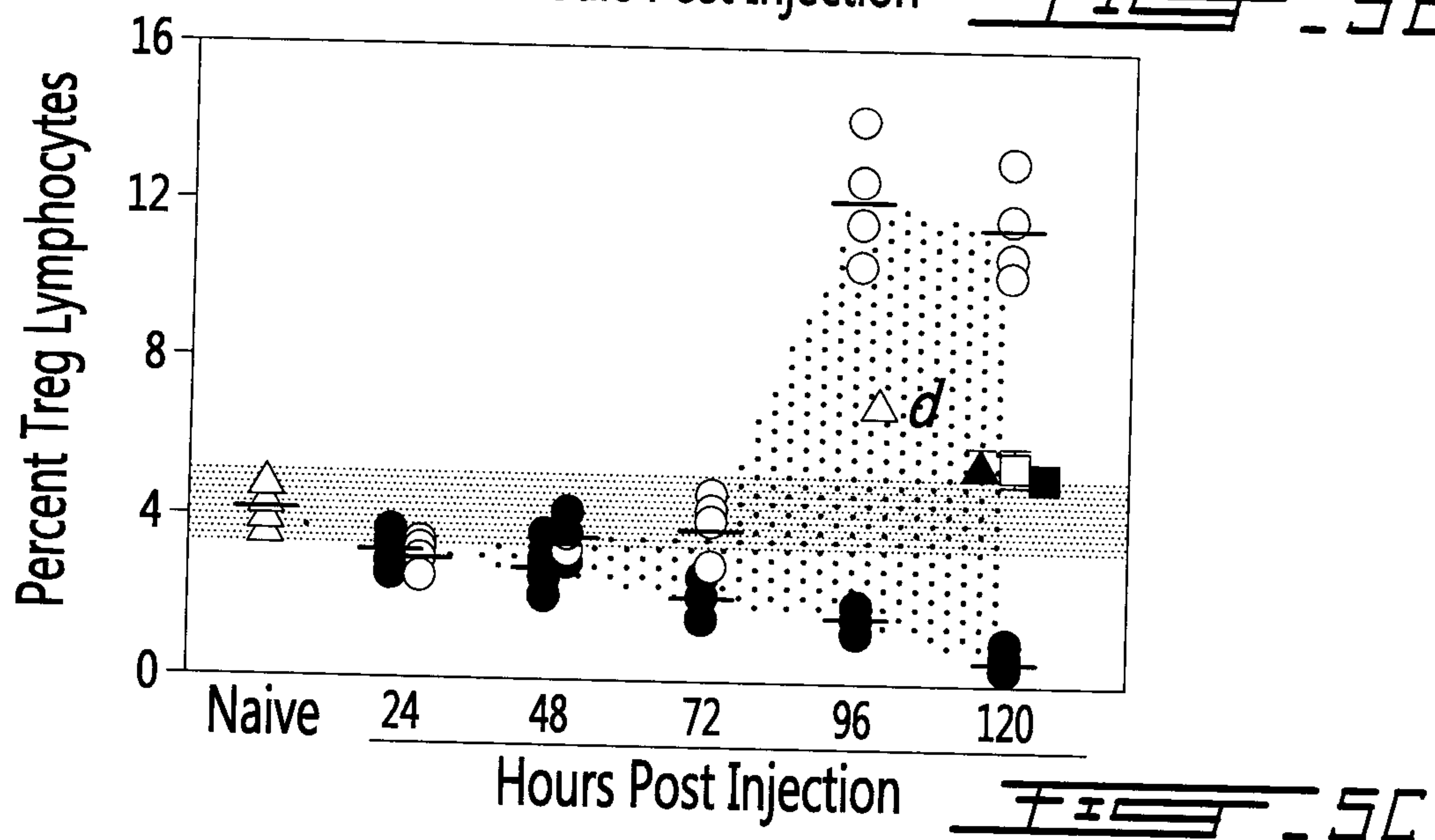
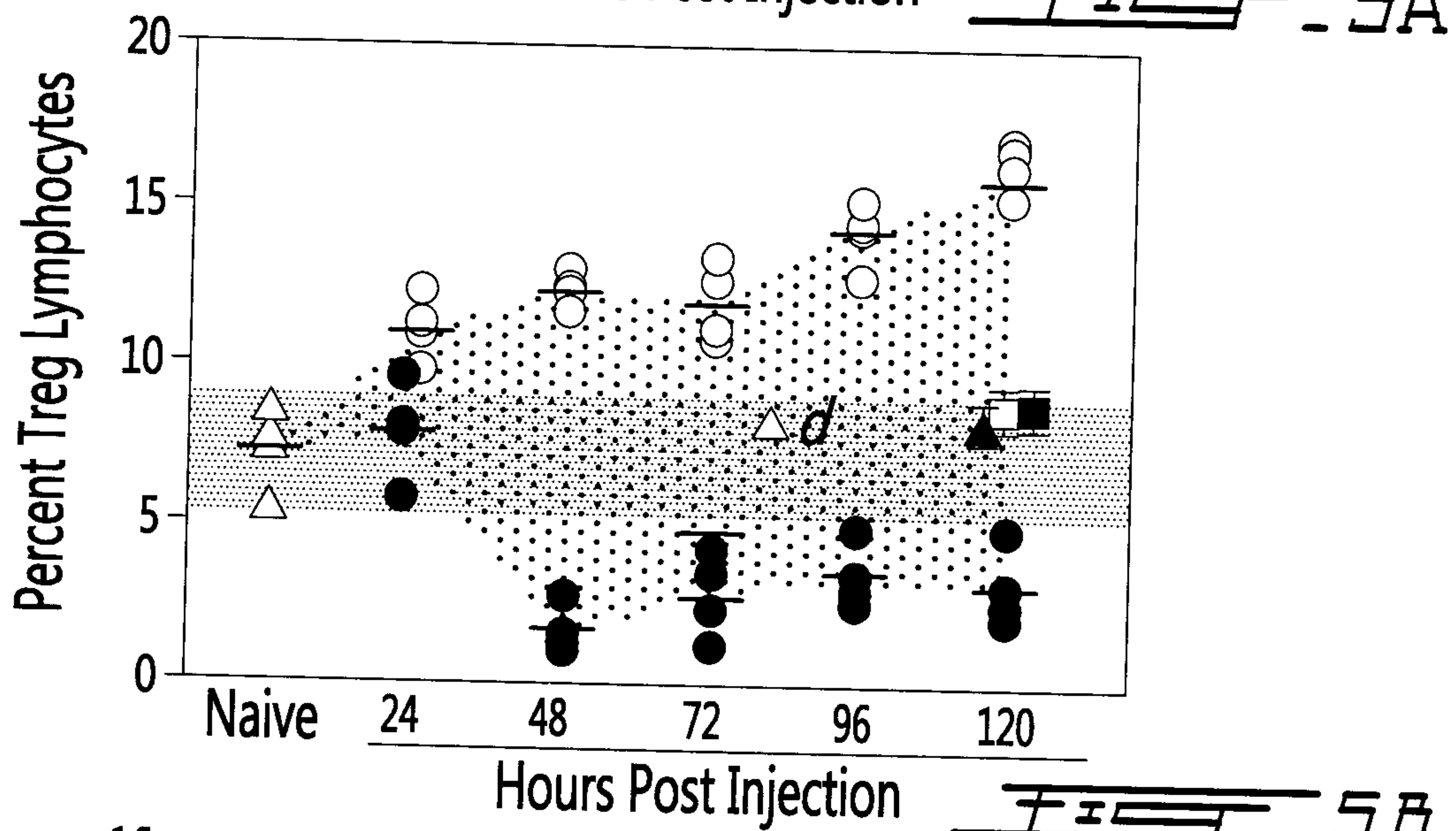
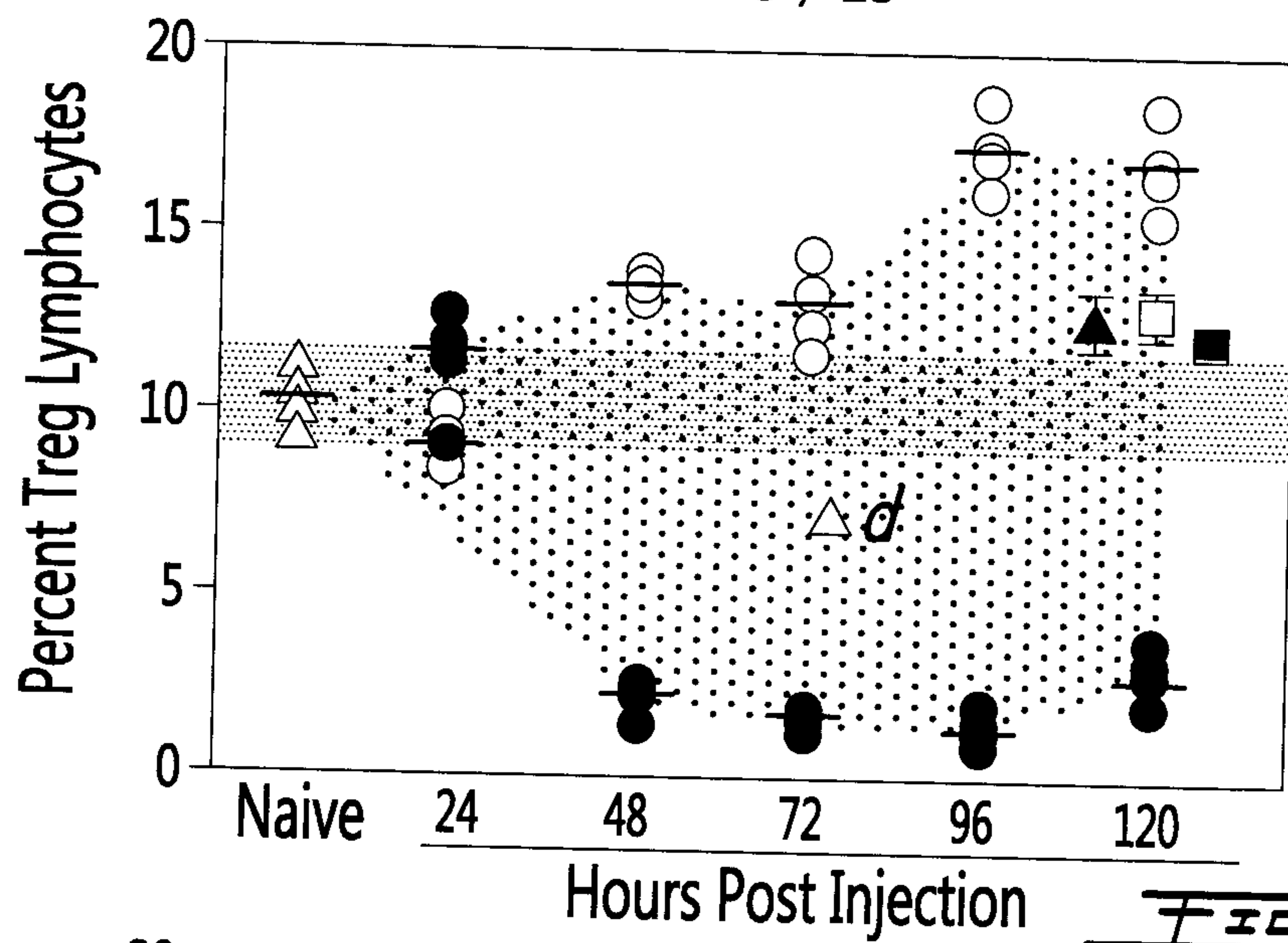


Figure 3

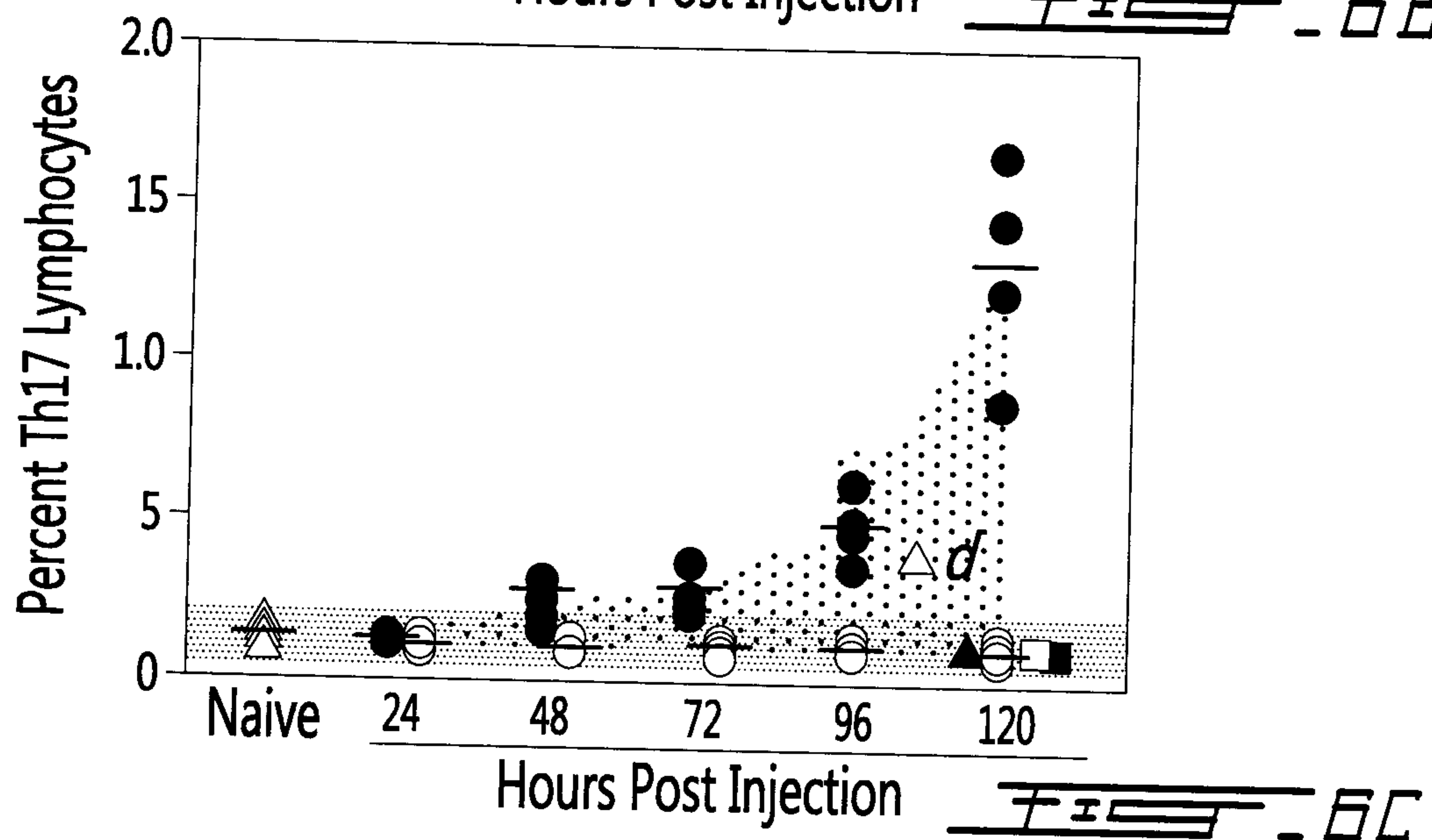
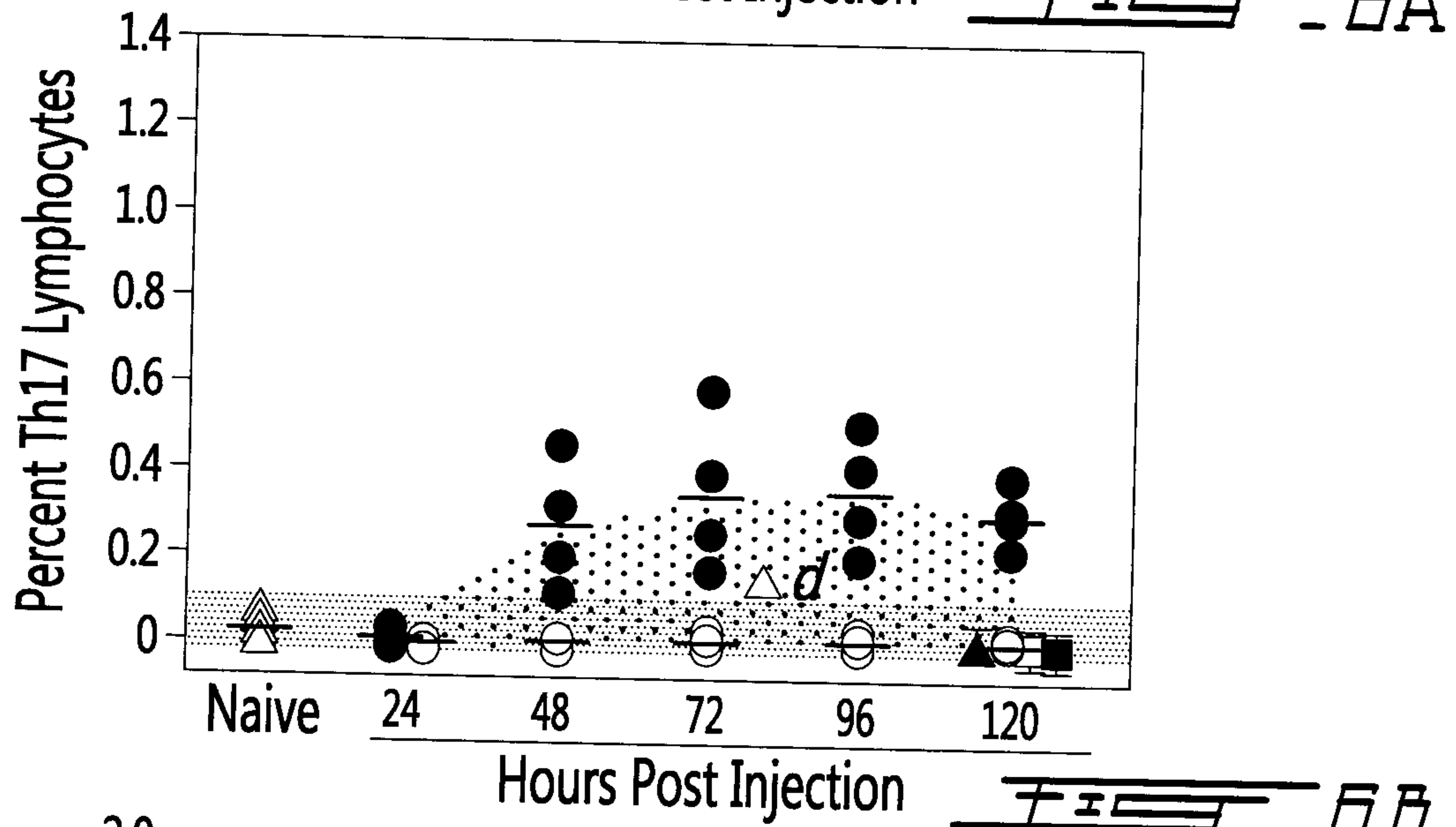
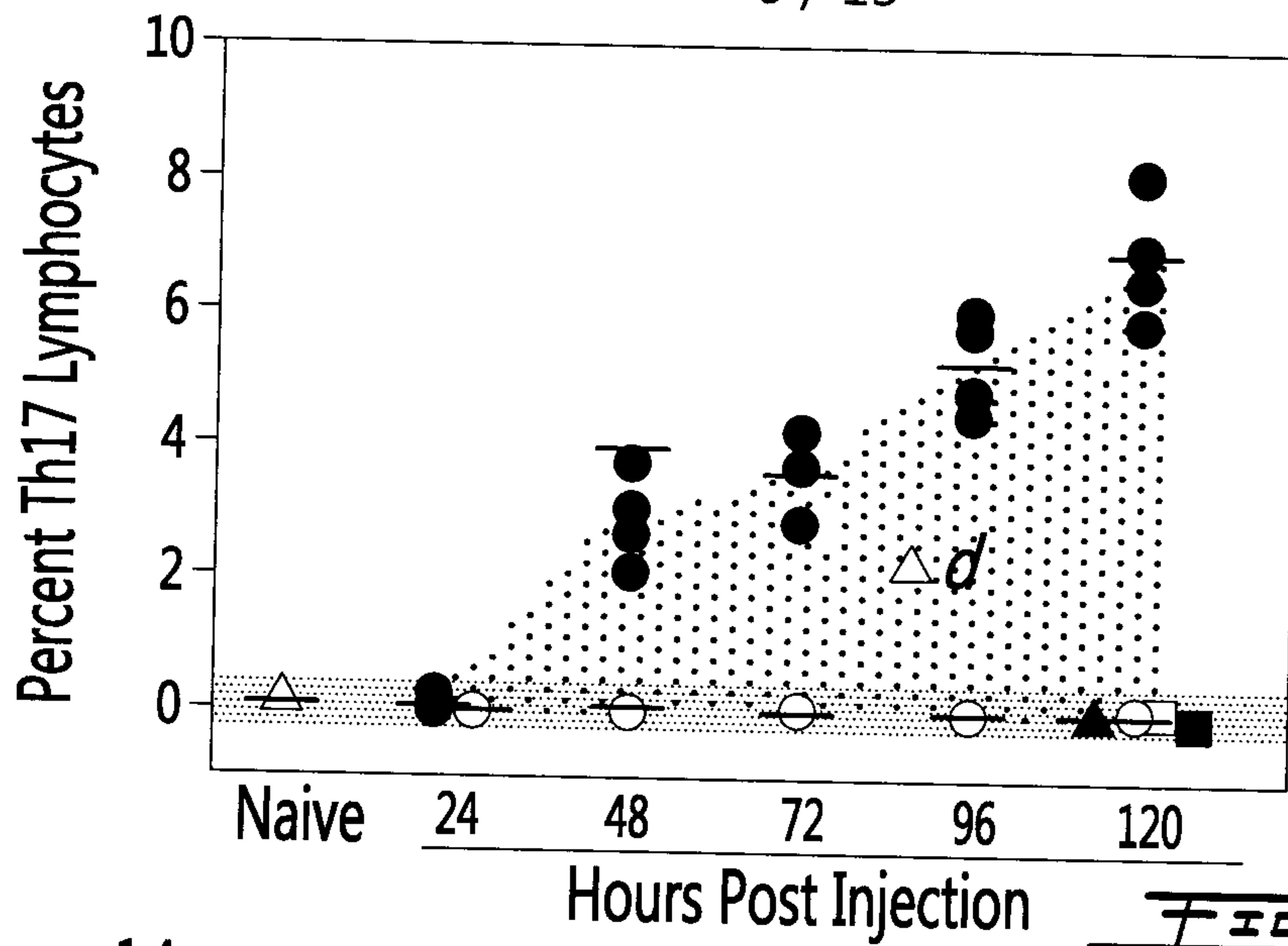
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Figure 4AFigure 4B

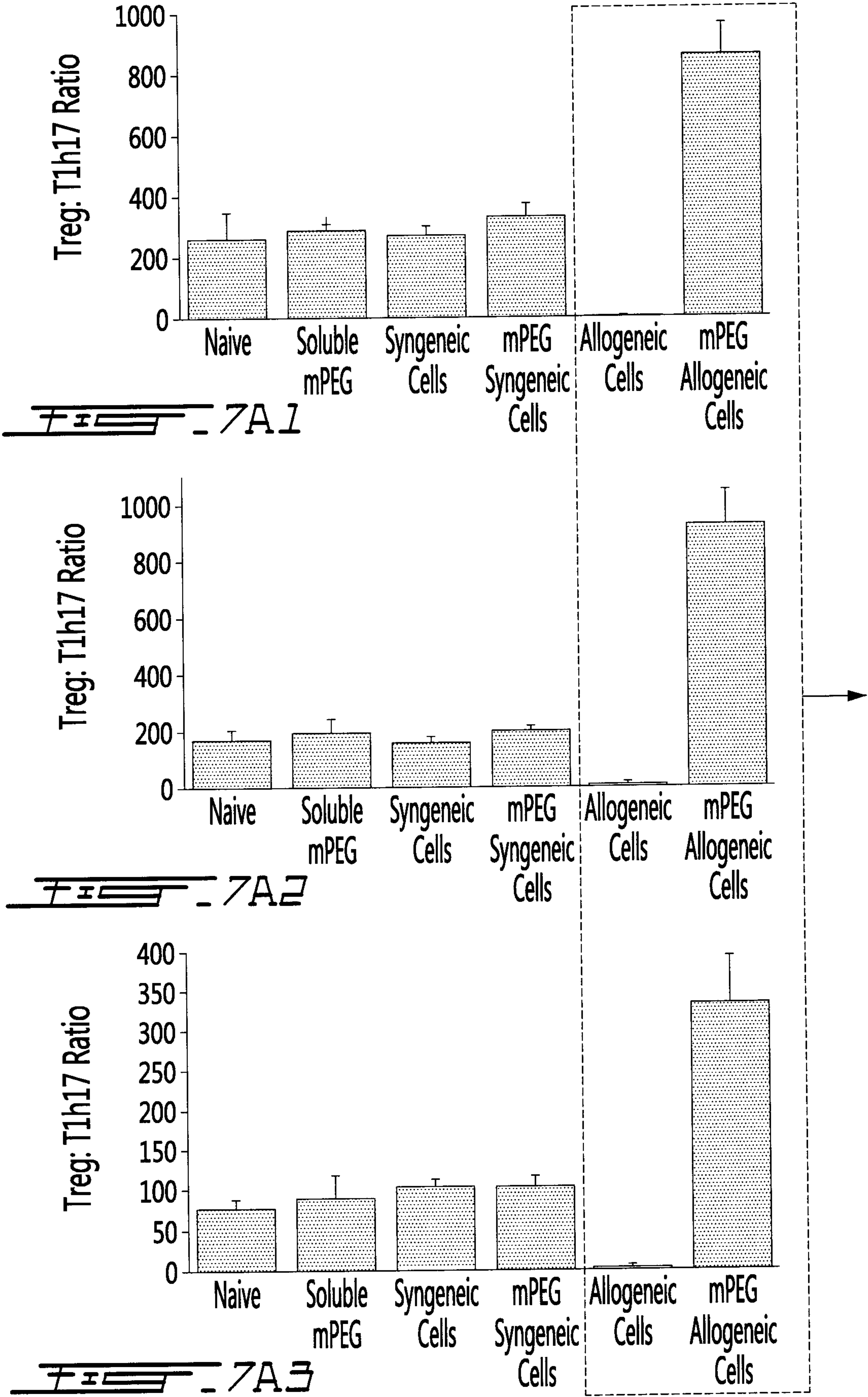
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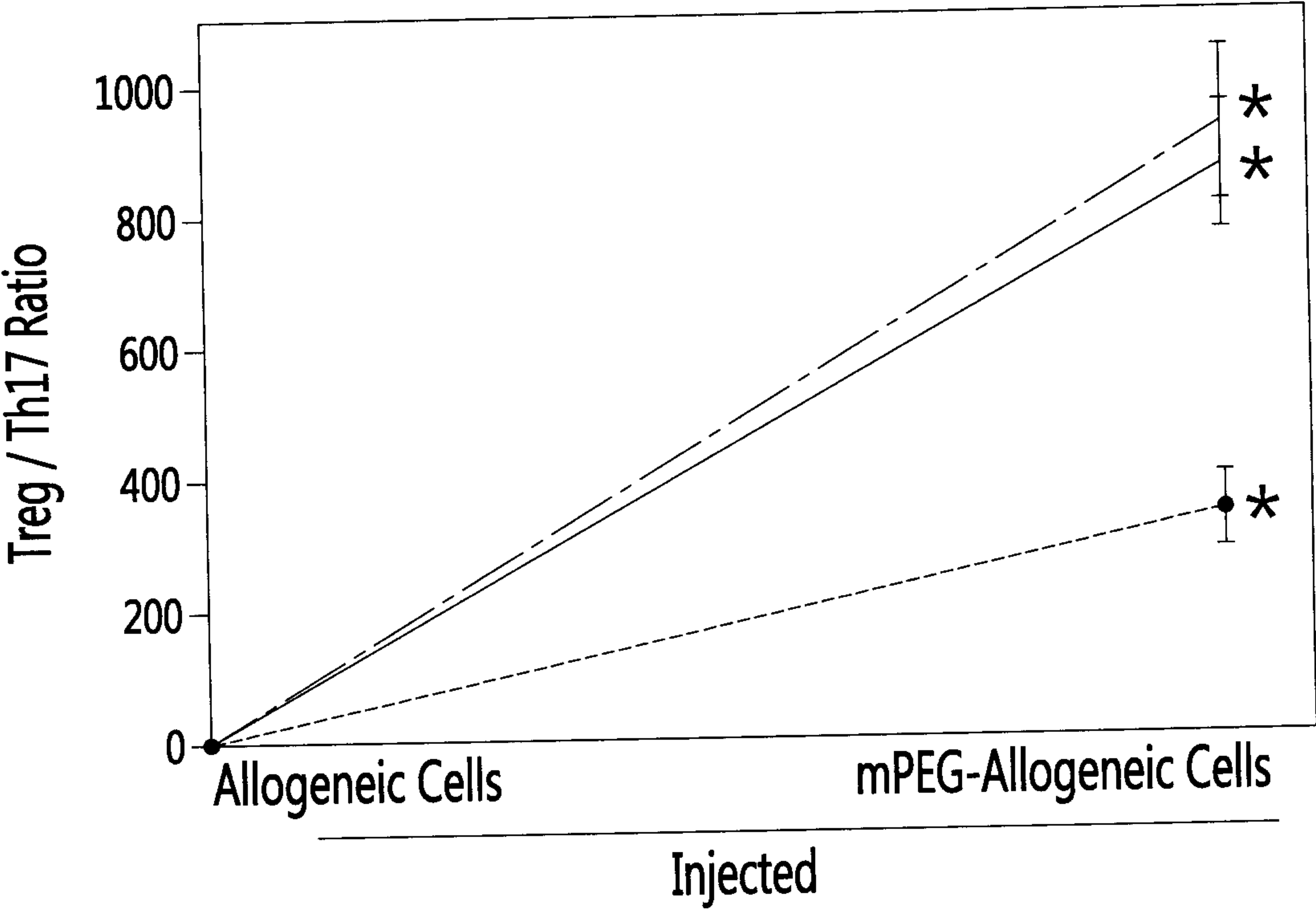
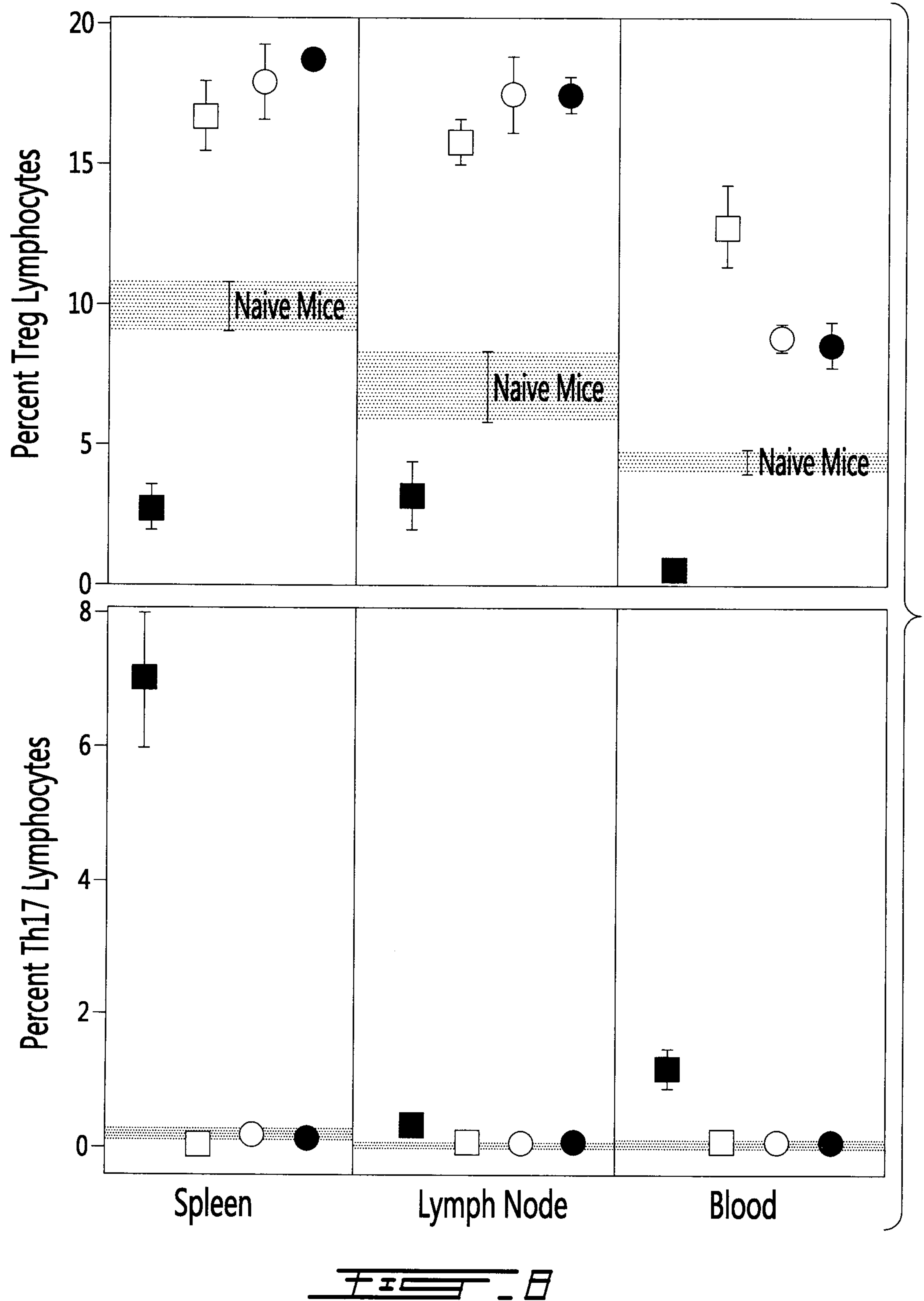
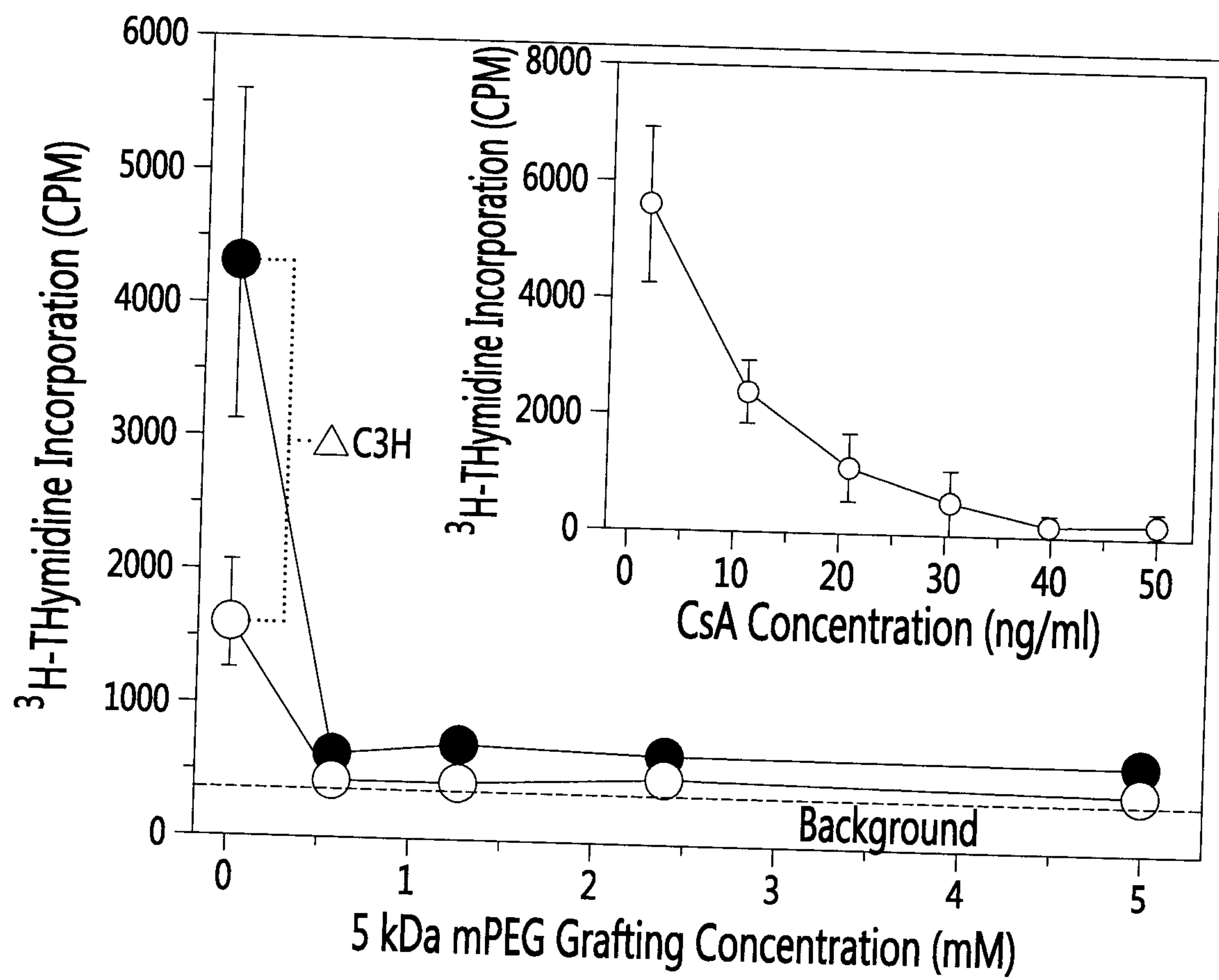
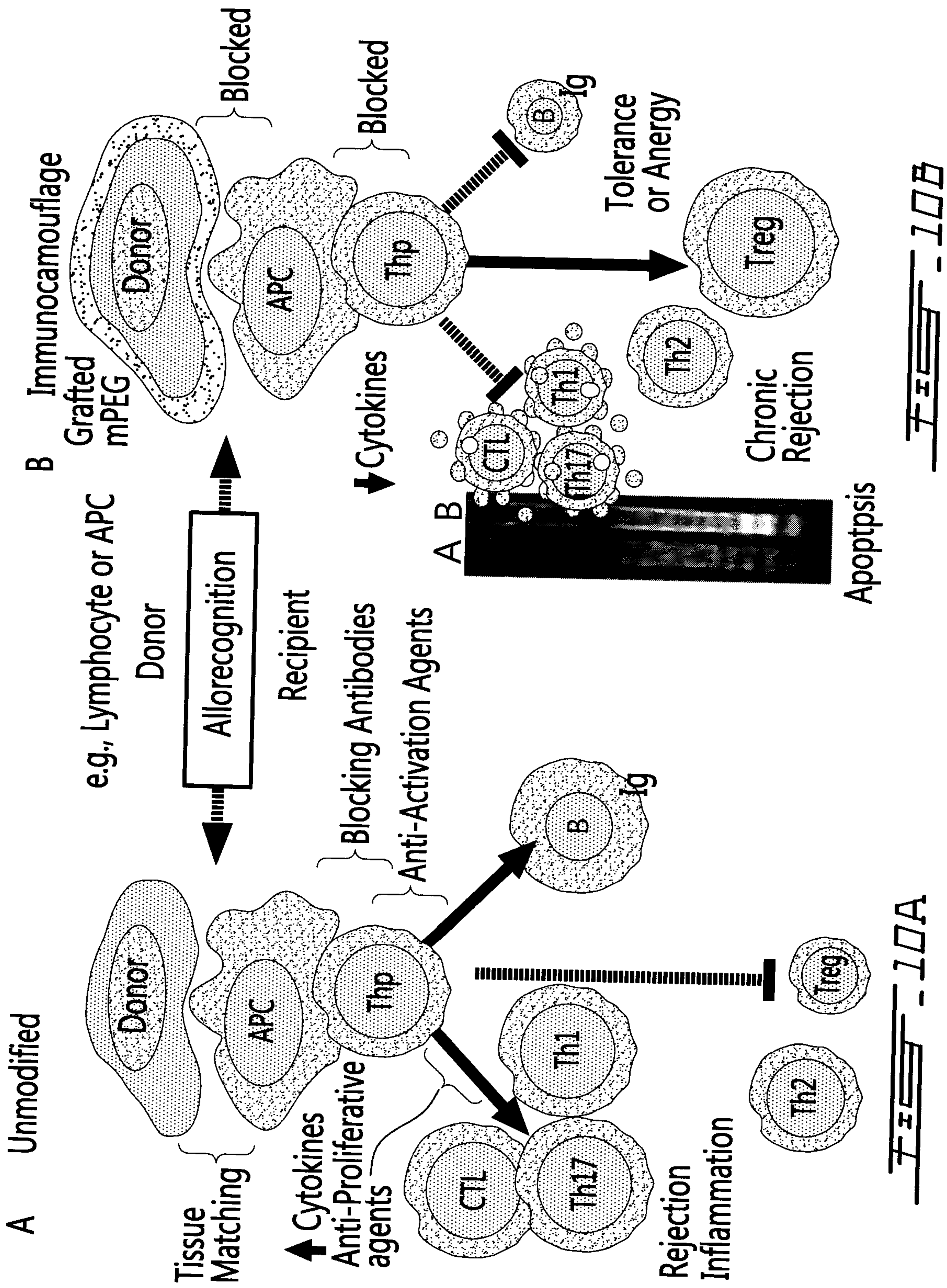


Figure 7B

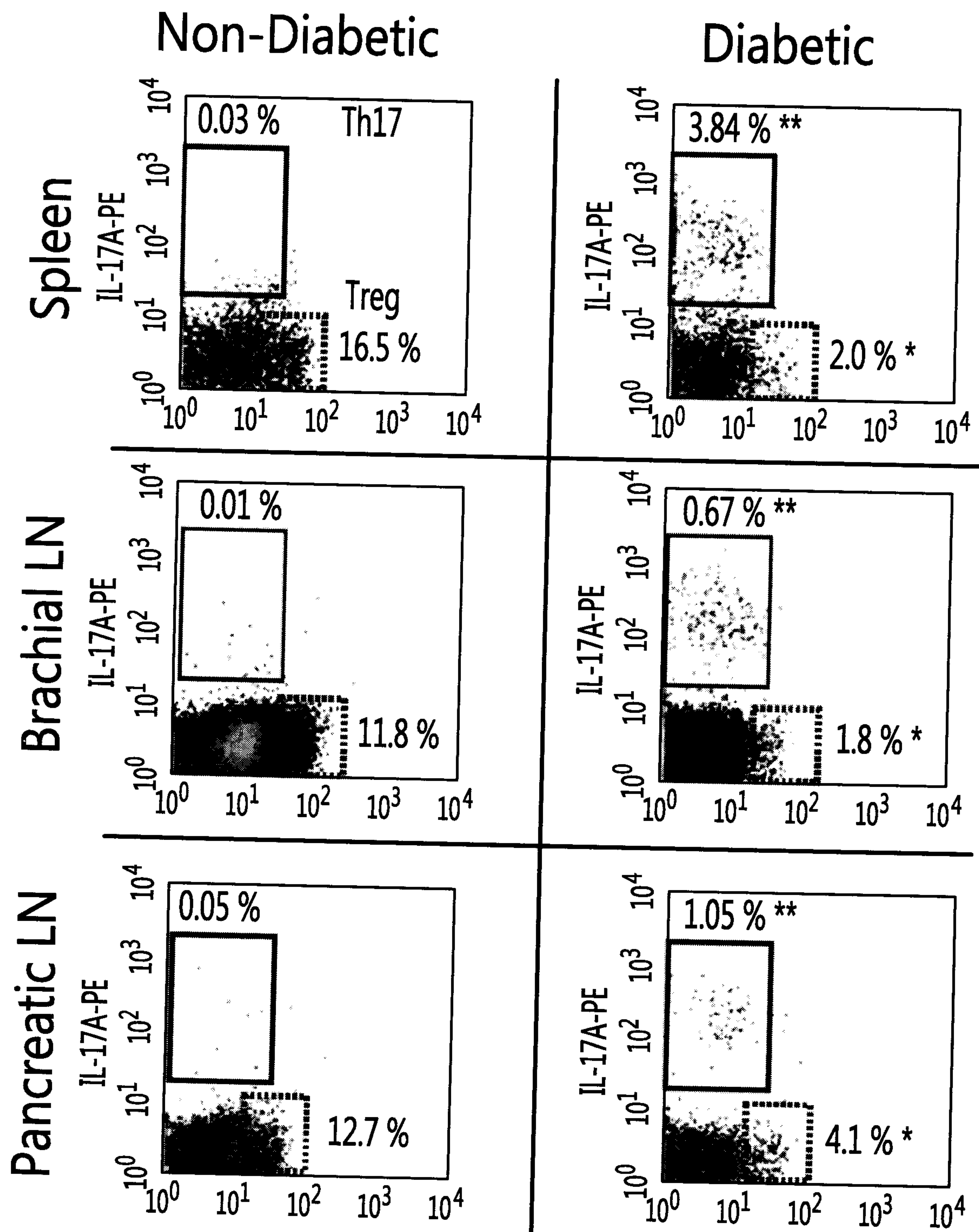


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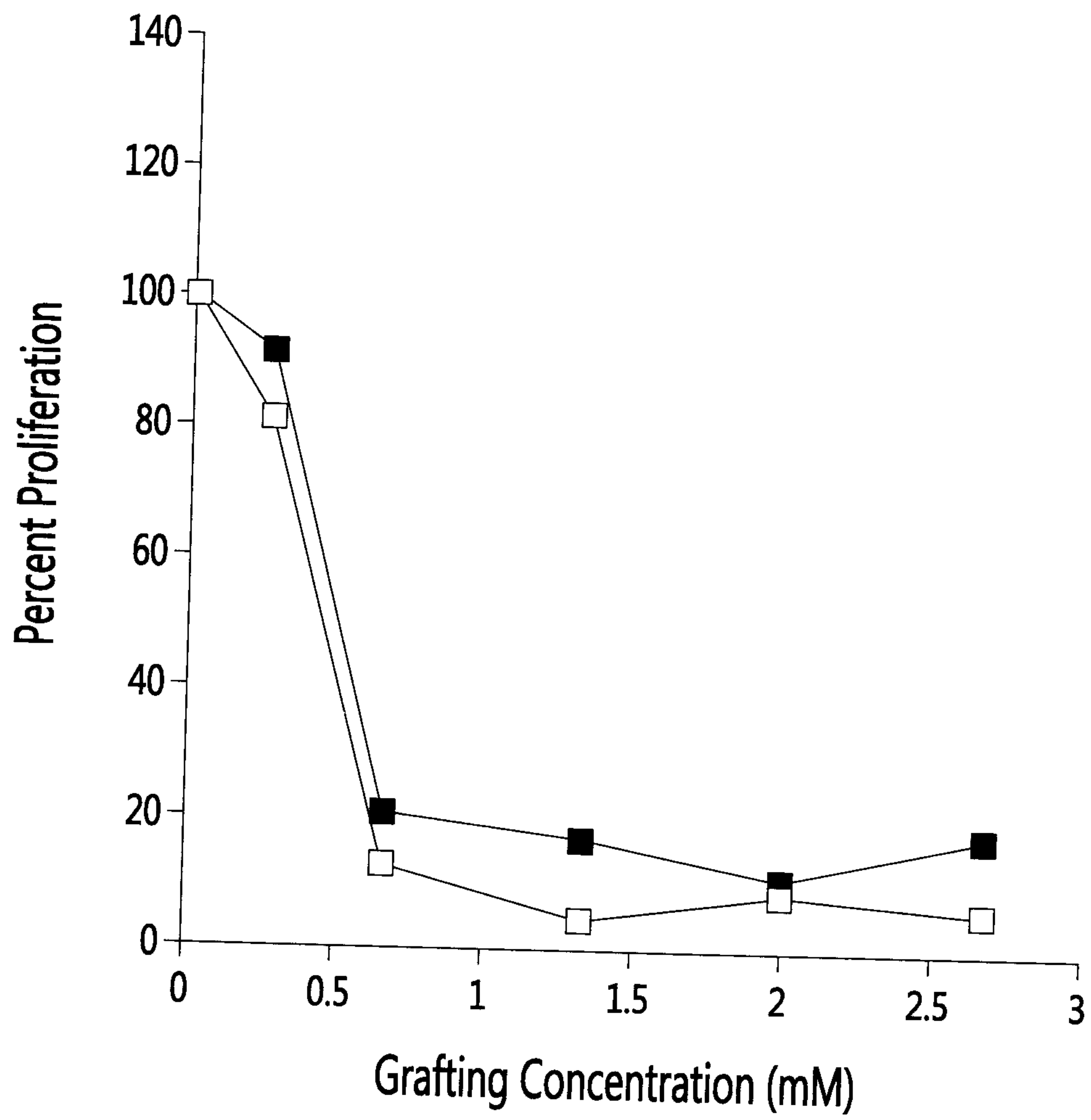
Figure 9



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Fig - 11

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Figure 12

