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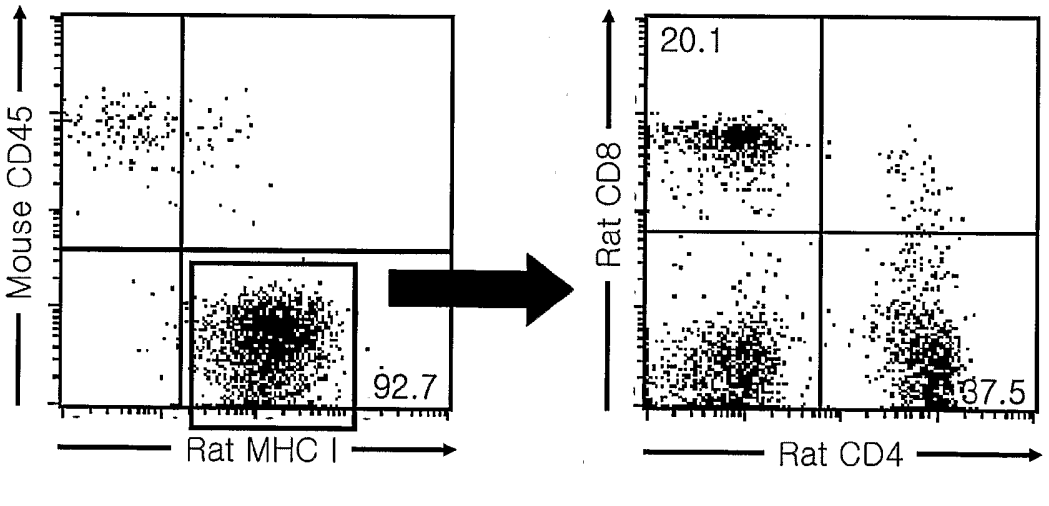
(54) Title: METHOD OF PRODUCING XENOGENIC CD4 T-CELL AND ANIMAL MODEL PRODUCING XENOGENIC CD4 T-CELL



(57) Abstract: The present invention relates to a method of producing CD4 T-cells and an animal model producing xenogenic CD4 T-cells. More specifically, the method concerns the production of CD4+CD8' T cells recognizing antigen presented by xenogenic major histocompatibility complex class (MHC-II) by transplantation of biological material containing cells having T cell differentiation capability and expressing xenogenic MHC II into immunodeficient animal. According to the present invention, because the animal including human can supplied with self immunocytes prepared by xenogenic animal, the present invention can be used for treating immune dysfunction-associated diseases such as infection, autoimmune disease, AIDS, and malignant tumor.

WO 2006/049439 A1

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METHOD OF PRODUCING XENOGENIC CD4 T-CELL AND ANIMAL MODEL PRODUCING XENOGENIC CD4 T-CELL

FIELD OF THE INVENTION

The present invention relates to a method of producing CD4 T-cells and an animal model producing CD4 T-cell. More specifically, the method concerns the production of CD4 T cells recognizing antigen presented by xenogenic major histocompatibility complex class (MHC-II) by transplantation of biological material containing cells having T cell differentiation capability and expressing xenogenic MHC II into immunodeficient animal, an animal model with the ability to produce the T cells, and clinical applications thereof.

BACKGROUND OF THE INVENTION

The human body defends itself from exposure to foreign substances by immunological reactions. The main component of the immune reactions is the lymphocytes, particularly the $CD4^{+}CD8^{-}$ T cells (CD4 T-cells), $CD4^{-}CD8^{+}$ T cells (CD8 T cells) and B-cells, which mediate antigen-specific immunity.

B cells mainly produce antibodies to antigens, and CD8 T cells specifically destroy infected cells. On the other hand, CD4 T cells, also known as helper T cells, serve a central role in the immune system by inducing and regulating activities of CD8 T cells and B cells. Malfunction of CD4 T cells is well known in immune-related diseases such as infection, acquired immune deficiency syndrome (AIDS), autoimmune disease and aging.

In contrast to B cells, T cells can only recognize its specific MHC-peptide complex. The T cell receptor (TCR) of T cells acquires the ability to recognize the specific peptide-MHC complex by positive selection of T cell precursors in the thymic cortex. Subsequently, positively selected T cells which recognize self antigens in the form of peptide-MHC complex as presented by thymic medullary epithelial cells and dendritic cells are negatively selected. Through this process, thymocytes recognizing MHC II differentiate to CD4 T cells and those recognizing MHC I differentiate to CD8 T cells. This process results in a T cell repertoire consisting of about 2.5 x 10^7 clonotypic T cells in the peripheral lymphoid organs of human beings.

With aging, production of T cells in the thymus declines, leading to reduced diversity of TCRs in the peripheral tissues and gradual loss of T cell function, which can increase susceptibility to infection, cancer, and autoimmune disease. Similarly, human immunodeficiency virus (HIV) impairs the immune system by selective destruction of CD4 T cells. Therefore, it is possible to treat aging-associated immune dysfunction, infection, autoimmune disease, and AIDS and to stimulate anti-tumor activity by replenishment with T cells having high TCR diversity. However, T cells are extremely species-specific, and therefore it is very difficult to produce self MHC-restricted T cells in other organisms or animals.

More specifically, established theory was that T cell development occurs in the thymus by positive selection via recognition of MHC II molecules present on thymic cortex epithelial cells. Therefore, it was believed that transplantation of human hematopoietic stem cells into an animal host will either lead to failure of T cell development or lead to development of CD4 T cells that recognize MHC II molecules of the host animal. Consequently, it was unlikely that such CD4 T cells

would have any therapeutic value in humans.

However, the inventors were the first to demonstrate in 1992 the expression of DR, a human MHC II molecule, in immature T cells of an embryonic thymus (Park et al., 1992, Hum Immunol, 33:294-8). In 1997, the inventors also demonstrated the ability of immature T cells to induce positive selection by self-education (Choi et al., 1997, Hum Immunol, 54:15-20). These developments gave rise to the possibility of producing xenogenic T cells.

SUMMARY OF THE INVENTION

To resolve the aforementioned problems, an object of the present invention is to provide a method of production for self CD4 T cells educated by self MHC II-expressing T cells in xenogenic organism, CD4 T cells and an animal model for producing the CD4 T cells, which can be used in the treatment of infection, autoimmune disease, AIDS and malignant tumors.

Another embodiment of the present invention is to provide a method of producing a xenogenic T cell in an animal comprising the steps of:

transplanting xenogenic biological material containing cells capable of differentiating into T cell and expressing endogenous MHC II of a xenogenic donor into an immunodeficient animal which are incapable of expressing endogenous MHC II and therefore unable to mount an immune response to xenogenic T cell,

The still embodiment of the present invention is to provide CD4 T cells prepared by the method.

The further embodiment of the present invention is to provide an animal which produces a xenogenic (donor) CD4 T cell being capable of recognizing an

antigen presented by MHC II of a xenogenic donor, prepared by transplantation of biological material containing a cell which have T cell differentiation capability and expressing its endogenous MHC II of the xenogenic donor into an immunodeficient animal which doest not express endogenous MHC II, and is incapable of mounting an immune response to xenogenic T cells.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows a DNA construct that induces expression of CIITA (MHC Class II transactivator) in mouse thymocytes, which results in expression of MHC II.

Figure 2 shows expression levels of MHC II for transgenic mice (Plck-CIITA Tg) transfected with DNA construct of Figure 1 and for normal B6 mouse as analyzed by flow cytometry.

Figure 3 shows the development of mouse thymocytes in Plck-CIITA^{tg+}CIITA$^{0/0}$ mouse as compared to normal (CIITA$^{+/0}$) and CIITA-deficient mouse (CIITA$^{0/0}$).

Figure 4 shows the development of mouse thymocytes in normal (MHC II$^{+/0}$), MHC II deficient (MHC II$^{0/0}$), and Plck-CIITA^{tg+}MHC II$^{0/0}$ mouse as analyzed by flow cytometry.

Figure 5 shows development of mature CD4 and CD8 T cells in the peripheral blood of MHC II gene deficient mouse transplanted with bone marrow of both Plck-CIITA Tg and B6. PL mice as compared to control (both wild type B6 and B6.PL bone marrow).

Figure 6 shows the diversity of TCR receptors of normal B6 mouse, Plck-CIITA Tg, and Plck-CIITA^{tg+}CIITA$^{0/0}$ mice as analyzed by flow cytometry.

Figure 7 shows a comparison of CD4^{+}CD8^{-} T cell function in the periphery

of normal B6, Plck-CIITA Tg, and Plck-CIITA^{tg+}CIITA$^{0/0}$ mice.

Figure 8 shows rat T cells within the blood of RAG-1, IL-2 receptor γ chain, MHC II gene deficient (RAG1$^{-/-}$IL-2Rγ$^{-/-}$MHC II$^{-/-}$) mouse at 15 weeks after rat bone marrow transplantation as analyzed by flow cytometry.

Figure 9 shows human T-cells in the blood of RAG1$^{-/-}$IL-2Rγ$^{-/-}$MHC II$^{-/-}$ mouse at 18 weeks after human bone marrow transplantation as analyzed by flow cytometry.

DETAILED DESCRIPTION

The present invention relates to a method of production for self-MHC class II-restricted CD4 T cells using xenogenic animals.

The term "xenogenic animal" means another organism or taxonomical species as classified differently from the donor of the CD4 T cells. For example, a xenogenic animal would show an immunological reaction to the donor's transplant of blood, lymphocytes, or organs.

In the present invention, the term "immunodeficient animal" means a mutant, either man-made or natural, that has been rendered incapable of immune reaction. The appropriate immunodeficient animal for the invention is an animal lacking T cells, B cells, NK cells, and NK-T cell, and therefore does not mount an immunological response against xenogenic T cells.

The aforementioned animal does not express endogenous MHC II proteins, so that MHC II is not expressed or MHC II expression itself is blocked in the thymic epithelia of such animals. As the method of inhibiting expression of MHC II molecules, any of the following is possible: where the gene encoding the MHC II

molecule is lacking; where MHC II gene transcription is unfunctional; where MHC II mRNA translation is unfunctional; or most preferably, the MHC II gene is knocked out. An example of mice incapable of MHC II gene transcription, the gene encoding CIITA (MHC class II transactivator) is missing, but examples of such animals are not limited thereto. The aforementioned animals can be produced by mating immunodeficient animals with animals whose MHC II expression or function has been blocked. In this invention, "MHC II" denotes all types of MHC II molecules naturally expressed in immunodeficient animals. More specifically, I-A and I-E are found in mice and HLA-DP, HLA-DR and HLA-DQ in humans.

Also, the immunodeficient animal lacks at least one gene selected from the group consisting of recombination activating gene (RAG)-1, RAG-2 and interleukin-2 receptor γ chain (IL-2Rγ), or is severe combined immunodeficiency (SCID) animal, which cannot mount an immune response to xenogenic cells.

The examples of aforementioned immunodeficient animals are mouse, rat or pig, but not limited thereto.

Specific examples of immunodeficient animals include SCID mice, RAG-1 knock-out, RAG-2 knock-out, RAG-1 or RAG-2 and IL-2Rγ gene knock-out animals, but are not limited thereto.

The transgenic animals, animal mating and immunodeficient animals can be readily produced or performed through conventional methods by those skilled in the technical field, and may also be obtained by purchase or gift.

As an example, knock-outs of RAG-1 and IL-2Rγ, and MHC-II were purchased and mated, resulting in mice deficient in RAG-1, IL-2γ and MHC-II ($RAG1^{-/-}IL\text{-}2R\gamma^{-/-}MHC\ II^{-/-}$).

The animal of the present invention does not express endogenous MHC II

and is incapable of mounting an immune response, therefore it can serve as an animal model for producing CD4 T cells capable of recognizing antigen as presented by self MHC II molecules of a xenogenic organism whose biological sample containing cells expressing MHC II and which can differentiate into T cells, has been transplanted into the animal model.

The method of producing xenogenic T cells according to this invention involves transplanting biological material including precursor cells with the potential to differentiate to MHC II^+ thymocytes, into an immunodeficient host followed by separation of developed CD4 T cells capable of recognizing antigen as presented by self MHC II from the blood, spleen and lymph nodes of the host.

The aforementioned xenogenic T cells can be either immature T cells in the thymus or mature T cells, particularly the thymic immature CD4 single positive T cells expressing MHC II on the cell surface.

The aforementioned biological material can be hematopoietic stem cells from bone marrow, or cord blood, or hematopoietic stem cells differentiated from embryonic stem cells, but are not limited thereto.

The aforementioned xenogenic animal can be human, rat, pig, monkey, or non-human primate.

The aforementioned transplantation of biological material can be performed by any conventional method known in the technical field of the present invention. As an example, the transplantation can be performed by intravenous (IV) injection, specifically by tail IV injection of bone marrow cells depleted of T cells (5×10^6 cells), CD34-positive cells of bone marrow or cord blood origin ($1\text{-}5 \times 10^5$ cells) in 4-12 week old mice.

The aforementioned transplantation is performed only once, but second and

third transplantation can be performed with a time interval of 1 to 4 weeks. The time interval is preferably controlled depending on the animal. In 4 to 20 weeks after the transplantation, CD 4 T cells developed from transplanted hematopoietic stem cells can be isolated from blood, spleen or lymph nodes.

The method for separating CD4 T cells derived from blood, spleen or lymph node from xenogenic animals may involve using antibodies with magnetic or fluorescent tags to CD4 T cells, but is not restricted thereto. For example, the aforementioned cells can be sorted magnetically following reaction with anti-CD4 antibodies conjugated with magnetic beads (Milteny Biotech, Auburn, CA), or can be sorted by flow cytometry after reacting the aforementioned cells with anti-CD4 antibodies conjugated with fluorescent tags.

As an example, this invention transplanted rat and human bone marrow cells into RAG-1, IL-2γ and MHC II deficient mice ($RAG1^{-/-}IL\text{-}2R\gamma^{-/-}MHC\ II^{-/-}$) and demonstrated that CD4 T cells of a rat or human donor could be produced. 15 Weeks after injection of 5×10^6 rat bone marrow cells resulted in CD4 T cells totaling 37.5% of rat lymphocytes within the mouse.

The method of producing xenogenic CD4 T cells in this invention is based on the fact that MHC II molecules present on the surface of immature T cells (thymocytes) can educate other immature T cells, and therefore positively select T cells that can recognize self MHC II molecules. This method is possible under the premise that education by MHC II molecules of the host is specifically blocked. Also, dendritic cells derived from donor cells can induce typical negative selection of self-reactive T cells.

Therefore, it is possible to produce self-CD4 T cells which do not induce immune rejection according to the methods of the present invention. Thus, the

produced CD4 T cells are capable of recognizing diverse antigens and therefore have therapeutic value for treating diseases caused by deterioration of the immune system, i.e. leukemia, AIDS, autoimmune disease and tumors, and aging-associated immune dysfunction.

Furthermore, the present invention relates to the animal model producing xenogenic CD4 T cells which is deficient in MHC II expression in all cell surfaces, does not induce immune response to xenogenic cells, and is transplanted with biological material containing xenogenic progenitor cells that are capable of differentiating into thymocytes expressing MHC II, ultimately producing the T cells of interest.

The present invention is further explained in more detail with reference to the following examples. These examples, however, should not be interpreted as limiting the scope of the present invention in any manner.

EXAMPLE 1

Induction of MHC II from mouse T cell

1-1: Preparation of DNA constructs

A fragment including a full-length human CIITA cDNA (a generous gift from Cheong-Hee Chang, Indiana University, USA) was introduced at the BamHI site of a p1017 vector containing the proximal promoter of lck (a generous gift from Jae Kyun Shin, Sungkyunkwan University, Korea) to produce transgenic mice designated as Plck-CIITA Tg. Figure 1 shows the vector structure that consists of the proximal promoter of lck, CIITA cDNA, and the poly A tail of hGH (human growth factor). CIITA is a critical transactivator required for the expression of MHC class II genes in most cells, and the proximal promoter of lck is known to be

responsible for the specific expression of the target gene in thymocytes and mature T cells.

Human CIITA cDNA construct shown in Fig. 1 was also introduced in the vector containing human CD2 promoter (a generous gift from Dr. Dimitris Kioussis, National Institute for Medical Research, London, UK) to produce transgenic mice designated as CD2-CIITA Tg.

1-2: Preparation of transgenic mice

The transgene was injected into the pronuclei of fertilized eggs of B6 mice (C57BL/6). The presence of the inserted human CIITA sequence was confirmed by polymerase chain reaction (PCR) of genomic DNA and flow cytometry of peripheral blood of transgenic mice. The transgenic mouse was designated as Plck-CIITA Tg mice.

1-3: Flow cytometry

The expression of MHC II in Plck-CIITA Tg mice and normal B6 mice was investigated by flow cytometry.

Fresh cell suspensions of thymocytes, splenocytes, lymph node cells, and peripheral blood leukocytes of the transgenic mouse were prepared in phosphate buffered saline (PBS) solution including BSA (bovine serum albumin). The erythrocytes removed by lysis with RBC lysis buffer (DiNonA Inc.) for 5 minutes, centrifuged at 1,000 rpm for 10 minutes to obtain cell pellet, and then suspended in phosphate buffered saline solution containing 5% BSA to produce 1 X 10^6 cell. To compare MHC II expression in each peripheral leukocytes and thymocytes, one million cells were suspended in 100 μl PBS, and distributed into test tubes, and an antibody recognizing mouse MHC II, and a combination of antibodies recognizing

each leukocytes were diluted and distributed in 10μl test tube. The antibodies were FITC (fluoroscein isothiocyanate)-, PE (phycoerythrin)- or APC (allophycocyanin)-conjugated antibodies. Anti-CD3 antibody, anti-CD4 antibody and anti-CD8 antibody were used for analyzing T cell and immature T cell in each developing stage, B220 antibody was used for selecting B cell, and MAC-1 antibody was used for selecting macrophage.

The suspended solution and antibodies were incubated for 30 min at 4°C, and then centrifuged at 1,000 rpm for 10 minutes to obtain cell pellets. The pellet was washed twice with PBS to remove the unreacted antibody. Finally 200 μl of PBS was added to the cell pellet after centrifugation, and the flow cytometric analysis was performed using FACS Calibur (Becton-Dickinson, Mountain View, CA). Anti-mouse antibodies used in this study were as follows: anti-I-A^b (AF6-120.1), anti-I-A/I-E (2G9), and anti-CD3 (145-2C11) were purchased from Pharmingen (San Diego, CA), and anti-CD4 (GK 1.5), anti-CD8a (53-6.7), anti-B220 (RA3-B2), and anti-Mac-1 (M1/70) mAbs were purchased from DiNonA Inc (Seoul, Korea).

In Figure 2, the expression of MHC class II molecules on the surface of thymocytes and splenocytes of Plck-CIITA Tg mice is compared with that of wild type B6 mice. The thick solid line represents the staining of cells from Plck-CIITA Tg with anti-MHC class II antibody, and the thin solid line represents the staining of cells from wild type B6 mice with anti-MHC class II antibody. Thymocytes are classified into four subsets according to the expression pattern of CD4 and CD8 (that is, $CD4^-$ $CD8^-$ double negative thymocytes, $CD4^+CD8^+$ double positive thymocytes, and $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive thymocytes). Splenocytes are classified into $CD3^+$ mature T cells, $B220^+$ B cells and Mac-1^+ macrophages. In Plck-CIITA Tg mice, MHC class II molecules (I-A^b) are expressed on $CD4^+CD8^+$ DP thymocytes,

and expression persisted in the mature CD4 and CD8 T cells of the spleen and lymph nodes, while all the different types of immature and mature T cells from wild-type littermates did not show persistent expression of I-A^b. The expression of I-A^b on B cells and macrophages from the spleens of Plck-CIITA^{Tg} mice was not affected by Plck promoter-driven CIITA transgene expression.

EXAMPLE 2

MHC II-mediated positive selection in mouse T cells

2-1: Preparation of Plck-CIITA^{tg+}CIITA$^{0/0}$ mouse

To directly address the question of whether MHC II-positive thymocytes mediate positive selection, Plck-CIITA^{Tg+}CIITA$^{0/0}$ mice, in which thymocytes act as the only thymic antigen presenting cells, were produced by backcrossing Plck-CIITA^{Tg} mice to CIITA-deficient mice (CIITA$^{0/0}$; B6.129S2-C2ta^{tm1Ccum}/J; The Jackson Laboratory, Bar Harbor, ME), and thymic differentiation was evaluated. In CIITA-deficient mice, MHC class II expression is near-totally suppressed, and it had been known that the development of mature CD4 T cells is severely defected in theses mice (Chang et al, 1996, Immunity, 4, 167-178). Thus, MHC class II expression in Plck-CIITA^{Tg+}CIITA$^{0/0}$ mice is restricted to thymocytes and mature T cells, and thymocytes do not develop into mature CD4 T cells via thymocyte-thymic epithelial cell interaction.

Figure 3 shows the development status of thymocytes in Plck-CIITA^{tg+}CIITA$^{0/0}$ and wild type (CIITA$^{+/0}$) mice. While differentiation into CD4 SP cells was almost completely abrogated by the lack of MHC II-expressing cells in the thymi of the CIITA$^{0/0}$ control mice (0.8%), a substantial fraction of the thymocytes (8.5%) developed into CD4 SP cells in the Plck-CIITA^{Tg+}CIITA$^{0/0}$ mice.

These results imply that thymocytes could be positively selected by thymocyte-thymocyte interaction.

2-2: MHC II function for T-cell interaction

To verify that MHC molecules on the surface of thymocytes are essential for the generation of CD4 SP thymocytes in Plck-CIITA^{tg+}CIITA$^{0/0}$ mice, we generated MHC class II-deficient mice (MHC II$^{0/0}$) in which the expression of the CIITA transgene was limited to T-lineage cells. CD4 T cell selection of the resulting Plck-CIITA^{Tg+}MHC II$^{0/0}$ mice was then assessed by flow cytometric analysis, based on the assumption that if the CD4 SP cells observed in Plck-CIITA^{Tg+}CIITA$^{0/0}$ mice were solely the result of CIITA transgene expression, then the development of CD4 SP thymocytes might occur even in the absence of MHC class II expression.

Figure 4 shows the results of flow cytometric analysis of thymocytes from wild-type (MHC II$^{+/0}$), MHC class II-deficient (MHC II$^{0/0}$) and Plck-CIITA^{tg+}MHC II$^{0/0}$ mice. MHC class II-null mice fails to generate CD4 SP thymocytes, irrespective of whether they have the Plck-CIITA transgene (Plck-CIITA^{Tg+}MHC II$^{0/0}$) or lack the Plck-CIITA transgene (MHC II$^{0/0}$), unlike the MHC II$^{+/0}$ control. These data confirm that the differentiation of DP thymocytes into CD4 SP thymocytes in the presence of Plck-CIITA transgene (Plck-CIITA^{Tg+}CIITA$^{0/0}$) is not due to thymic differentiation induced by the CIITA transgene itself.

2-3: Positive selection by thymocyte-thymocyte interaction

It has been reported that residual MHC II is found on a subset of cortical thymic epithelial cells (cTECs) in the thymi of CIITA$^{0/0}$ mice (Chang et al, 1996, Immunity, 4, 167-178). To exclude the possibility that CIITA-independent MHC II molecules might be responsible for the selection of the CD4 SP cells observed in

Plck-CIITA^{Tg+}CIITA$^{0/0}$ thymi, irradiation bone marrow (BM) chimeras were generated by injecting mixed BM cells from Plck-CIITA^{Tg} and B6.PL mice into irradiated (800 cGy) MHC II-deficient mice (Plck-CIITA^{Tg}+B6.PL→MHC II$^{0/0}$). Thymocytes and T cells of B6.PL mice express Thy1.1 antigen on their surface, while Thy1.2 antigen is expressed on T-lineage cells of Plck-CIITA Tg and wild-type B6 mice. Thus, thymocyte and T cells derived from B6.PL BM could be distinguished from those from Plck-CIITA Tg and wild-type B6 mice.

Peripheral blood was taken from the retro-orbital venous plexus of the chimeric mice at 8 weeks after engraftment, and stained with anti-CD4, anti-CD8, anti-thy1.1 and anti-thy1.2 antibodies.

Figure 5 shows the result of flow cytometric analysis of peripheral blood after antibody staining. Control chimeras (B6+B6.PL→MHC II$^{0/0}$), which are reconstituted with mixed BM from normal B6 and B6.PL mice, are unable to produce CD4 T cells of either the B6.PL (Thy1.1) or B6 (Thy1.2) BM origin. In contrast, a substantial fraction (17.3%) of mature peripheral CD4 T cells is generated in Plck-CIITA^{Tg}+B6.PL→MHC II$^{0/0}$ chimeras, in which positively selecting MHC II molecules are provided exclusively by immature thymocytes of Plck-CIITA^{Tg} BM origin. Moreover, CD4 T cells in Plck-CIITA^{Tg}+B6.PL→MHC II$^{0/0}$ chimeras are derived from both Thy1.2^{+} (Plck-CIITA^{Tg} donor and MHC II$^{0/0}$ host) and Thy1.1^{+} (B6.PL donor) BM. It is clear from the proper generation of Thy1.1^{+} CD4 cells that MHC class II-positive thymocytes could induce the positive selection of other thymocytes via thymocyte-thymocyte interaction.

EXAMPLE 3

TCR diversity and function of CD4 T cells produced

by thymocyte-thymocytes interaction

3-1: TCR Diversity

The T cell receptor (TCR) is a heterodimer of α and β chains, and various α and β chains are generated by gene rearrangement. Development of antibodies against the variable region of TCR β chain (TCR Vβ) makes it possible to estimate the TCR diversity.

To examine the TCR repertoire of the CD4 T cells generated in the Plck-CIITA^{Tg+}CIITA$^{0/0}$ mice, CD4 SP thymocytes and peripheral CD4 T cells were typed for TCR Vβ usage, and compared to the corresponding cells in Plck-CIITA Tg or normal mice.

Thymocytes and splenocytes were extracted from each mouse, and stained with anti-CD4, anti-CD8, and anti-TCR Vβ.

Figure 6 shows the results of flow cytometric analysis of thymocytes and splenocytes from wild type B6, Plck-CIITA Tg, and Plck-CIITA^{tg+}CIITA$^{0/0}$ mice. Although the percentages of each TCR Vβ family in the CD4 SP thymocytes and mature CD4 T cell show marginal differences, the overall TCR Vβ profiles are similar for the three mouse types, which indicates that MHC II-expressing thymocytes are able to select CD4 T cells with diverse TCR Vβ subsets.

3-2: Function of CD4 T cells

The question as to whether CD4 T cells selected on MHC II-positive thymocytes are functionally competent was addressed using mixed lymphocyte cultures *in vitro*. The function of the CD4 T cells from Plck-CIITA^{Tg+}CIITA$^{0/0}$ mice was compared with those from Plck-CIITA Tg and normal mice in mixed lymphocyte reactions. CD4 T cells from spleens and lymph nodes of each mouse were isolated by magnetic cell sorting using MACS with anti-CD4 (GK1.5)

microbeads.

After determining the purity (95~97%) of the collected CD4 T cells by flow cytometry using the anti-CD4 mAb (RM4-5)-FITC, the isolated CD4 T cells (1×10^5) were stimulated for 3 days with irradiated (3,000 cGy) B6 or BALB/c splenocytes (1×10^5 or 4×10^5) in DMEM medium (Gibco, Carlsbad, CA) that was supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and 50 μM β-mercaptoethanol. The cultures were pulsed with 1 μCi/well [^{3}H]-thymidine (Amersham Bioscience) for the final 16 hrs of incubation and the mean incorporation of thymidine in DNA were measured in quadruplicate wells by liquid scintillation counting.

Figure 7 shows the results of thymidine uptake after mixed lymphocyte reaction. The CD4 T cells from Plck-CIITA^{Tg+}CIITA$^{0/0}$ mice give more vigorous responses to allogeneic antigen presenting cells (BALB/c) than do those from Plck-CIITA Tg or normal mice. Moreover, the CD4 T cells from Plck-CIITA^{Tg+}CIITA$^{0/0}$ mice show proliferative responses in the presence of syngeneic stimulator cells (B6), while the CD4 T cells from CIITA Tg or wild-type mice do not respond in this way. These results imply that CD4 T cells selected by MHC class II-positive thymocytes are functionally intact in terms of response to polyclonal antigen. More vigorous response of CD4 T cells from Plck-CIITA^{Tg+}CIITA$^{0/0}$ mice may reflect the defect in negative selection by medullary thymic epithelial cells and dendritic cells, compared with Plck-CIITA Tg and wild type B6 mice.

EXAMPLE 4

Production of rat CD4 T cells in mice

RAG-1 gene knock out mice (B6.129S7-Rag1^{tm1Mom}/J, Jackson Laboratory)

were backcrossed with an IL-2 receptor common γ chain-deficient mice (B6.129S4-Il2rg^{tm1Wjl}/J, Jackson Laboratory) and MHC class II-deficient mice (B6.129-H2$^{dlAb1-Ea}$/J, Jackson Laboratory) to generate RAG1$^{-/-}$IL-2Rγ$^{-/-}$MHC II$^{-/-}$ mice, to which bone marrow cells of Sprague-Dawley (SD) rats were transferred. Bone marrow cells were collected from both femur and tibia of SD rats, and low-density bone marrow cells were obtained after centrifugation (1,000 g, 30 min) onto a 28% bovine serum albumin cushion as previously described by Prakapas et al. (Prakapas et al., 1993, Immunol Lett, 37:63-71). Low-density bone marrow cells were suspended in PBS containing 5% fetal bovine serum, and incubated with biotin-conjugated anti-rat CD4 antibody (Pharmingen, San Diego, CA) at 4℃ for 20 minutes. After washing with PBS, cells were incubated with anti-biotin magnetic bead (Milteny Biotech, Auburn, CA)) at 4℃ for 20 minutes, and then mature CD4 T cells were depleted by magnetic cell sorting using MACS system (Milteny Biotech, Auburn, CA). All RAG1$^{-/-}$IL-2Rγ$^{-/-}$MHC II$^{-/-}$ mice were irradiated with 2.5 Gy using a cobalt radiation source 1 day before cell transfer. Five million rat bone marrow cells were intravenously inoculated into mice. Peripheral blood was taken from the retro-orbital venous plexus at intervals of 1 or 2 weeks, and stained with anti-mouse CD45, anti-rat MHC class I, anti-rat CD4, and anti-rat CD8 antibodies. Rat bone marrow-derived leukocyte population was determined by flow cytometry.

Figure 8 shows the results of flow cytometric analysis of peripheral blood taken at 15 weeks after bone marrow transplantation. The percentage of rat MHC class I^{+} cells in the peripheral blood mononuclear cells of RAG1$^{-/-}$IL-2Rγ$^{-/-}$MHC II$^{-/-}$ mouse is 92.7% (Figure 8, left panel). Rat CD4 and CD8 T cells in rat MHC class I^{+} population reach 37.5% and 20.1%, respectively (Figure 8, right panel). These results imply that rat CD4 T cells could be generated in MHC class II-deficient

mouse host.

EXAMPLE 5

Production of human CD4 T cells in mice

Human CD4 T cells were generated in RAG1$^{-/-}$IL-2Rγ$^{-/-}$MHC II$^{-/-}$ mice after the xeno-transplantation of human hematopoietic stem cells. Human cord blood cells were collected during normal full-term deliveries. Mononuclear cells were separated by Ficoll-Hypaque density-gradient centrifugation and suspended in PBS containing 5% fetal bovine serum. Human CD34^{+} cells were isolated by incubation of cord blood cells with magnetic bead-conjugated anti-human CD34 antibody at 4°C for 20 minute, followed by magnetic sorting using MACS system. All RAG1$^{-/-}$IL-2Rγ$^{-/-}$MHC II$^{-/-}$ mice were irradiated with 2.4 Gy using a cobalt radiation source 1 day before cell transfer. One hundred thousand human CD34^{+} cells were intravenously inoculated into mice. Peripheral blood was taken from the retro-orbital venous plexus every other week, and stained with anti-mouse CD45, anti-human CD45, anti-human CD4, and anti-human CD8 antibodies. Human leukocyte population was determined by flow cytometry.

Figure 9 shows the results of flow cytometric analysis of peripheral blood taken at 18 weeks after bone marrow transplantation. The percentage of human CD45^{+} cells in the peripheral blood mononuclear cells of RAG1$^{-/-}$IL-2Rγ$^{-/-}$MHC II$^{-/-}$ mouse is 80.7% (Figure 9, left panel). Human CD4 and CD8 T cells in human CD45^{+} population reach 5.5% and 5.6%, respectively (Figure 9, right panel). These results imply that human CD4 T cells could be generated in MHC class II-deficient mouse host.

As shown above, this invention pertains to the production of CD4 T cells in

xenogenic animals. Thus produced CD4 T cells are capable of recognizing diverse antigens and therefore have therapeutic value for treating diseases caused by deterioration of the immune system, i.e. aging, leukemia, AIDS, autoimmune disease and tumors.

WHAT IS CLAIMED IS:

1. A method of producing xenogenic T cells in an animal comprising the steps of:

transplanting xenogenic biological material containing cells capable of differentiating into T cell and expressing endogenous MHC II of a xenogenic donor, into an immunodeficient animal which is incapable of expressing endogenous MHC II and therefore unable to mount an immune response to xenogenic T cells, and

isolating the CD4 T cells recognizing an antigen presented by xenogenic MHC II from blood, spleen and lymph nodes of the transplanted animal.

2. The method of Claim 1, wherein the immunodeficient animal is deficient in the gene encoding MHC II protein, MHC II gene transcription or MHC II mRNA translation.

3. The method of Claim 2, wherein the immunodeficient animal is an MHC II gene knock-out or is deficient in the CIITA (Major Histocompatibility Complex Class II transactivator) gene.

4. The method of Claim 1, wherein the immunodeficient animal is inhibited in expression of at least one selected from the group consisting of RAG (recombination activating gene)-1, RAG-2, and interleukin-2 receptor γ chain (IL-2Rγ); or is a SCID (severe combined immunodeficiency) animal.

5. The method of Claim 3 or Claim 4, wherein the immunodeficient

animal is selected from the group consisting of:

(a) A SCID animal deficient in MHC II gene;

(b) An animal deficient in MHC II and RAG-1 genes;

(c) An animal deficient in MHC II and RAG-2 genes;

(d) An animal deficient in RAG-1, MHC II, and IL-2R γ chain genes;

(e) An animal deficient in RAG-2, MHC II, and IL-2R γ chain genes;

(f) A SCID animal deficient in CIITA gene;

(g) An animal deficient in CIITA and RAG-1 genes;

(h) An animal deficient in CIITA and RAG-2 genes;

(j) An animal deficient in RAG-1, IL-2Rγ chain and CIITA genes; and

(k) An animal deficient in RAG-2, IL-2Rγ chain and CIITA genes.

6. The method of Claim 1, wherein the immunodeficient animal is mouse, rat or pig.

7. The method of Claim 1, wherein the xenogenic donor is human, rat, pig, or non-human primate.

8. The method of Claim 1, wherein the biological material is bone marrow, cord blood or hematopoietic stem cells differentiated from embryonic stem cells.

9. The method of Claim 1, wherein the step of isolating the CD4 T cell is performed by using antibodies to CD4 T cells followed by magnetic or flow cytometric sorting.

10. A T cell with a T cell receptor (TCR) which is produced by the method according to any one of Claims 1-4, and the T cell receptor is able to recognize antigen presented by xenogenic (donor) MHC II, but does not recognize self (donor) antigen of xenogenic donor.

11. The T cell of Claim 10, wherein the T cell is a CD4 single positive thymocyte or a mature CD4 T cell.

12. The T cell of Claim 10, wherein the T cell is developed in an immunodeficient animal selected from the group consisting of:

(a) A SCID animal deficient in MHC II gene;

(b) An animal deficient in MHC II and RAG-1genes;

(c) An animal deficient in MHC II and RAG-2 genes;

(d) An animal deficient in RAG-1, MHC II, and IL-2R γ chain genes;

(e) An animal deficient in RAG-2, MHC II, and IL-2R γ chain genes;

(f) A SCID animal deficient in CIITA gene;

(g) An animal deficient in CIITA and RAG-1 genes;

(h) An animal deficient in CIITA and RAG-2 genes;

(j) An animal deficient in RAG-1, IL-2Rγ chain and CIITA genes and

(k) An animal deficient in RAG-2, IL-2Rγ chain and CIITA genes.

13. An animal which produces a xenogenic (donor) CD4 T cell being capable of recognizing an antigen presented by MHC II of a xenogenic donor, prepared by transplantation of biological material containing cells which are

capable of differentiating into T cells and express endogenous MHC II of the xenogenic donor, into an immunodeficient animal which does not express endogenous MHC II, and is incapable of mounting an immune response to xenogenic T cells.

14. The animal of Claim 13, wherein the immunodeficient animal is an MHC II gene knock-out or is deficient in CIITA (Major Histocompatibility complex Class II transactivator) gene.

15. The animal of Claim 13, wherein the immunodeficient animal is inhibited in expression of at least one selected from the group consisting of RAG (recombination activating gene)-1, RAG-2, and interleukin-2 receptor γ chain (IL-2Rγ); or is a SCID (severe combined immunodeficiency) animal.

16. The animal of Claim 14 or 15, wherein the immunodeficient animal is selected from the group consisting of:

(a) A SCID animal deficient in MHC II gene;

(b) An animal deficient in MHC II and RAG-1 genes;

(c) An animal deficient in MHC II and RAG-2 genes;

(d) An animal deficient in RAG-1, MHC II, and IL-2R γ chain genes;

(e) An animal deficient in RAG-2, MHC II, and IL-2R γ chain genes;

(f) A SCID animal deficient in CIITA gene;

(g) An animal deficient in CIITA and RAG-1 genes;

(h) An animal deficient in CIITA and RAG-2 genes;

(j) An animal deficient in RAG-1, IL-2Rγ chain and CIITA genes and

(k) An animal deficient in RAG-2, IL-2Rγ chain and CIITA genes.

17. The animal of Claim 13, wherein the immunodeficient animal is a mouse, rat or pig.

FIG. 1

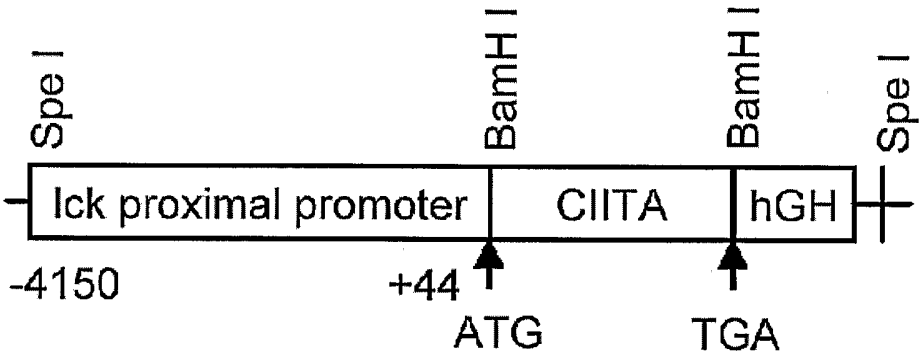

Spe I
BamH I
BamH I
Spe I
lck proximal promoter
CIITA
hGH
-4150
+44
ATG
TGA

FIG. 2

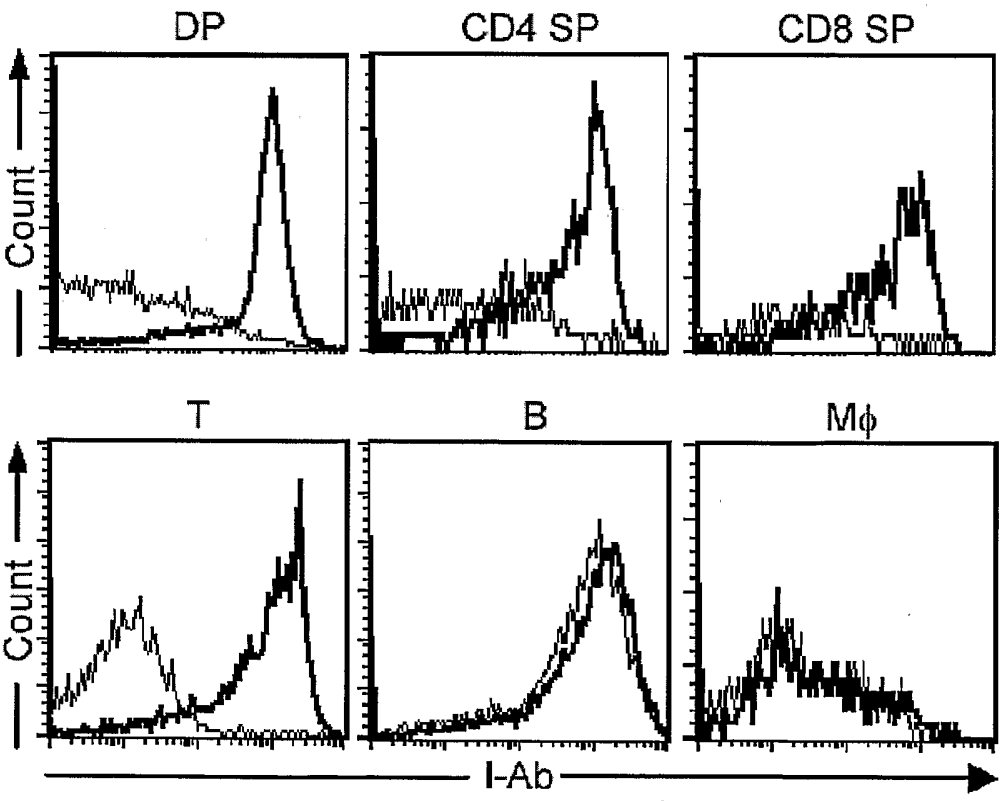

DP
CD4 SP
CD8 SP
Count
T
B
Mϕ
Count
I-Ab

FIG. 3

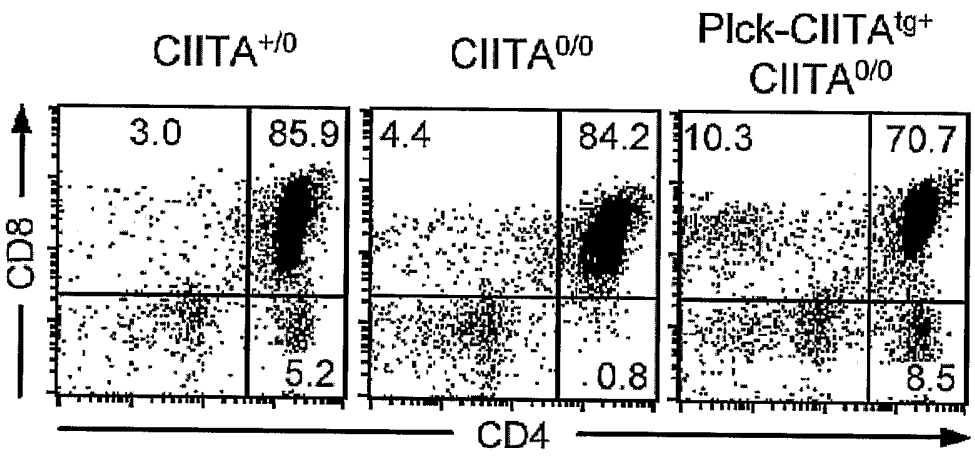

CIITA+/0
CIITA0/0
Plck-CIITAtg+
CIITA0/0
3.0
85.9
5.2
4.4
84.2
0.8
10.3
70.7
8.5
CD8
CD4

FIG. 4

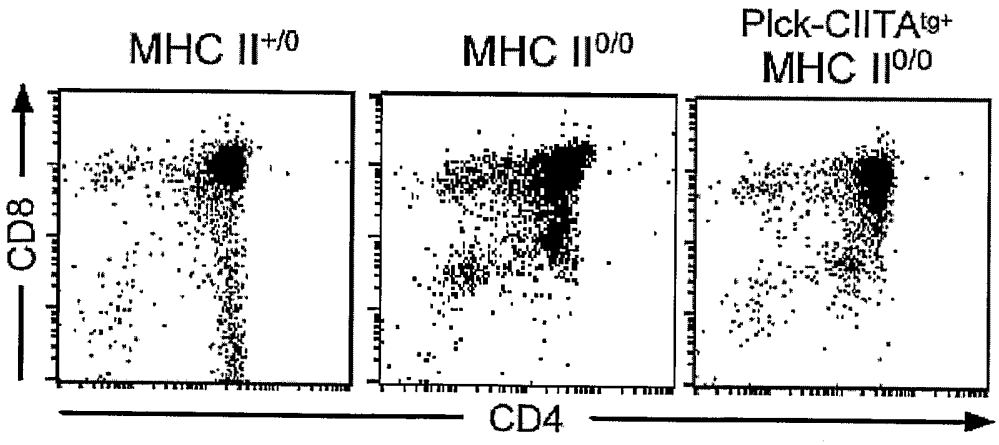

MHC II+/0
MHC II0/0
Plck-CIITAtg+
MHC II0/0
CD8
CD4

FIG. 5

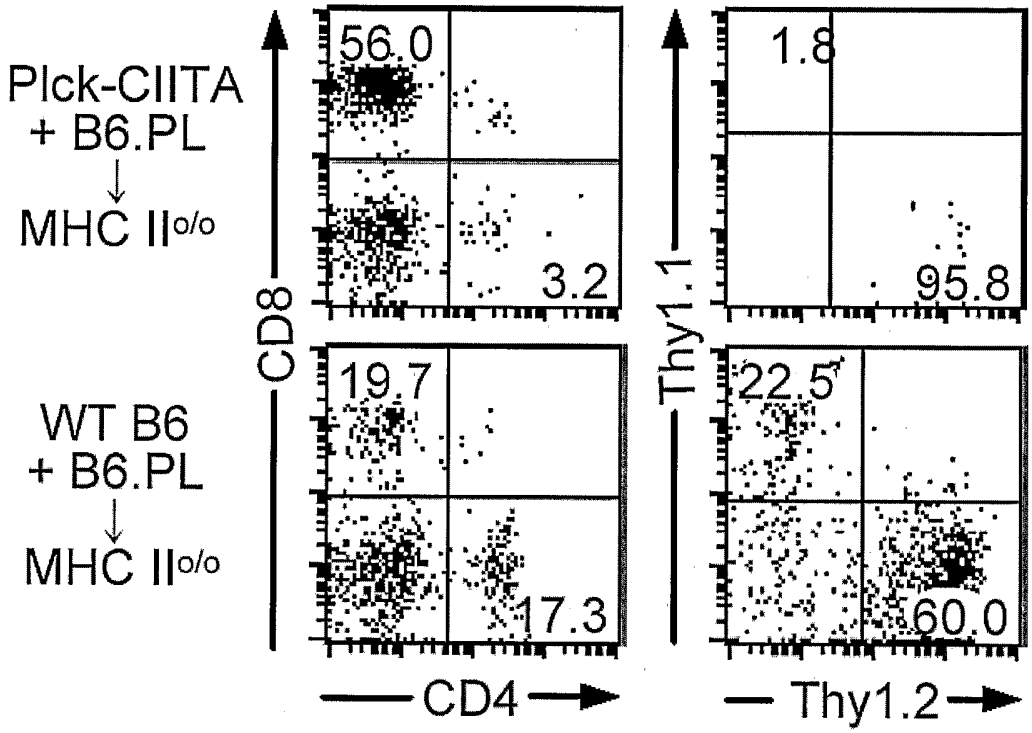

Plck-CIITA
+ B6.PL
↓
MHC II o/o
WT B6
+ B6.PL
↓
MHC II o/o
CD8
CD4
Thy1.1
Thy1.2
56.0
3.2
1.8
95.8
19.7
17.3
22.5
60.0

FIG. 6

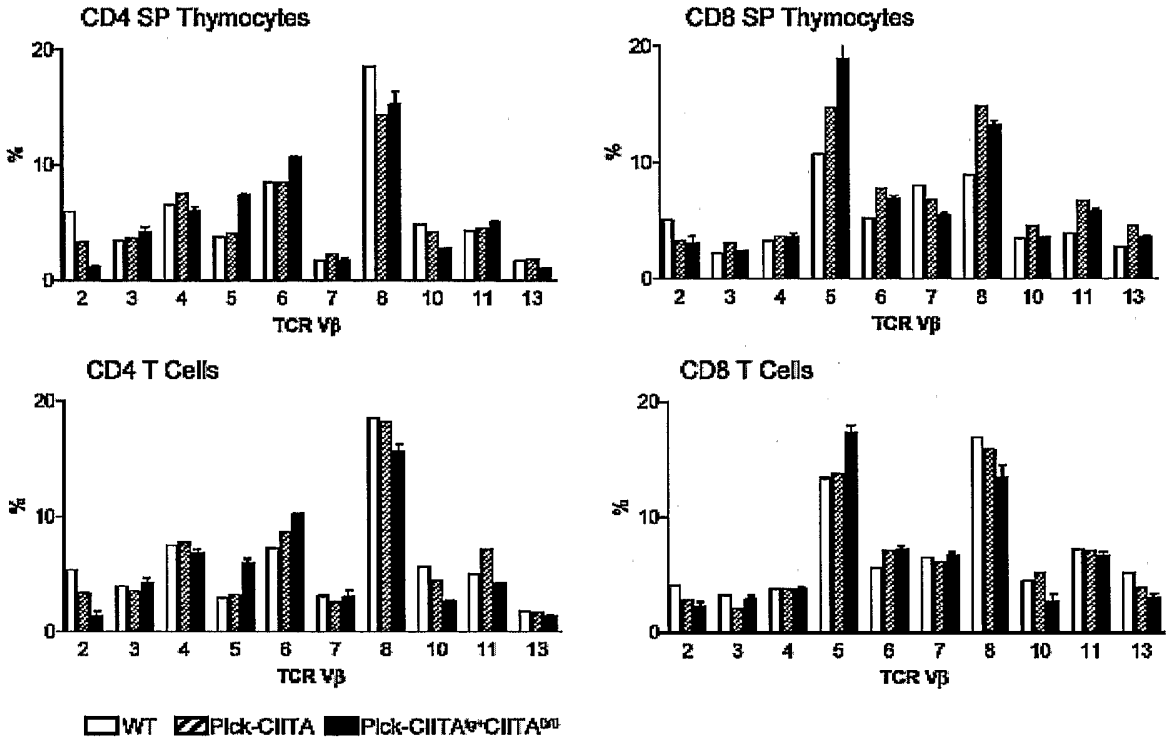

CD4 SP Thymocytes
CD8 SP Thymocytes
CD4 T Cells
CD8 T Cells
%
TCR Vβ
2 3 4 5 6 7 8 10 11 13
0 10 20
WT
Plck-CIITA
Plck-CIITA^hi CIITA^int

FIG. 7

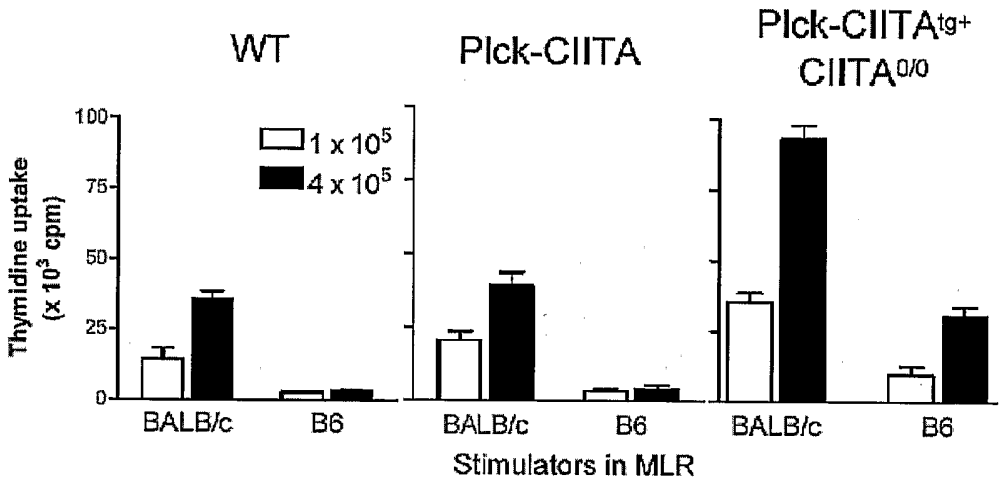

WT
Plck-CIITA
Plck-CIITA^{tg+} CIITA$^{0/0}$
Thymidine uptake (x 10^3 cpm)
100
75
50
25
0
1 x 10^5
4 x 10^5
BALB/c
B6
BALB/c
B6
BALB/c
B6
Stimulators in MLR

FIG. 8

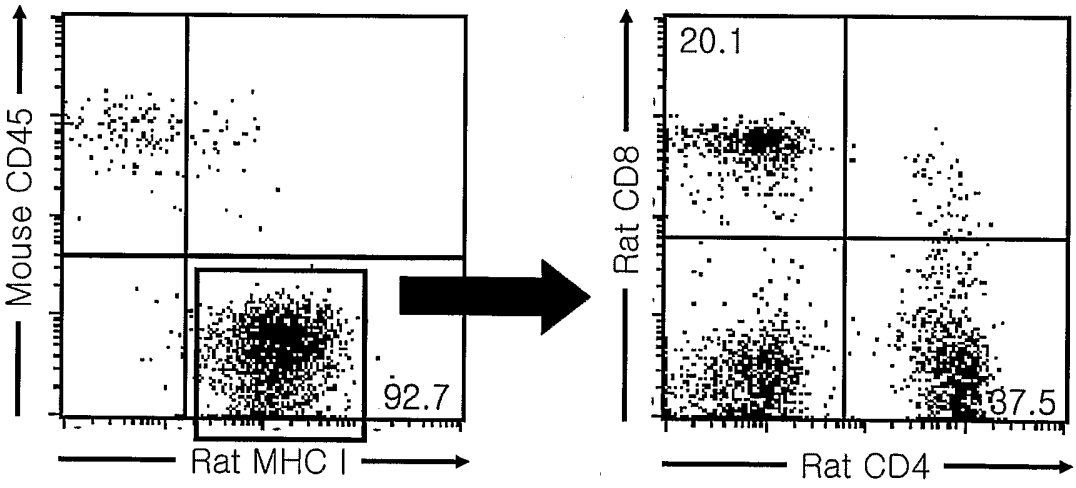
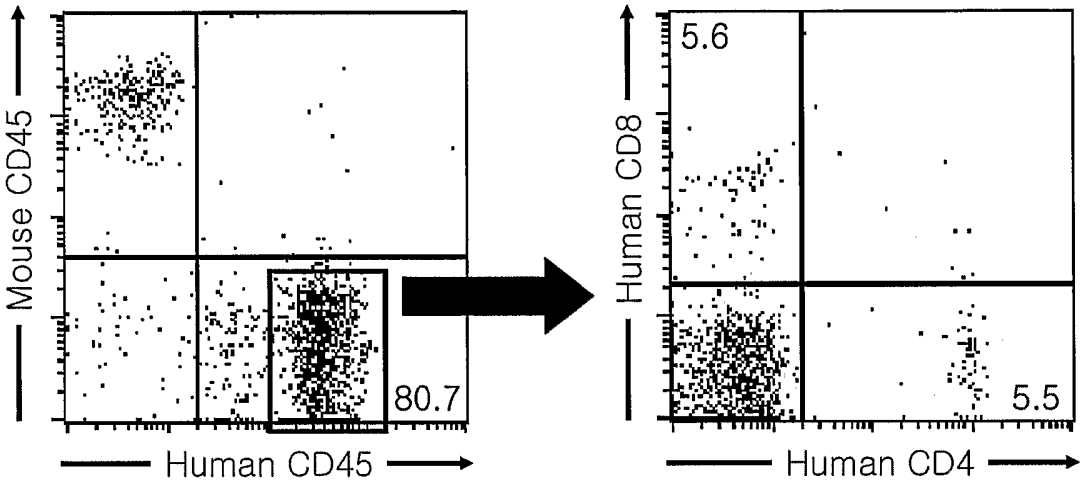

Mouse CD45
Rat MHC I
92.7
Rat CD8
Rat CD4
20.1
37.5

FIG. 9



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2005/003693

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/06(2006.01)i, A01K 67/027(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 5/06(2006.01)i, A01K 67/027(2006.01)i

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean Patents and applications for inventions since 1975
Korean Utility models and applictions for Utility models since 1975
Japanese Utility models and application for Utility models since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)

eKIPASS, Delphion, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Holscher, C. et al., 'Long term substitution and specific immune responses after transfer of bovine peripheral blood lymphocytes into severe combined immunodeficient mice' In: Vet. Immunol. Immunopathol., Vol.70(1-2), pp.67-83, 1 September 1999 See the whole document.	1-17
A	Watier, H. et al., 'Anti-pig xenogeneic response by human CD4+ T-lymphocytes' In: Pathol. Biol., Vol.48(4), pp.372-376, May 2000 See the whole document.	1-17
A	Chang, C.H. et al., 'Mice lacking the MHC class II transactivator (CIITA) show tissue-specific impairment of MHC class II expression' In: Immunity, Vol.4(2), pp.167-178, February 1996 See the whole document.	1-17
A	JP 04325087 A (TERUMO KABUSHIKI KAISHA) 13 November 1992 See the whole document.	1-17
A	WO 2004/060052 (GENENTECH INC.) 22 July 2004 See the whole document.	1-17

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 MARCH 2006 (08.03.2006)	**09 MARCH 2006 (09.03.2006)**

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Republic of Korea
Facsimile No. 82-42-472-7140

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LEE, CHUNG HO
Telephone No. 82-42-481-8160

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR2005/003693

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP0497275A2	05.08.1992
		EP497275A2	05.08.1992
		EP497275A3	10.02.1993
		JP04325087	13.11.1992
		JP4325087A2	13.11.1992
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		US5374549A	20.12.1994
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		AU2003301079A1	14.07.2004
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		AU2004256042A1	20.01.2005
		BR200316779A	01.11.2005
		CA2507880AA	22.07.2004
		CA2507898AA	08.07.2004
		CA2507898A1	08.07.2004
		EP01572744A2	14.09.2005
		HR20050649A2	31.12.2005
		KR1020050084317	26.08.2005
		KR1020050084318	26.08.2005
		KR1020050086913	30.08.2005
		NO20053435A	31.08.2005
		US2005095243A1	05.05.2005
		US2005095243AA	05.05.2005
		US2005163775A1	28.07.2005
		US2005163775AA	28.07.2005
		US2006024300AA	02.02.2006
		WO2004056312A2	08.07.2004
		WO2004056312A3	26.05.2005
		WO2004060052A2	22.07.2004
		WO2004060052A3	23.09.2004
		WO2004060053A2	22.07.2004
		WO2004060053A3	27.01.2005
		WO2005000351A2	06.01.2005
		WO2005005462A2	20.01.2005
		WO2005005462A3	26.01.2006