การตอบสนองของทีเซลล์ที่จำเพาะต่อยาในผู้ติดเชื้อเอชไอวีที่มีอาการแพ้ยา

นายภัทรวัจน์ ตันติวรสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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DRUG SPECIFIC T-CELL RESPONSES IN HIV PATIENTS WITH CLINICAL DRUG HYPERSENSITIVITY

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ภัทรวัจน์ ตันติวรสิทธิ์: การตอบสนองของทีเซลล์ที่จำเพาะต่อยาในผู้ติดเชื้อเอชไอวีที่มีอาการแพ้ยา. (DRUG SPECIFIC T-CELL RESPONSES IN HIV PATIENTS WITH CLINICAL DRUG HYPERSENSITIVITY) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นพ. เกียรติ รักษ์รุ่งธรรม , 49 หน้า

ปฏิกิริยาภูมิแพ้ยาเป็นปัญหาสำคัญในทางการแพทย์ โดยเฉพาะในผู้ป่วยที่ติดเชื้อเอชไอวีซึ่งมีโอกาสเกิดได้มากกว่าคนปกติ 100 เท่า ปัจจุบันวิธีการวินิจฉัยทางห้องปฏิบัติการที่ถือเป็นมาตรฐานหลักคือ การตรวจการตอบสนองต่อยานั้นๆด้วยการวัดการแบ่งตัวของลิมโฟไซต์ ที่เรียกว่า Lymphoproliferation assay มีรายงานการตรวจการแสดงออกของ CD134 บนผิว CD4 เซลล์ สามารถ ใช้ในการวิเคราะห์การตอบสนองของ Memory T-cell ที่จำเพาะต่อแอนติเจนของโรคติดเชื้อ การศึกษาครั้งนี้มีจุดประสงค์เพื่อวินิจฉัยปฏิกิริยาภูมิแบบล่าต่อยาต้านไวรัสเอชไอวีชนิด Nevirapine โดยอาศัยการทดสอบการกระตุ้นทีเซลล์โดยมี CD134 เป็นเครื่องบ่งชี้ในการวินิจฉัย การทดลองนี้ทำการศึกษาในกลุ่มผู้ป่วยที่เคยมีประวัติการเกิดปฏิกิริยาภูมิแพ้แบบล่าจากยา Nevirapine 10 คน ผู้ป่วยเอชไอวีที่ได้รับยา Nevirapine แต่ไม่พบอาการไม่พึงประสงค์จากยา 10 คน และอาสาสมัครที่มีสุขภาพปกติ 10 คน ได้ทำการทดสอบการกระตุ้นทีเซลล์ โดยเปรียบเทียบกับการใช้ และไม่ใช้ Monocyte derived dendritic cells ช่วยในการกระตุ้น โดยเปรียบเทียบกับวิธีมาตราฐานที่นิยมใช้คือ CFSE lympoproliferation assay ผลพบว่าการทดสอบการกระตุ้นโดยใช้ Monocyte derived dendritic cells ช่วยให้ผลที่ดีกว่าไม่มี Monocyte derived dendritic cells กล่าวคือ ร้อยละ 70 และ 10 มีการตอบสนองต่อยา ตามลำดับ นอกจากนี้เมื่อนำผลการทดสอบการกระตุ้นทีเซลล์โดยวินิจฉัยจาก CD134 เทียบกับวิธีมาตราฐานพบว่าให้ค่าของการตอบสนองเท่ากันทั้งในกลุ่มที่ใช้ และไม่ใช้ MDCs ช่วย

ดังนั้นการทดสอบการกระตุ้นทีเซลล์ในห้องปฏิบัติการน่าจะมีประโยชน์ในการวินิจฉัยช่วยผู้ป่วยที่ เกิดปฏิกิริยาภูมิแพ้แบบช้าจากยา Nevirapine ได้

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS: CD134 / NEVIRAPINE / DELAYED TYPE HYPERSENSITIVITY / CFSE / MONOCYTE DERIVED DENDRITIC CELLS (MDCs)

PATTARAWAT THANTIWORASIT: DRUG SPECIFIC T-CELL RESPONSES IN HIV PATIENTS WITH CLINICAL DRUG HYPERSENSITIVITY. ADVISOR: PROF. KIAT RUXRUNGTHAM, M.D., 49 pp.

Introduction: HIV patients have a higher risk of drug allergy than general population. Nevirapine (NVP) is one of the commonly prescribed NNRTIs in Thailand and other developing countries. Approximately 20% of patients developed NVP-rash, 2-5% has hypersensitivity reaction (fever, rash, hepatitis) and approximately 0.3% has Steven-Johnson syndrome (SJS) or Toxic epidermal necrolysis (TEN). Multiple drug exposure in advanced immunocompromised patients has made the diagnosis of allergy to what culprit drug is much more challenging. There is no any gold standard test to assist the diagnosis, thus we examined a flowcytometry-based drug-specific CD134+/CD25+ T cell assay and also investigated the roles of adding MDCs to enhance the sensitivity of this T cell assay.

Methods: Peripheral blood mononuclear cell (PBMC) samples from 10 HIV-infected patients who had clinical allergic to nevirapine (median duration after NVP-allergy was 31 months, range 1-99 months) and from 10 control healthy donors and from other 10 HIV+ patients subjects who were treated with nevirapine but had no clinical hypersensitivity, were tested *in vitro* for NVP-induced CD134+/CD25+ up regulation of CD4+ T cells. The assays were also compared between adding and not adding MDCs as antigen presentating cells on the sensitivity of the responses to NVP.

Results: With and without MDCs added, NVP induced positive CD134+/CD25+/CD4+ responses were detected in 7/10 (70%) vs 1/10 (10%) (P=0.003), respectively.

Conclusion: Although other drug-specific T cell *in vitro* tests such as CD69, CD107a, cytokines have been investigated to diagnose T-cell mediated drug allergy, this is the first evidence to support the potential role of CD134 or OX40 expression in drug-specific T cell test. Our results also supported the useful role of MDCs to enhance the sensitivity of *in vitro* drug-specific T cell test particularly in patients who have history of the specific drug allergy for years.

Field of Study : Medical Science Student's Signature .....

Academic Year : 2011 Advisor's Signature .....

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LISTS OF ABBREVIATIONS

%	Percent
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µl	Microlitre
µM	Micromolar
ºC	Degree Celsius
-ve	negative result
+ve	positive result
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cm^2	Centimeter
DMSO	Dimethyl sulfoxide
DTH	Delay type hypersensitivity
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
GM-CSF	Granulocyte-macrophage colony-stimulating Factor

ICS	Intra cellular cytokine staining assay
IgG	Immunoglobulin G
IL-4	Interleukin-4
MDCs	Monocyte derived dendritic cells
ml	Millilitre
mg	Milligram
min	Minute
mM	Millimolar
NSAIDS	Non steroidal anti-inflammatory drugs
PBL	Peripheral blood lymphocyte
PBS	Phosphate-buffered saline
pH	Potential of Hydrogen ion
Rpm	Revolutions per minute
RCM	Radiocontrast media

CHAPTER I

INTRODUCTION

Adverse drug reaction has been classification to two groups 1). Predictable reaction that involved with pharmacologic action of the culprit drug and 2). Unpredictable reaction and is not involved with pharmacologic action. The first group found more common of up to 80% of overall adverse drug reaction(1-2). Drug allergy occurs by immune reaction, either humoral or cellular-mediated immunity or both, to drugs or the metabolite of drugs. Clinical drug allergy or drug sensitivity can occur in various onset that ranged from immediate type to delayed type hypersensitivity. More precisely, drug allergy is a term to specify for any reaction that caused by IgE-specific antibody mediated mechanism or also known as type I hypersensitivity. This type of drug allergy is always associated with immediate onset and may be with life-threatening symptoms such as anaphylaxis. In case of nevirapine, type I hypersensitivity reaction is rare. The most common clinical symptoms of nevirapine drug reactions include skin rashes that can be mild but more commonly will progress Stevens – Johnson Syndrome (SJS) or Toxic epidermal necrolysis (TEN). This type of reactions are mediated by cellular immunity against nevirapine or its metabolites.

In principle, all drugs, as chemical compounds, are monomeric cannot be recognized by T-cells same as other conventional protein antigens. Normally T-cells

recognize antigens by short peptide among 9-15 amino acid, therefore drugs has to change mechanism to be recognized by T-cells. There are three theories to explain how T cells recognize a chemical compound-based drug: 1. Hapten theory: Drugs in a hapten form, cannot recognize by T-cells, will bind with a carrier protein and enhance T cell recognition. 2. Pro-hapten theory: Some drugs need to be metabolized and will then bind to a carrier protein to be able to stimulate T-cells. 3. Pharmacological interaction with immune receptors: Some part of the drugs molecule may be able to bind to T-cell receptors and stimulate T-cells.(3-5)

To diagnose drug allergy and drug hypersensitivity reaction, clinical diagnosis based on history taking is commonly use. However, this approach is not applicable in patients who have been initiated and taken multiple drugs prior to the reaction(s) particularly it is a common setting among HIV patients with low CD4+ T cell count. In vitro test is therefore essential and relevant for patients in such settings. The advantages of drugs allergy test are 1. To prove of drug allergic reaction. 2. To find out the causative drug. Drug allergy test can be done both *in-vivo* and *in-vitro* test, but *in-vivo* test such as pick test and patch test have often been shown with low sensitivity to diagnose most type I and type IV drug hypersensitivity. Some advantages of the *In-vitro* test including it is safe, it prevent re-sensitization in patients, and can use with many types of specimens (depend on the assays) such as whole blood, serum, plasma or urine.(6-8)

For nevirapine (NVP) drug hypersensitivity which mainly caused by CMI or type IV hypersensitivity, in vitro test to detect NVP-specific T cell responses is needed. Currently there 4 methods have been reported for to assess T-cells specific response *in vitro*:

1. Flow cytometer analysis of different surface markers
2. Measurement of cytokine produce
3. Cell proliferation assays
4. Cytotoxic assays

However these drugs specific in-vitro tests have different specificity and sensitivity. Currently there is no any gold standard test to assist the diagnosis. In this study we examined the role of CD134 in the flowcytometry-based drug-specific T-cell assay and the role of monocyte-derived dendritice cells(MDCs)to enhance the sensitivity of this T-cell assay particular in patients who have a long duration after their last exposure to the culprit agent

CHAPTER II

OBJECTIVES

The objectives of this study were:

1. To evaluate nevirapine induced CD134+/CD25+ up regulation of CD4+ T-cells compared with cell proliferation assay by measuring CFSE dye dilution in HIV-infected patients with history of nevirapine drug reactions.

2. To investigate the roles of monocyte-derived dendritic cells in antigen presentation to improve the sensitivity of the responses to nevirapine among patients who have history of nevirapine drug reactions

CHAPTER III

LITERATURE REVIEW

Nevirapine

Since 1996nevirapine or dipyridodiazepinone(Fig1), a antiretroviral drugs in the non-nucleoside reverse transcriptase inhibitor (NNRTI) group has been approved for clinicaluse to treat an HIV patients. Nevirapine targets at the HIV-1 not HIV-2 reversetranscriptase enzyme (RT) by acting as an irreversible non-competitive inhibitor. Therefore drug in this group were strongly active than nucleoside reverse transcriptase inhibitor group (NRTI). Nevirapine is still widely prescribing as a first line antiretroviral therapy regimens in many developing countries including Thailand due to its low cost.

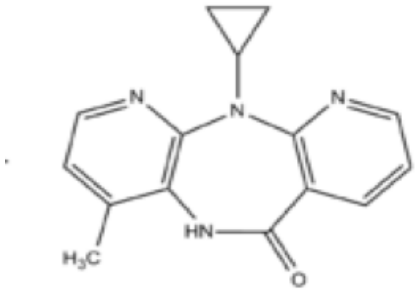


Figure 1. Chemical structure of nevirapine

Adverse events of nevirapine

HIV patients have a higher risk of drug hypersensitivity of up to 100 times than general population. Nevirapine (NVP) is one of the most common causes of drug allergy among HIV-infected patients. Navirapine has been reported to induce skin rashes and/or liver toxicity. Skin rashes found in 16% of patients. Approximately 2-5% had hypersensitivity reaction (fever, rash, hepatitis) and 0.3% has Steven-Johnson syndrome (SJS) or Toxic epidermal necrolysis (TEN). Reports of liver toxicity were found lower than skin rash which is 2.8%.(9-10)

Delayed-type hypersensitivity (Type IV)

Delayed-type hypersensitivity is one of four types of hypersensitivity (Table 1). In general the onset of symptoms (most common including maculopapular rash, but can be severe if progresses to life-threatening SJS or TEN) will take 7-10 days for the primary exposure or approximately 2 days for the subsequent exposure, therefore it is defined as delayed-type drug reaction. This type of reaction is mediated by either drug-specific CD4+ T cells or CD8+ T cells or both. More recent literatures have proposed type IV into 4 different subcategories: IVa, b, c and d. Type IVa and IVb are primarily mediated by CD4+ T cell which is Th1 and Th2, respectively, Type IVc is mediated mainly by CD8+ T cells or cytotoxic T cells. Type IVd is a Th17 CD4+ T cell-mediated and therefore predominantly associated with neutrophil infiltration.

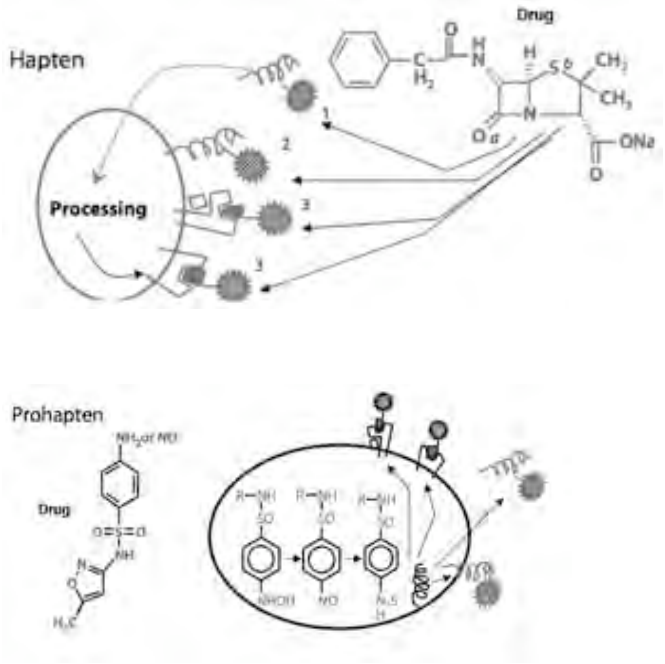
Table 1. Difference type of delayed type hypersensitivity.

	Type I	Type II	Type III	Type IV
Immune reactant	IgE	IgG	IgG	IFn-γ, TNF-α, IL-5, IL-4, Perforin/ granzyme B
Antigen	Soluble antigens	Cell or matrix associated antigens	Soluble antigens	Cell-associated antigen or direct T-cell stimulation
Effectors	Mast cells	Phagocytes, NK cells	Fc receptor+ cells/complement	T-cells
Reactions	Allergic rhinitis, asthma, systemic anaphylaxis	Hemolytic anemia, thrombocytopenia	Serum sickness, Arthus reaction	Contact dermatitis, Maculopapular and bullous exanthema, Hepatitis

Mechanism were occur after receives antigen again, reaction will occur in 10 hours after receive then highest at 24-72 hours. Pathology of this type occur by re-stimulation of antigen with T lymphocyte after that T lymphocyte were change to blast

cell proliferation and cytokine release especially chemotactic factor and Macrophage migration inhibitory factorcause of lymphocyte and macrophage congregate then tissue were damage by lysosomal enzymes and lymphotoxin from lymphocyte.

All drugs are chemical products cannot recognize by T-cells. T-cells normally recognize antigens by short peptide among 9-15 amino acid, therefore drugs has to change mechanism to recognize by T-cells. There are three theories to explain how drugs can recognized by T-cells 1. Hapten: Drugs in hapten form cannot recognize by T-cells will bind with protein and enhance T-cell recognition. 2. Pro hapten: Some drugs need to metabolize and bind protein then present with APCs to stimulate T-cells. 3. Pharmacological interaction with immune receptors: Drugs design some part similar with protein or enzyme can be bind T-cell receptors and stimulate T-cells (Fig 2).(1-2, 11-12)



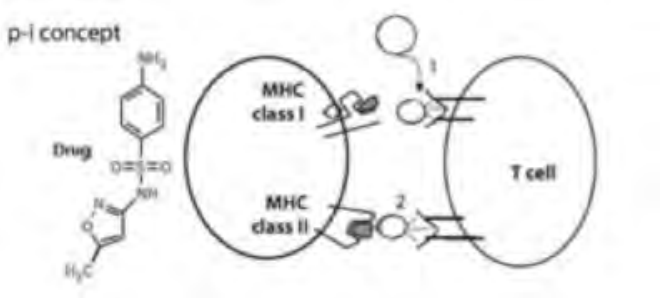


Figure 2. Theories to explain how drugs can recognize by T-cells.(1-2, 11-12)

Drug hypersensitivity test

Many drugs can induce hypersensitivity such as Anaphylaxis, Urticaria, Rhinitis, Hemolytic anemia, Thrombocytopenia, Maculopapular. Thereby advantage of drugs allergy test are 1. To prove true drug allergic reaction. 2. To find out drug cause of adverse reaction. Drug allergy test can use both *In-vivo* and *In-vitro* test, but *in-vivo* test such as pick test often show negative results in patients who allergic with drug many times or differentiation of immune presentation. *In-vitro* test are safe and prevent re-sensitization in patients and can use many type of specimens such as whole blood, serum, plasma or urine by test with specimen directly or stimulate patient cells.(4-5, 13-14)

In-vitro test for T-cells specific response to drug are principle use in four methods.

1. Flow cytometer analysis of different surface markers
2. Measurement of cytokine produce
3. Cell proliferation assays
4. Cytotoxic assays

The gold standard test for *in vitro* drug test is cell proliferation assay, this assay perform by investigated specific cell proliferation to suspect drug. But this test has been used for long time and used 6-7 days for turnaround time and give only 70% of sensitivity.(15-17) Thereby we perform the new approach for drug specific in vitro test.

Table 2. *In vitro* test of drug specific T-cell response

Test	Measured	Methods
Flow cytometer analysis of different surface markers	Surface marker eg. CD25, CD69, CD40L, CD70, CD134and HLA-DR	Flowcytometer
Measurement of cytokine produced	Cytokine release eg. IFN-g, IL-2, TNF-a and IL-4	PCR, RT-PCR, ELISA, ELISpot or Flowcytometer
Cell proliferation assays	Proliferated cells	H_3 thymidine, CFSE dye dilution
Cytotoxicity assay	Cytotoxic T cell kill target cell	Cytotoxicity assay, PCR

Dendritic cells

Dendritic cells are immune cells play role as antigen presenting cells process an antigen then present on the surface to other immune cells. Dendritic cellsat advancement stage they appeared pseudopodscalls “dendrite” of name dendritic cells. Dendritic cells are found with small amount in tissues mainly the skin called Langerhans cells. They can also be found in an immature state in the blood. At activated stage dendritic cells were migrate to lymphoid tissue, interaction with T lymphocyte and B-lymphocyte to induce adaptive immune response.

Dendritic cells in-vivo has common 2 types are Plamacytoid and Myeloid (Table 3).Dendritic cells can also generate by *in-vitro* cultured, used as antigen presenting cells for many research experiments even though they are not show behavior as dendritic cells from *in-vivo*.(18-19)

Dendritic cells played role in drug hypersensitivity through parent's drugs or their metabolites bind protein then process and presented by antigen presenting cells such as dendritic cells. Normal dendritic cells in peripheral are immature with low surface markers and incompetence to stimulate T lymphocyte. Dendritic cells maturation induced by specific antigen, they were up-regulate co stimulatory molecules then migrate to lymphoid tissue. Mature dendritic cells were present antigen to T lymphocyte as danger signals.(20-21) A recent study of dendritic cells in drug hypersensitivity to β-lactamshas shown dendritic cells maturation and increase activation markers. Furthermore used mature dendritic cells as antigen-presenting cells can induce higher up-regulation of activation marker and cell proliferation of T lymphocyte in β-lactam hypersensitivity patients than used other antigen presenting cells such as B lymphocyte or monocyte.(21)

Table 3. Differential types of dendritic cells.

Type	Description	Secretion	Toll-like receptors
Myeloid dendritic cell	Most similar to monocytes. mDC are made up of at least two subsets: (1) the more common mDC-1, which is a major stimulator of T cells (2) the extremely rare mDC-2, which may have a function in fighting wound infection	IL-12	TLR 2, TLR 4
Plasmacytoiddendritic cell	Look like plasma cells, but have certain characteristics similar to myeloid dendritic cells.	Produce high amounts of interferon-alpha and thus became known as IPC (interferon-producing cells) before their dendritic cell nature was revealed.	TLR 7, TLR 9

CD134 or OX-40

CD134 or OX-40 is an activation marker, a member of tumor necrosis factor receptors, It is found in both CD4+ T lymphocyte and CD8+ T lymphocyte, but not found in naïve or resting memory T lymphocyte(22). CD134 is up regulation after a stimulation since first hour and is highest at 24 hours till 5 days(22-24). CD134 up regulation were reports to help CD4+ T lymphocyte expansion and survival of both CD4+ T lymphocyte and memory T-cells.(24)De Smedt T. etal found relationship between CD134 and dendritic cells in T lymphocyte stimulation, due to naïve T cells stimulated through dendritic cells enhance response when stimulated with OX-40 ligand.(25)Zaunders J. et al have demonstrated that CD134 can be used as an activation marker of memory CD4+ T lymphocyte to detect infectious antigen specific responses. The proposed test was found comparable with the lymphoproliferation assay and intra cellular cytokine staining assay. CD134 up-regulation test gave response be equal to cell proliferation assay but more rapidly and gave highest response than Intra Cytokine Staining (ICS).(26)

Table 4. Comparison of *in vitro* tests to detect delayed drug hypersensitivity(13)

Test (functional phenotypic marker of DHR)	Method (readout systems)	Duration (day)	Advantages	Disadvantage	Remarks
LTT (proliferation of drug-specific T cells)	3H-thymidine incorporation harvesting radioactivity, Measurement -CSFE or non-reactive methods also possible	6-7	Many drugs and drug concentrations testable; automation and many replicates possible	Long-lasting; radioactivity and expensive equipment; experience needed; not sensitive in SJS/TEN	Most widely used test; with well-documented sensitivity ; best for DRESS, severe MPE
CD69 up-regulation on cell surface	Flow cytometry	2-3	Rapid; identification of reactive cell subset	Flow cytometry is difficult to standardize; automation would need improvement	Results are similar to LTT
Cytokine synthesis and secretion (e.g. IL-2, IL-5, IL-13, IFN-g)	ELISA, ELISPOT, flow cytometry	2-3	Rapid; sensitive; informative regarding patho-mechanism	Expensive, minimally 2 cytokines needed; specificity lower (dependent on cytokines)	May be most sensitive, but reduced specificity
Cytotoxicity (perforin, granzyme B, granulysin, CD107a)	ELISA, ELISPOT, flow cytometry	3	Rapid; may address additional mechanism	Sensitivity in severe DHRs needs more evaluation	Possibly suitable for severe reactions; CD8 and NK cells focused

CHAPTER IV

MATERIALS AND METHODS

Patients And Controls

Patients from the King Chulalongkorn Memorial Hospital and Thai Red Cross Research Center with clinical history of nevirapine delayed-type hypersensitivity were enrolled and their blood samples were studied for nevirapine-specific cell proliferation assay and CD134 up regulation with and without dendritic cells pulsed. The study was reviewed by the Faculty of Medicine, Chulalongkorn University IRB. All patients provided a signed written informed consent.

Inclusion criteria

Adult patients (age more than 20 years) with history in nevirapine delayed- type hypersensitivity and had current CD4 above 100 cells /mm^2.

Exclusion criteria

Patients had current CD4 below 100 cells /mm^2.

Patients who have only the clinical reaction of type I hypersensitivity

Sample size

A total of 10patients with history of nevirapine-delayed type hypersensitivity attending at King Chulalongkorn Memorial Hospital were included in the study as experimental group. And 10 control healthy donor and from other 10 HIV+ patients subjects who were treated with nevirapine with no clinical hypersensitivity were tested in

vitro for NVP-induced CD134+/CD25+ up-regulation of CD4+ T cells and CFSE dye dilution assay. The assays were also compared between adding and not adding MDCs as antigen presentation on the sensitivity of the responses to NVP.

Nevirapine

This studies were used nevirapine diluted in phosphate buffered saline final concentration at 3 µg/ml and 6 µg/ml

Peripheral blood mononuclear cell isolation

Collected fresh blood from each patient among 40 mL in ACD-C tube. PBMCs were isolated from fresh blood by Ficoll–Hypaque density gradient centrifugation. Briefly, treated tube was diluted with equal volume of RPMI-1640 and transferred to a 50 conical ml tube. Then, it was underlayed with 13 milliliters (ml) of IsoPrep in diluted blood. Next, the tube was centrifuged at 1,500 rpm for 30 minutes at 22ºC with no intervention. After centrifugation, Cells from lymphocyte layer were collected and washed two times with RPMI-1640 by centrifugation at 2,000 rpm for 10 minutes at room temperature.

Dendritic cells separation and generation

20 x 10^6 cells of PBMC, was adjusted to 1 mL of 5% fetal bovine serum in IMDM medium plated in 6 wells plate. Culture mononuclear cells for 2 hours at 37^oC 5% CO_2.Monocyte were adherent on tissue culture plate, Non-adherence peripheral lymphocyte (PBL) were washed out carefully, then the adhered monocytes were collected and freezed at liquid nitrogen for use later. MDCs were generated by incubating the thawed monocytes with recombinant human GM-CSF (150 ng/ml) and recombinant human IL-4 (150 ng/ml) at 37^o C in 5% CO_2 incubator for 5 days. Immature

monocyte derived dendritic cells (MDCS) were heaves and check phenotypic of MDCS. MDCs were stain with fluorescents monoclonal antibody to CD83-FITC, CD80-PE, CD209-PE-cy5, and CD11c-APC. After that immature MDCs were pulse for 48 hours with Nevirapine at final concentration 3, 6 µg/mL and phytohemagglutinin (PHA), Staphylococcal Enterotoxin B (SEB) as positive controls and cytomegalovirus,Epstein–Barr virus, Flu truncated peptide (CEF peptide) plus CMV lysate as a recall memory antigen.

CD134 up regulation assay

Freeze non-adhered PBL were thaw in 37^{O}C gently drop 10% of fetal bovine serum in RPMI 1640 medium and mixed all process must be on ice. Centrifuge 1,500 RPM at 4 ^{O}C for 5 minutes then pour supernatant and re-suspend with 10% of fetal bovine serum in RPMI 1640 medium leave cell solution at least 30 minutes. Count number of cells then seed cell at 200,000 cells per well in 96 wells U bottom plate. Added navirapine, PHA, SEB, CMV lysate treated MDCs in each well with ration of MDC per peripheral blood lymphocyte equal 1:10 or navirapine, PHA, SEB, CMV lysate directly used same amount with stimulate dendritic cells culture for 2 days at 37^{o} C in 5% CO2 incubator. Mixed cells were remove at day 2 after stimulation by pipette to BD Falcon 12x75 mm Tube, wash with washing buffer then centrifuge at 1,500 rpm, room temperature for 10 minutes. Discard the supernatant cells were then stained with monoclonal anti-body CD4-PerCP, CD134-PE and CD25-APC and analyze by flowcytometer.

CFSE dye dilution assay

Resuspended lymphocytes, at a concentration of 1-5×10^6 cells in 1 ml PBS containing 5% (v/v) heat-inactivated fetal calf serum (HI-FCS).Dilute CFSE either directly into the cell suspension or prepare a dilution in aqueous solution and add this in an appropriate volume to give a final concentration of 3 µM/ml of cell suspension. Recommended diluting 10 mM CFSE Stock solution in DMSO with PBS to acquire 1 mM CFSE in PBS, then pipette 3 µl of 1 mM CFSE in 1 ml of cell's suspension. Mix immediately on a vortex for 4 minutes, the vortex speed should not be lower than a half of maximal speed to ensure single cell suspension, but should not exceed two-third of it to ensure viability of cells. After thorough mixing, leave cells to label with CFSE by incubating for 4 min at room temperature. Exposure to light should be avoided.

Optimum labeling of the cells is complete within only a few minutes at room temperature, and incubation for longer periods can result in toxicity to cells that can affect cell proliferation. Wash the cells by diluting with ten volumes of PBS containing 20% (v/v) HI-FCS, sediment by centrifugation at 1500 rpm for 5 min at 4 °C and discard the supernatant. Wash the cell pellet in the same manner at least one more time. Re-suspend with 10% of fetal bovine serum in RPMI 1640 medium. Count number of cells then seed cell at 200,000 cells per well in 96 wells U bottom plate. Added navirapine, PHA, SEB, CMV lysate treated MDCs in each well with ration of MDC per peripheral blood lymphocyte equal 1:10 or navirapine, PHA, SEB, CMV lysate directly used same amount with stimulate dendritic cells culture for 2 days at 37^o C in 5% CO2 incubator.

Statistics

All patient and donors data were comparing responses percentage between added and non MDCs added. A nonparametric Wilcoxon rank sum test were used for subgroup analysis.A p value of less than 0.05 was considered statistically significant.

CHAPTER V

RESULTS

Patient and control subjects

A total of 10HIV-infected patients with history of nevirapine induced delayed type hypersensitivity, 10 control healthy donors and the other 10 HIV+ patients who were well tolerated to nevirapine were included from the King Chulalongkorn Memorial Hospital.

Table 5. Characteristics of the study population

Characteristics	Patients with nevirapine allergy	Patients without nevirapine allergy	Controls
Number of subject	10	10	10
Male/Female	3/7	8/2	5/5
Age, years (Mean ± SD)	38.3 ± 9.06	40 ± 6.03	45.4 ± 11.83
Time interval (month) after navirapine hypersensitivity	31	NA	NA

Table 6. Detail characteristics of each patient

Subjects	Sex	HIV status	CD4 (cells/mm2)	Age	**Time interval** (month) After navirapine hypersensitivity
1	M	Positive	244	29	20
2	F	Positive	795	44	64
3	F	Positive	303	27	24
4	F	Positive	503	48	31
5	F	Positive	999	29	99
6	M	Positive	302	38	45
7	F	Positive	371	29	2
8	M	Positive	269	42	1
9	F	Positive	372	47	59
10	F	Positive	655	50	31
11	M	Positive	266	38	NA
12	M	Positive	396	42	NA
13	M	Positive	596	40	NA
14	M	Positive	408	37	NA
15	F	Positive	155	42	NA
16	M	Positive	192	31	NA
17	M	Positive	403	43	NA
18	M	Positive	801	50	NA
19	M	Positive	330	31	NA
20	F	Positive	520	46	NA
21	M	Negative	NA	57	NA
22	F	Negative	NA	58	NA
23	M	Negative	NA	56	NA
24	F	Negative	NA	36	NA
25	F	Negative	NA	34	NA
26	M	Negative	NA	45	NA
27	M	Negative	NA	48	NA
28	M	Negative	NA	50	NA
29	F	Negative	NA	21	NA

Subjects	Sex	HIV status	CD4 (cells/mm2)	Age	Time interval (month) After navirapine hypersensitivity
29	F	Negative	NA	21	NA
30	F	Negative	NA	49	NA

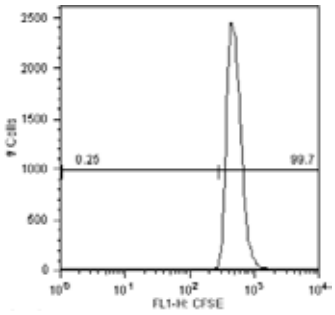
CFSE Dye dilution assay set up

To define the positive cut-off for this assay, the lymphoproliferative results after stimulated with PHA were expressed in percentage of cells division histogram plot using the FlowJo program (Tree Star,Inc. Asland). To optimize CFSE dye dilution test in term of incubation time, cells were harvested to analyzed at day 3, 4 and 5 compared with the baseline at day 0.

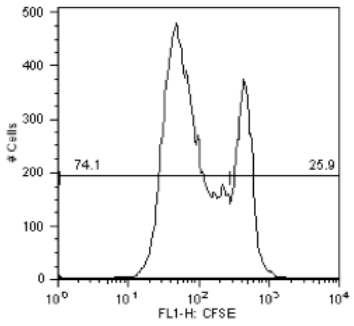
In figure 3-4, the results showed that PHA stimulation could increase the level of cell proliferation in 5 day higher than others, i.e., 74%, 82.8% and 86.3% of proliferating cells were obtained, respectively.

Figure 3. Percentage of CFSE proliferating cells after stimulated with PHA mitogen for 3-5 days

Day 0



Day 3



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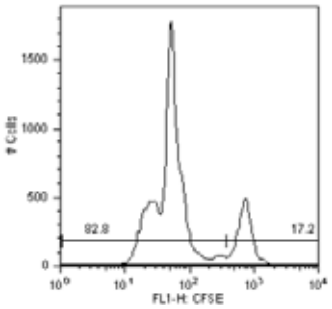

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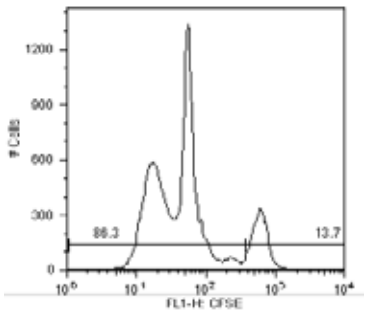


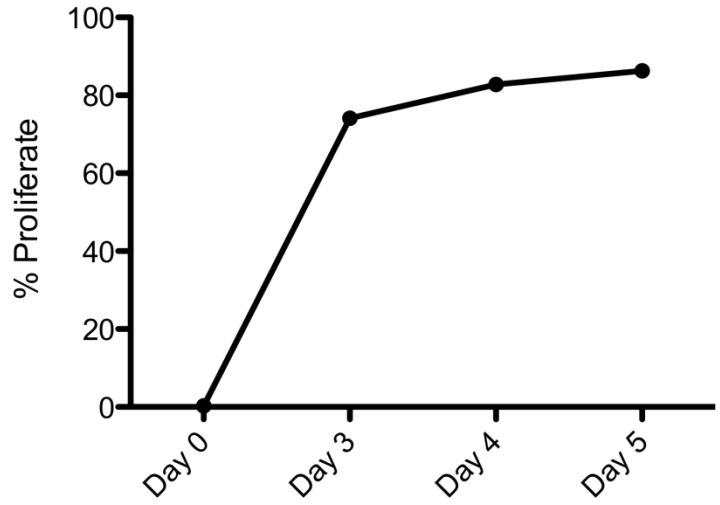
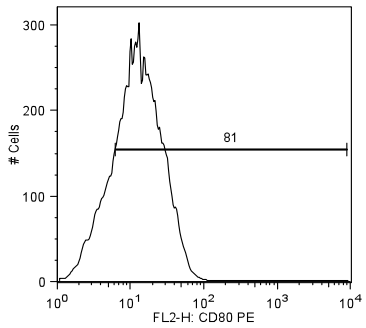


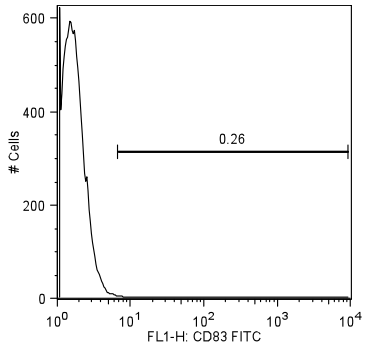
Figure 4. The kinetic profile of CFSE-stained cell proliferation after stimulated with PHA

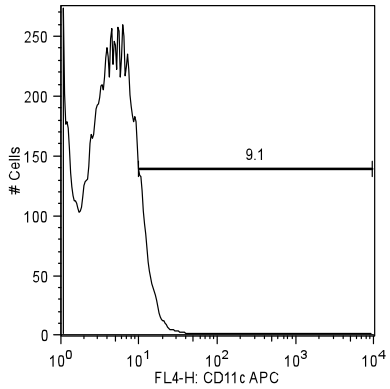
Phenotypic changes of MDCs by Nevirapine

To study whether nevirapine induces any phenotypic changes on imDCs in subjects who had a T-cell mediated nevirapine DTH response, nevirapine was added to into the culture media. Results showed that a increase of the expressions of DC maturation and activation markers i.e., CD80, CD83 and CD11c.(Fig 5 A and B) Furthermore, nevirapine-treated imDCs obtained a higher T-cell stimulatory capacity, illustrated by in- creased CD134 up regulation of allogeneic T-cells.

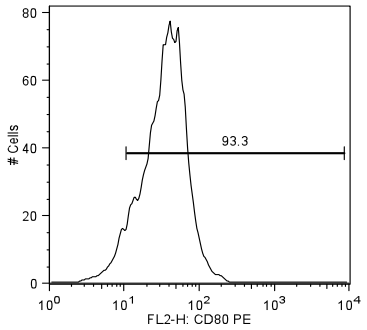
MDCs + Culture Medium

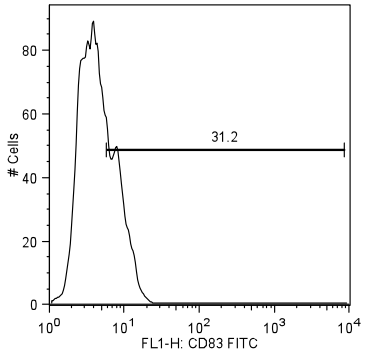


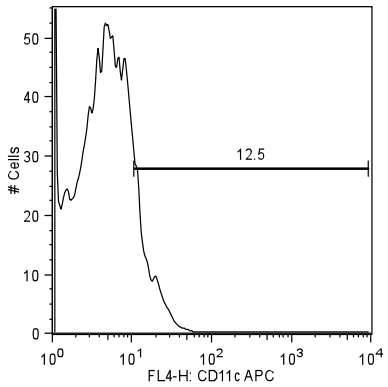




MDCs + Nevirapine (6µm)







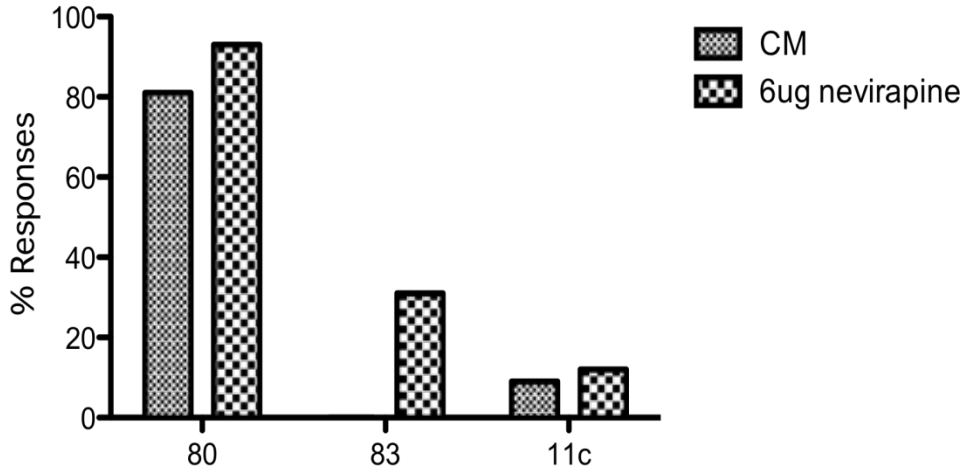


Figure 5. MDCs phenotypic change after stimulated with nevirapine. (CM= culture media only)

Lymphoproliferation test with nevirapine using the CFSE dye dilution assay

Lymphoproliferation measurement by the use of CFSE dye dilution assay as a gold standard were performed. PBMC samples were stimulated with nevirapine, and two positive control reagents: PHA, SEB and recall memory antigens CMV, EBV, Flu Truncated peptide plus CMV lysate, and results were expressed asa proliferation index (FlowJo program was used for the calculation). The positive cut-off was defined as mean +2 standard deviation of the negative samples. In this case, the cut-off proliferation index is 2.

As shown in figure 8 and 9, none of the HIV+ patients who were well tolerated to NVP had shown any positive responses to NVP either in assay of which had or had no MDCs included in the culture systems. However, in figure 10 and 9, 1 of 10 (10%) NVP-hypersensitivity HIV+ patients has a positive result in respond to 6 μg/ml of nevirapine with no MDCs added.In the parallel experiments with MDCs added, there were 7 of 10 or 70% of patients showed positive results. The specificity of this test was 100%.

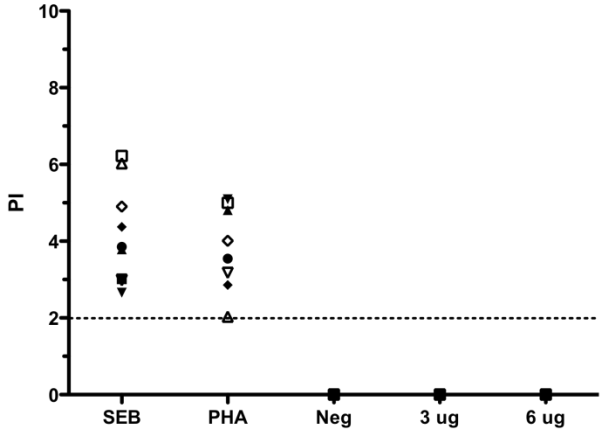


Figure 6. CFSE assay results of healthy volunteers. This assay was without MDCs added into the culture system while stimulating with nevirapine for 5 days.

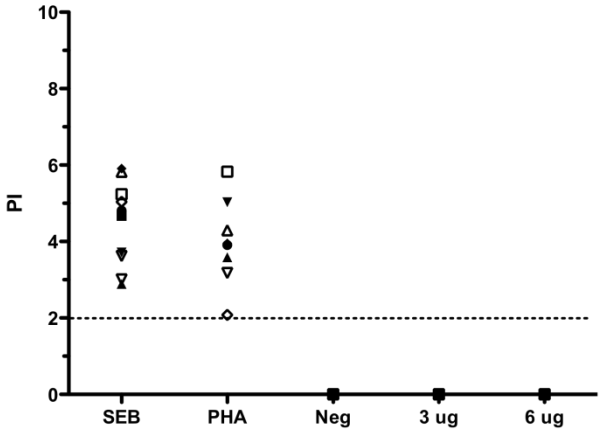


Figure 7. CFSE assay results of healthy volunteers. This assay was MDCs added into the culture system while stimulating with nevirapine for 5 days.

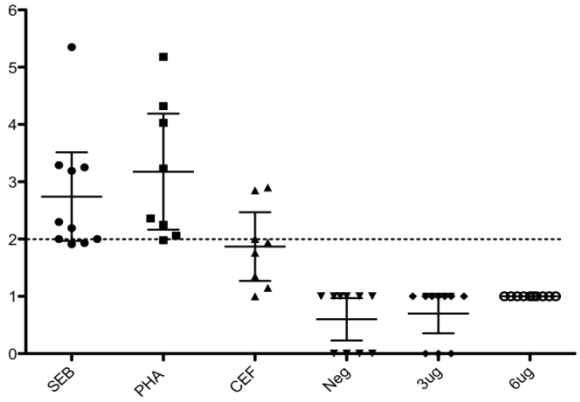


Figure 8. CFSE assay results of nevirapine-well tolerated patients. This assay was without MDCs added.

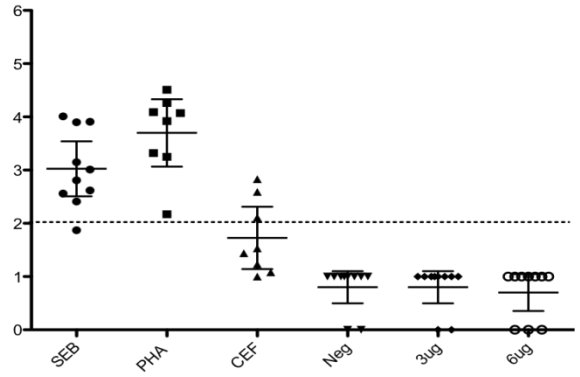


Figure 9. CFSE assay results of nevirapine-well tolerated patients the results were based on the assay that MDCs were added.

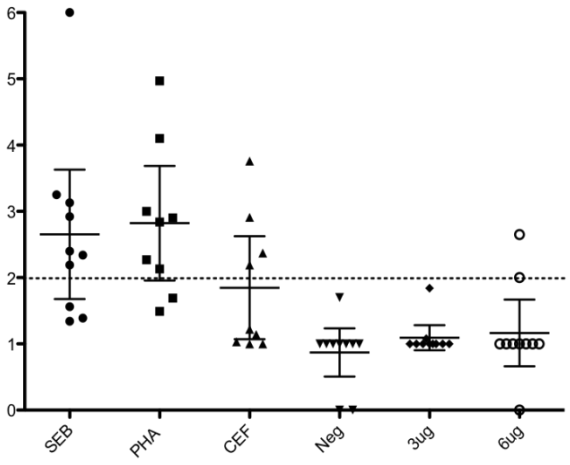


Figure10. CFSE assay results of nevirapine-hypersentive HIV+ patients when MDCs were not added into the culture system.

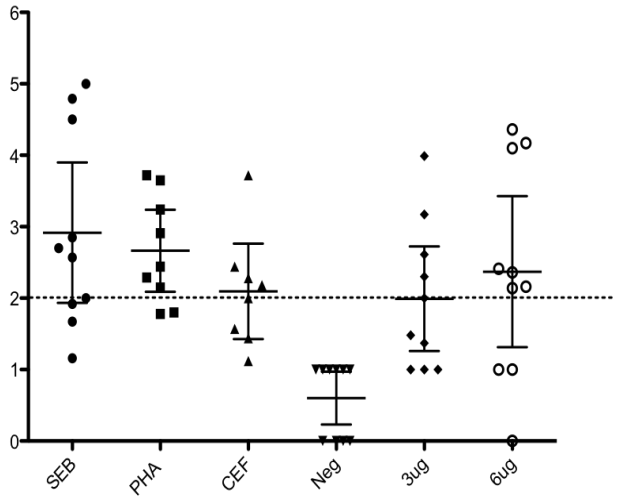


Figure 11. CFSE assay results of nevirapine-hypersentive HIV+ patients when MDCs were not added into the culture system.

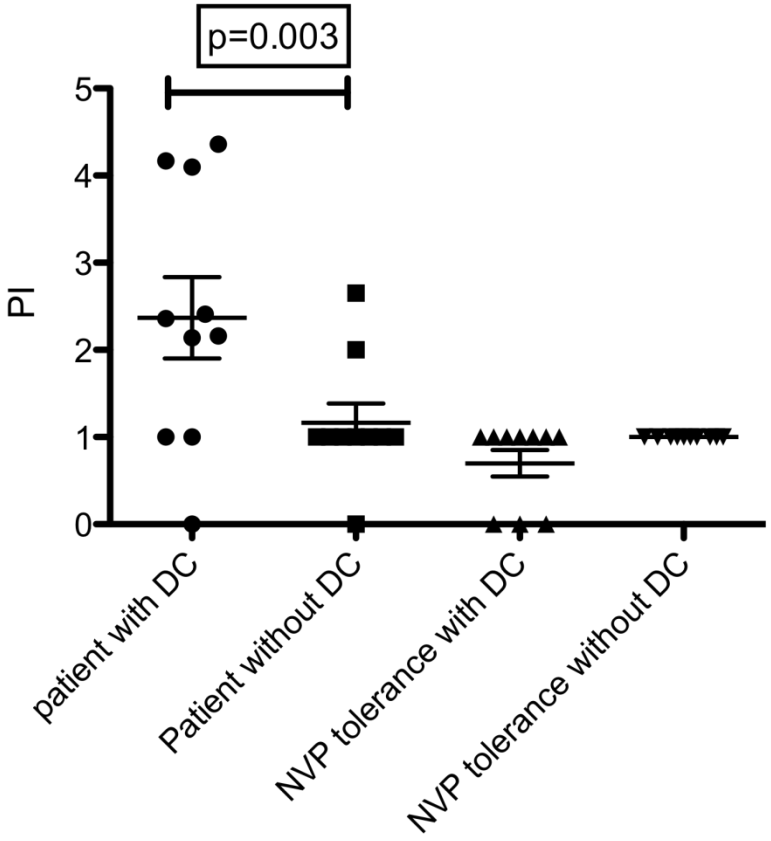


Figure 12. Comparative analyses of the results between the NVP-hypersensitivity and NVP-well tolerated HIV+ patients, and also between without and with MDCs in the culture system

Nevirapine specific T-cells test using the CD134 expression assay

Positive cut off for CD134 expression test were established based on the dataset of 20 control subjects (10 were healthy, 10 were HIV+ who were well tolerated when taking nevirapine) and the cut-off defined by the % CD134+/CD25+/CD4+ that greater than the mean+ 2SD values of this control dataset which was 0.49%. Similar to the finding when using CFSE test, all HIV+ patients who were well tolerated to NVP clinically showed no any positive response to NVP stimulation regardless of having or not having NVP-pulsed MDCs included in the culture system. (Figure 15 and 16)

For patients who had NVP hypersensitivity, the results showed in figure 17, only 1 of 10 (10%) considered as having a positive CD134+/CD25+/CD4+ response to 6 µg/ml (but not to 3 µg/ml) of nevirapine when the test did not include NVP-pulsed MDCs. When NVP-pulsed MDCs were added into the system, 7 of 10 (70%) patients found to have a positive NVP responsesat 6 µg/ml concentration (p value = 0.003) (Fig 19). At 3 µg/mL of nevirapine assay, 6 of 10 patients (60%)showed a positive result. For those 3 patients who did not response to NVP in both CFSE and CD143 assays, their time interval after the last navirapine hypersensitivity were over 2 years.According to the positivity criteria defined, the results of CD134 measurements were compared between patients and healthy controls, and the sensitivity and specificity of these assays were calculated using their clinical history to nevirapine hypersensitivity as a gold standard. Inthis study, none of the control cases either healthy donors or NVP-well tolerated patients showed any positive results, while 7 out of 10 patients with a history of nevirapine reactions (70%) had increased CD134 expression. Thus the sensitivity of this assay is 70%, and specificity is100%.

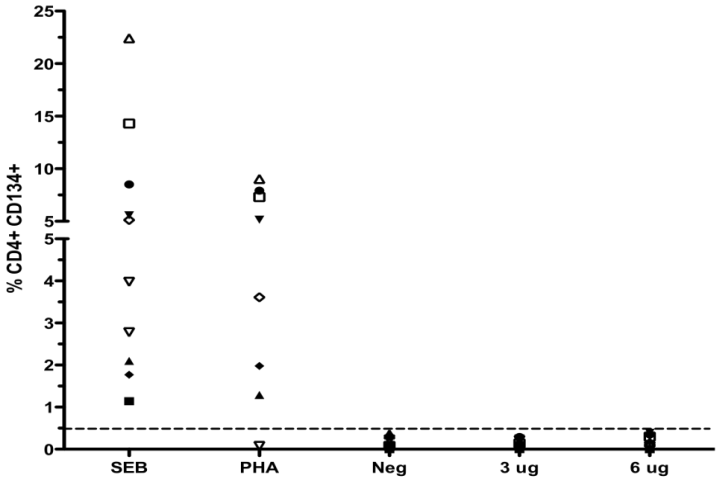


Figure 13. NVP-specific CD134+/CD4+ T-cell response of healthy volunteers without NVP-pulsed MDCs

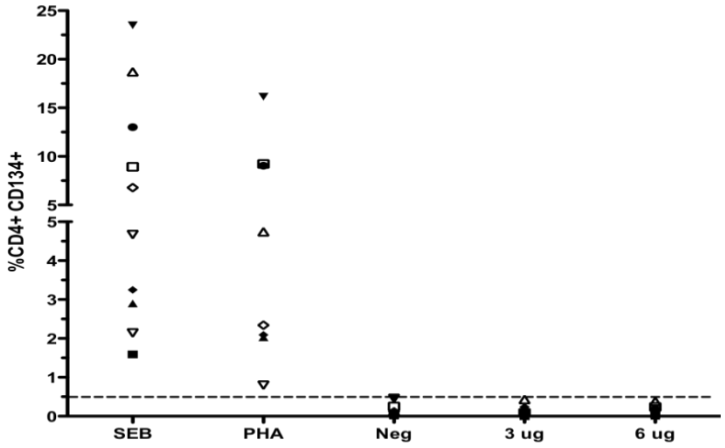


Figure 14. NVP-specific CD134+/CD4+ T-cell response of healthy volunteers with NVP-pulsed MDCs

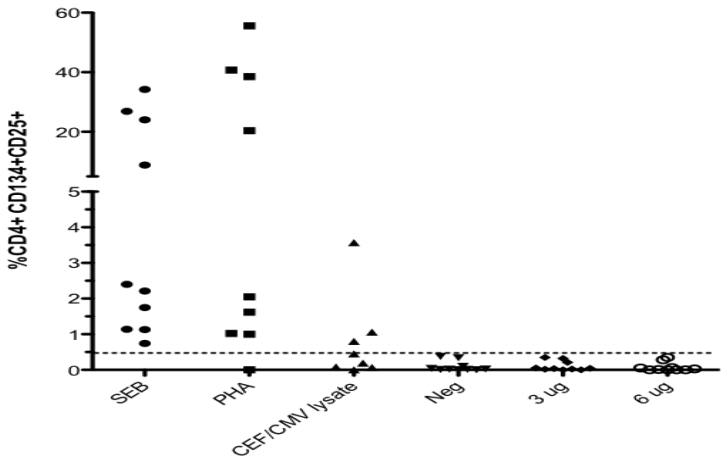


Figure 15. NVP-specific CD134+/CD4+ T-cell response of nevirapine-well tolerated patients without NVP-pulsed MDCs

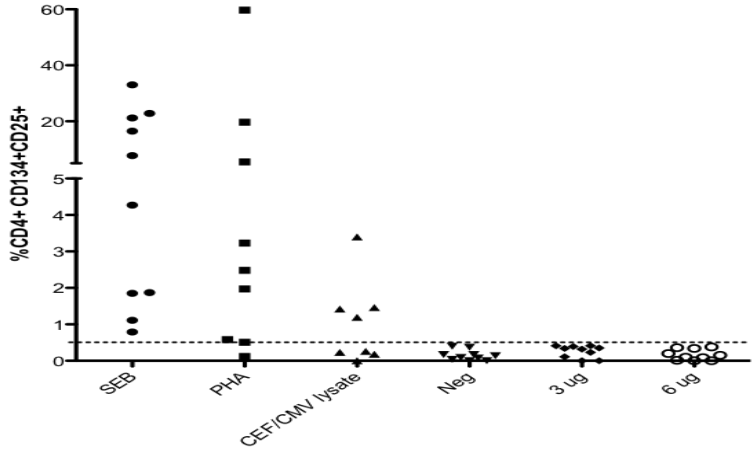


Figure 16. NVP-specific CD134+/CD4+ T-cell response of nevirapine-well tolerated patients with NVP-pulsed MDCs added

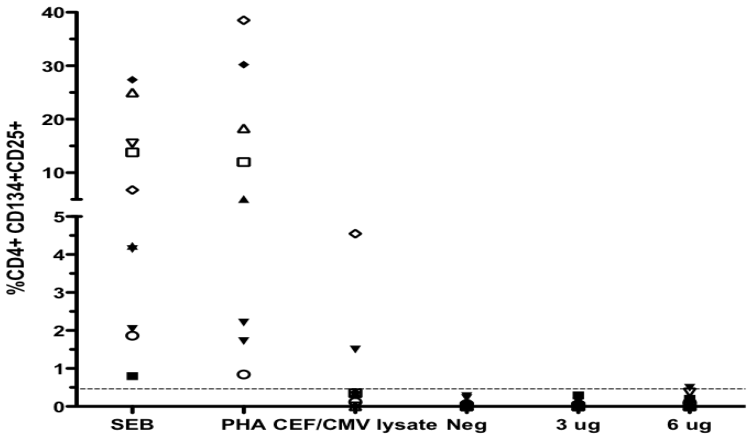


Figure 17. NVP-specific CD134+/CD4+ T-cell response of nevirapine-hypersensitive patients without NVP-pulsed MDCs

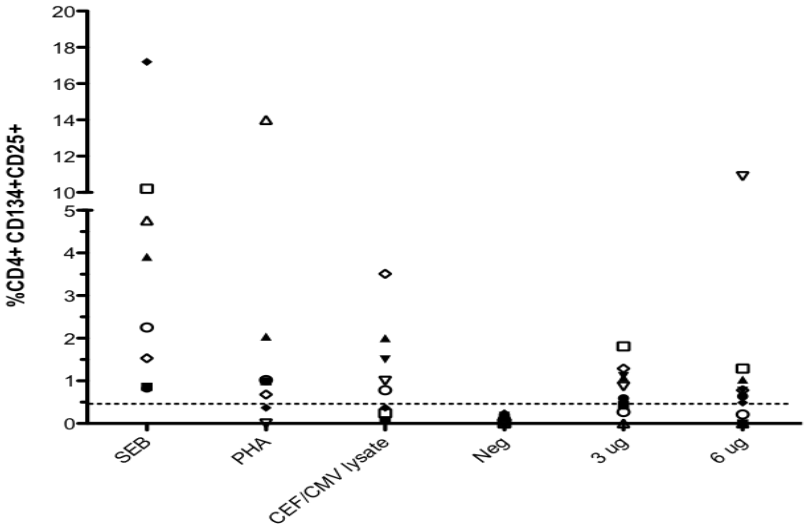


Figure 18. NVP-specific CD134+/CD4+ T-cell response of nevirapine-hypersensitive patients with NVP-pulsed MDCs

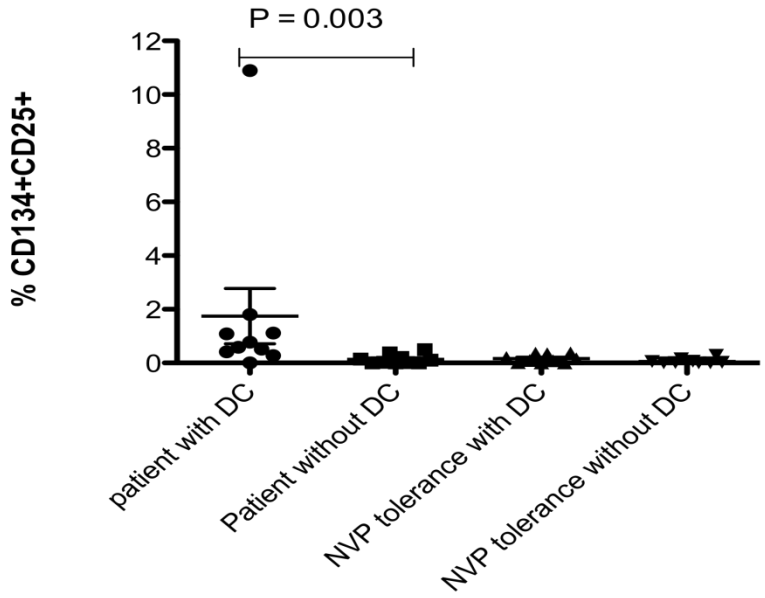


Figure 19. Comparison between the nevirapine-specific CD134+/CD25+/CD4+ responses of nevirapine-hypersentive and well-tolerated patients

Comparison between CFSE dye dilution assays and CD134 up regulation assay

There were no differences in terms of percentage of responses between CFSE dye dilution tests and CD134 up regulation test.

CHAPTER V

Discussions

*In vitro*drug-specific T-cell tests such as the detection of CD69, CD107a, or cytokine expressions have been investigated to diagnose T-cell mediated drug allergy. This is the first evidence to support the potential role of CD134 or OX40 expression in drug-specific T-cells. Our results have also supported a previous report of the useful role of adding antigen presenting cells, in this case MDCs, to enhance the sensitivity of this *in vitro* drug-specific T-cell test particularly in patients who have history of the specific drug allergy for several years.

Previous studies have shown CD134 or OX-40 up-regulation only in stimulated human CD4 T-cell, we have also confirmed in drug allergy testing system. CD4 T-cell both from patient and patients or healthy controls showed response of CD134+/CD25+ after stimulated with mitogen or recall antigens but not in media alone. Stimulated T-cells when activated will produce interleukin 2 (IL-2) and further promote T cell proliferation. Up-regulation of CD25 or IL-2 receptor to is therefore is a one of the useful markers of T cell activation. Normally T-cells show small amount of CD25 up-regulation in human peripheral blood samples, but express in a higher % and density in activated T-cell similar to CD134. Previous report shown results combination up-regulation of CD25, and CD134 as markers for identifying disease-associated memory T-cell after stimulation.J. Endl et al. compared between the expression of CD25 alone and CD25 with CD134

responses from Tetanus toxoidin active memory T cells, they found CD25 and CD134 response higher than when measure CD25 alone.(26-27)

In this study, the nevirapine-specific CD134+/CD25+/CD4+ assay has shown that the specificity and sensitivity based on 10 subjects per group are comparable between this new assay and the CFSE-based assay i.e., none of healthy subjects or NVP-well tolerated subjects had positive responses to NVP regardless of additional NVP-pulsed antigen presenting cells in this case we used monocyte-derived dentritic cells. When the test was performed in nevirapine-hypersenitive patients, adding NVP-pulsed MDCs had significantly enhanced the percent positivity from 10% to 70%. When compared to previous reports on in vitro drug-specific T cell assays, shown in table 7, the enhancement effect of drug-specific-pulsed dendritic cells on the sensitivity of the test have also been found in lymphoprolifraitve test (LLT) and in CFSE assays.(28-29)

Dendritic cells are professional presenting cells, recent study of dendriticcellsin drug hypersensitivity to β-lactams and heparins has shown dendritic cells maturation and increase activation markers. Furthermore used mature dendritic cells as antigen-presenting cells can induce higher up-regulation of activation marker and cell proliferation of T lymphocyte in β-lactam hypersensitivity(28, 29), results were similar with ourexperiment when compared CD134 up-regulation with monocyte derived dendritic cells (MDCs).

In this study, interestingly one of the patients who had history NVP reaction for 99 months, when NVP-pulsed MDCs were added to the assay, we are able to detect his NVP-specific T cell responses using this new CD134/CD25 test.

This CD134-based assay has been validated with the gold standard CFSE dye dilution assays when they studied a number of recall antigens or with mitogen such as PHA, SEB, CEF plus CMV lysate. Although the CFSE dye dilution assay has been used as a gold standard to replace the conventional lymphoproliferation assay in drug specific T-cell in-vitro test for several years, the main limitation of test is the requirement of long duration culture (6 days) and the sensitivity was only 70%. In this study, CD134-based assay while proven comparable to CFSE in term of the specificity and sensitivity, but it required only 2 days instead of 6 days culture. (Table 7)

The other advantages of CD134-based assay is the flexibility of type samples, similar to other surface marker analyses, both whole blood assay or peripheral blood mononuclear cell (PBMC) sample can be used. With whole blood method, it will be more robust to perform the test.

Table 7. Arguments of difference type drug specific T lymphocyte testing (30)

Drug allergy test	Pro	Con	Type of drug	% Responses	
				With MDCs added	Without MDCs added
LTT	Gold standard	Use H^3thymidine (Isotope)	- β-lactam, sulfanilamide, antiepileptic, quinolones	88.8	22.2
CFSE	Has been confirmed with gold standard for drug allergy.	-6 days for culture -Toxic in high concentration	phenytoin	100	NA
CD69	2 day for culture	-Negative results if immune response was mainly directed to a metabolite. -T cell not activated strongly enough and noprevious memory response had been generated.	- β-lactam, sulfanilamide, antiepileptic, quinolones	NA	NA
CD134 (This study)	-2 day for culture -Has been proved with ICS and CFSE in other antigen. -Can use with whole blood	Has not been proved in drug allergy		70	10

Table 8. Arguments ofprevious drug specific T lymphocyte testing and Drug specific CD134up regulation with MDCs used.

	Standard Cell proliferation assays	Drug specific CD134 expression assay
Study	Reported by Rodriguez-Pena R, et al. J Allergy Clin Immunol.2006 Oct;118(4):949-56.	Present study
Assays	Tritiated thymidine used in cell proliferation assays	CD 134 up regulation
Monocyte derived dendritic cells added	Yes	Yes
Population	9 Patients	10 Patients
Drug type	Amoxicillin	Nevirapine
Immune status	Normal	HIV infection, Immunocompromised host (Median CD4= 371.5 cell/mm^2)
Median time interval after the last reaction (months)	132	31 (range 1-99)
% Positive	90	70

Even though the new approach to predict nevirapine induced hypersensitivity by genetic testing such as HLA-B*3505 or HLA-Cw*04 may be useful tests to predict nevirapine hypersensitivity but they are higher cost than other in vitro test i.e., 3,000 bath for 1 locus compared with 1,000 bath with our test.(31-32)

One must take into a consideration in regard to the study population in this study of whom were HIV infected patients, although they all have been treated with antiretroviral therapy for a median of 3.5 years and have CD4 count more than 300 cell/cu.mm, a number of studies have shown that patients may not have a complete immune recovery. Of note, MDCs have been shown can improve memory response in HIV patients to Gag and Nef.(33)It is likely that this CD134-based assay if imply in drug allergy individuals who have normal immune function may provide a better yield on sensitivity which is warranted for further investigation.

CHAPTER VI

Conclusions

This is the first evidence to support the potential role of CD134 or OX40 expression in drug-specific T cell response to be use for the diagnosis of drug allergy. In addition our results have also supported the previous report of the useful role of MDCs to enhance the sensitivity of in vitro drug-specific T cell test particularly in patients who have history the specific drug allergy for years.

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