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Characterization of plasmacytoid dendritic cells in the CD4C/HIV transgenic mouse model

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Université de Montréal
Faculté des études supérieures

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Characterization of plasmacytoid dendritic cells in the CD4C/HIV transgenic mouse model

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CHARACTERIZATION OF PLASMACYTOID DENDRITIC CELLS IN THE CD4C/HIV TRANSGENIC MOUSE MODEL

Plasmacytoid dendritic cells (pDCs), or interferon (IFN) producing cells (IPCs), participate in innate and adaptive immune responses against viral infections. Type I interferons exert antiviral effects by direct inhibition of viral replication and stimulation of a variety of immune cell types. Murine pDCs are $CD11c^{lo}B220^{+}CD11b^{-}Gr1^{lo}IL3R^{lo}LY6C^{hi}$ while human pDCs are $CD4^{+}IL3\text{-}R^{hi}CD45RA^{+}(B220)HLA\text{-}DR^{+}$. They are both identified as cells that show plasmacytoid morphology, and reside in T cell zones of peripheral lymphoid organs and in blood at a low frequency.

To study HIV-1-mediated pathogenesis, the $CD4C/HIV^{mutG}$ transgenic (Tg) mice have been constructed in our laboratory. In this model, no viral infection and replication occurs but the expression of HIV-1 *nef* gene in $CD4^{+}$ T cells and in macrophages/DC lineages induces an AIDS-like disease with several hallmarks of AIDS. These common features include $CD4^{+}$ down-regulation on T cells, depletion of of $CD4^{+}$ T cells, lymphoid tissue atrophy and some non-lymphoid organ pathologies. The immune system in this model is impaired in many aspects and is under investigation. DCs other than pDCs display functional, maturational and homing impairments in these Tg mice.

Here, we show that 25-40% of pDCs express HIV-1 gene *nef* in Tg mice. Compared to non-Tg, Tg-derived pDC show alterations in their frequency, maturation, localization and in vitro response to stimulation. Also, based on preliminary results splenic pDCs from Tg mice produce more IFN-α than their equivalents from nTg mice.

Considering the major immunoregulatory functions of pDCs, the observed changes in their characteristics suggest their implication in the pathogenesis of the AIDS-like disease in CD4C/HIV Tg mice.

Key words: AIDS, IFN-α, Antiviral immunity, Dendritic cells, Nef, Lymphoid organs, CpG, FLT3L.

Caractérisation des cellules dendritiques plasmacytoides chez la souris transgénique CD4C/HIV

Les cellules dendritiques plasmacytoides, aussi appelées cellules productrices d'interferon, participent dans les réponses immunitaires innées et acquises contre les infections virales. Les interferons de type I accomplissent des fonctions antivirales par l'inhibition directe de la réplication virale et par la stimulation de plusieurs types de populations cellulaires du system immunitaire. Le phénotype des cellules dendritiques plasmacytoid chez la souris est $CD11c^{lo}B220^{+}CD11b^{-}Gr1^{lo}IL3R^{lo}LY6C^{hi}$, alors que chez l'homme leur phénotype est $CD4^{+}IL3R^{hi}CD11c^{-}B220^{+}HLA\text{-}DR^{+}$. Les cellules dendritiques plasmacytoid sont identifiées par leur morphologie similaire à celles des plasmocytes autant chez la souris que chez l'homme. Elles représentent un faible pourcentage de la population totale de cellules dendritiques et sont localisées dans les zones de cellules T des organes lymphoïdes périphériques et dans le sang.

Afin d'étudier les mécanismes impliqués dans la pathogenèse du virus d'immunodéficience humaine (VIH), la souris transgénique (Tg) $CD4C/HIV^{mutG}$ qui exprime le gène nef du VIH, a été mise au point dans notre laboratoire. Dans ce modèle murin, il n'y a ni l'infection ni la réplication virale, cependant une maladie ressemblante au SIDA est induite par l'expression du gène *nef* du VIH-1 dans les cellules T $CD4^{+}$ et les cellules des lignées macrophage/dendritique. Cette maladie et le syndrome d'immunodéficience humaine ont plusieurs caractéristiques communes incluant la régulation négative de l'expression de la molécule CD4 à la surface des cellules T, la déplétion des cellules T $CD4^{+}$, l'atrophie des tissus lymphatiques ainsi que des maladies des organes non-lymphoïdes. Dans ce modèle, le système immunitaire est défectueux à plusieurs niveaux et ces altérations sont actuellement sous investigation. Une étude récente a démontré que chez la souris Tg, les cellules dendritiques excluant les cellules dendritiques plasmacytoides, ont des anomalies au niveau de leur fonction, état de maturation et localisation.

La présente étude démontre que 25-40% des cellules dendritiques plasmacytoides chez la souris Tg expriment Nef. Les cellules dendritiques plasmacytoides chez la souris Tg présentent des altérations dans leur fréquence, maturation, localisation et leur réponse *in vitro* aux stimulants. De plus, selon des résultats préliminaires, les cellules

dendritiques plasmacytoides issues de la rate de la souris Tg produisent plus d'IFN-α que leur équivalentes chez la souris non-Tg.

En considérant d'une part les multiples fonctions immuno-régulatrices que possèdent les cellules dendritiques plasmacytoides et d'autre part les changements observés dans leur caractéristiques principales chez la souris Tg, on peut suggérer que ces cellules sont impliquées dans la pathogenèse de la maladie ressemblante au SIDA.

Mots clés : SIDA, L'immunité antivirale, Cellules dendritiques, Nef, Organes lymphoïdes, CpG, Flt3L.

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ABBREVIATIONS LIST

Ab.: Antibody

Ag.: Antigen

AIDS: Acquired immunodeficiency syndrome

BM: Bone marrow

BrdU: Bromodeoxyuridine labeling

CC: Chemokine

CCR: Chemokine receptor

CDC: Conventional dendritic cells: lymphoid DCs ($CD8^{+}DEC205^{+}CD11c^{hi}B220^{-}$) and myeloid DCs

$CD11c^{lo/int}$: CD11c expressed at low to intermediate level

CD40L: CD40 ligand

CpG: Unmethylated, phosphorylated oligodeoxynucleotide rich in CG motifs

CTL: cytotoxic T lymphocyte

ER: Endoplasmic reticulum

FDC: Follicular dendritic cell

Flt3L: Fms-like tyrosine kinase Ligand

GC: Germinal center

GM-CSF: Granulocyte-monocyte colony stimulating factor

hIPC: Human interferon producing cell

HIV: Human immunodeficiency virus

IDC: Interstitial dendritic cells (DCs residing in all peripheral tissues except in the skin)

IFN-α: Interferon alpha

IHC: Immunohistochemistry

IL: Interleukin

IntDC: Interdigitating dendritic cells (residing in T cell zones of lymphoid organs)

IPC: Interferon producing cell

ISH: *In situ* hybridization

Kda: Kilo-dalton

LC: Langerhans cells (DCs found in stratified epithelia such as skin)

LN: Lymph node

MA: Medium alone

MC: Monoclonal

MDC: Myeloid dendritic cells ($CD11b^{+}CD11c^{hi}B220^{-}$)

MFI: Mean fluorescence intensity

MHC: Major histocompatibility complex

MIPC: Mouse interferon-producing cells

MLR: Mixed leucocyte reaction

MZ: Marginal zone

NIPC: Natural interferon producing cell

NK: Natural killer cells

nTg: Non-transgenic, normal mice

ODN: Oligodeoxynucleotide

O.N.: Overnight

PALS: Periarteriolar lymphoid sheets (in white pulp of spleen)

PAMP: Pathogen associated molecular pattern

PBMC: Peripheral blood mononuclear cells

PDC: Plasmacytoid dendritic cells

PDGF: Platelet derived growth factor

PG: Proteoglycan

PKR: Protein kinase R

pLN: Peripheral lymph node

PRR: Pathogen recognition-receptor

RBC: Red blood cells

rIL-3: Recombinant interleukine-3

SC: Subcapsular (in this texte refers to pLN subcapsular zone)

Ss: Single-stranded

TCR: T-cell receptor

Tg: Transgenic, can refer to $CD4C/HIV^{mutG}$ or $CD4C/HIV^{mutA}$ mice

Th: Helper T cell

TLR: Toll-like receptor

TNF: Tumor necrosis factor

Tr: Regulatory T cell

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CHAPTER 1: INTRODUCTION

1. INNATE AND ADAPTIVE IMMUNE RESPONSES

The immune system utilizes innate and adaptive responses in order to recognize and clear pathogens from the host [1]. Innate immunity, which is antigen (Ag) non-specific, includes natural barriers to pathogens such as skin and mucous membranes and specialized effector cells such as phagocytes and natural killer (NK) cells [1]. Macrophages, neutrophils and dendritic cells (DCs) at certain stage of their differentiation, show phagocytic functions. NK cells are cytotoxic cells participating in innate immunity because of their ability to lyse virus-infected and tumor cells [1, 2, 3]. Phagocytes and cytotoxic NK cells, as the main players of innate immunity, can discriminate between pathogens and self by utilizing signals upon ligation of their cell surface pathogen recognition receptors (PRRs) [4, 5]. Innate immunity is closely linked to and influences adaptive responses [6]. For example, proinflammatory cytokines produced by cells involved in innate immunity, enhance the Ag-presenting capacity of DCs [6]. Adaptive immunity refers to Ag-specific responses including humoral and cell-mediated defense mechanisms, which depend on B cells and T cells respectively [1]. The acquired immune response has also a memory component that recognizes a pathogen that has previously infected the host [1].

In adult, B and T cells develop from pluripotent stem cells in bone marrow (BM). While B cells mature in BM, T cells complete their maturation in thymus where after undergoing a selection process, only 5% of them survive and leave this organ. Negative selection induces central tolerance through deletion of self-reactive T cells in order to avoid immune response against self-tissues [1].

DCs process Ags and present them to T cells, in association with peptide-binding proteins i.e. major histocompatibility molecule (MHC) for recognition by T cell receptors (TCRs). Intracellular Ags, processed into peptides in the cytosol of the antigen-presenting cell (APC), bind to MHC class I while extracellular Ags that have entered the endocytic pathway of the APC associate with MHC class II molecules [7]. $CD4^+$ T cells, also called helper T cells, recognize Ag presented by MHC Class II and upon activation have profound immune-regulatory effects on processes such as antibody (Ab) responses, cytotoxic T lymphocytes (CTL) responses and generation of memory cells. $CD8^+$ T cells are mainly CTLs that recognize Ags presented by MHC class I. These cells participate in cell-mediated immune defense [1, 8]. However, particular subsets of $CD4^+$ and $CD8^+$ T cells have also been

attributed with major roles in inducing peripheral tolerance that together with central tolerance taking place in thymus maintain the homeostasis of the organism [9, 10].

When $CD4^+$ T cells are primed upon Ag recognition, they become activated, expand and release cytokines [1]. At this stage, helper T cells are categorized based on the cytokine profile they secrete [11]. $CD4^+$ T cells polarized as Th1 produce interleukin IL-2 and IFN-γ stimulating cell-mediated immune responses [1, 12]. Type 2 helper T cells (Th2) secrete IL-4, IL-5, IL-6 and IL-10 and induce Ab secretion by B cells [11, 12]. Elaboration of each class of cytokines, Th1 or Th2, inhibits the production of the other class of cytokines [12]. Previously, it was believed that each DC subset was specialized for priming either Th1 or Th2 adaptive responses (DC1 for Th1 inducers versus DC2 for Th2 inducers) [13]. However, all DC subsets can initiate adaptive Th1-Th2 cell responses and dictate the class of T cell immunity through the production of Th polarizing cytokines [11]. Indeed, the cytokine microenvironment is central in T helper cell polarization toward Th1 or Th2 cell type during immune responses [14].

DCs can direct a Th1 response, whenever the microbial stimuli that drive their maturation elicit IL-12 production [11, 15]. IL-12 is a cytokine produced by mononuclear phagocytes, DCs and activated NK cells inducing adaptive cell-mediated responses by promoting the generation of Th1 cells [1]. Also all DCs can suppress Th1 development and allow Th2 polarization upon exposure to IL-10 [1]. IL-10, produced by activated macrophages and Th2 polarized T cells down-regulates MHCII and costimulatory molecules on APCs [16]. It confers tolerogenic properties to DCs and drives the generation of mouse $CD4^+$ Tr cells [16]. IL-10 inhibits cell-mediated immunity favoring humoral responses [1]. Overall, a Th1 or Th2 response develops depending on antigen dose, the state of maturation of DCs together with parameters such as kinetics of activation and the nature of the maturational stimulus of DCs [11, 17, 18].

2. CHARACTERIZATION OF DCS AND THEIR ROLE IN THE IMMUNE SYTEM

DCs are a migratory group of BM-derived leukocytes with generally a short half-life and a fast turnover regenerating continuously from BM precursor cells through multiple developmental stages. In BM, DC progenitors develop into immature precursors that circulate in blood and home into various tissues throughout the organism. DCs represent a network of APCs specialized in the capture, processing, transport and presentation of Ag to T cells. They play a wide range of roles in initiation and regulation of immune responses against pathogens and in homeostatic regulation. They are implicated in innate and adaptive

immune responses and participate in the establishment of central and maintenance of peripheral self-tolerance [17, 19]. Central tolerance is established by DCs presenting self-Ags to T cells that undergo the selection process in the thymus [9, 20]. Moreover, in the absence of inflammation, DCs do not become fully activated and induce tolerance rather than immunity [6, 9]. Indeed, contrary to activated DCs that produce immunity, immature or quiescent DCs, i.e. mature DCs that are not fully activated, induce peripheral tolerance through multiple mechanisms. These include killing of T cells, paralyzing them (anergy) or/and generating regulatory T cells [20, 21]. The tolerogenic potential of DCs correlates with the absence of activation by proinflammatory signals [9, 21]. These signals are responsible for the switch from a quiescent to an activated state in some DC lineages [20].

Proinflammatory signals released by infectious agents attract DCs to the site of inflammation where they undergo a process called maturation that is required for their Ag-presenting function and for their migration into lymphoid organs. Once in lymphoid organs, matured DCs induce T cell activation and initiate Ag-specific acquired immune responses. Upregulation of cell-surface MHC class II and costimulatory molecules i.e. CD80, CD86 and CD40 characterize DC maturation [21]. This process also causes the secretion of cytokines and chemokines. MHC II and costimulatory molecule expression on APCs provide the two prerequisite signals to activate naïve T cells [1, 7]. Upon maturation, MHCII mainly residing in intracellular compartments, relocate from lysosomes to the cell surface [7]. Matured DCs upregulate cell surface chemokine receptors (CCRs), which play a role in their migration to T cell areas of lymphoid organs. Matured DCs also produce chemokines, which attract various subsets of T cells promoting DC-T cell interactions [18]. Thus, DC maturation is a key control point in converting an antigen into an immunogen [21, 22]. This central role of DC maturation in defense mechanisms is illustrated by the example of some tumor cells, which secrete factors inhibiting DCs maturation in order to escape the immune responses [6].

The interaction between NK cells and DCs may also be important in defense mechanisms. DCs acquire the ability of regulating NK cells function early post-infection allowing the production of cytotoxic and inflammatory responses that participates in the eradication of the pathogen [3]. In fact, cytokines produced by different DC subsets enhance NK cell activation and cytotoxic function. These effects are dependent on cytokines such as IFN-α and IL-12 for NK cell cytotoxicity and IFN-γ secretion while tumor necrosis factor (TNF)-α is required for CD69 expression. In addition, IL-2 activated NK cells interact with DCs and induce their maturation [2, 3, 23].

3. DC CLASSIFICATION IN MICE AND HUMANS

DCs are a heterogeneous population of cells sharing major functional and phenotypic features [17, 18]. Different DC subpopulations are distinguished based on ontogeny, phenotype, function, localization and migrational characteristics and accomplish complementary and overlapping functions in the immune system [18]. DCs can be obtained in large number, both in vitro and in vivo, in the presence of Fms-like tyrosine kinase-3 ligand (Flt3L) that is a hematopoietic growth factor [24, 25]. This is because most DCs develop from $Flt3^{+}$, hematopoietic progenitor cells in BM regardless of their myeloid or lymphoid origin [25, 26].

Mouse DCs are classified into three subsets. The majority of mouse DCs are called myeloid DCs (mDCs) because they originate from common myeloid precursors in BM. Lymphoid DCs derive from common lymphoid precursors, as do T cells. The myeloid DCs have a characteristic cell surface phenotype, namely $CD8\alpha^{-}CD11b^{+}CD11c^{hi}$, they are widely distributed in lymphoid and non-lymphoid tissues. Lymphoid DCs are $CD8\alpha^{+}CD11b^{-}CD11c^{+}$ cells residing mostly in the thymus, but are also present in the periphery at a lower frequency than mDCs [15, 27].

More recently, plasmacytoid DCs (pDCs) with a particular morphology mimicking immunnoglobulin (Ig)-secreting plasma cells have been characterized as a distinct DC subset in mice and humans. Contrary to what was believed previously, mouse pDCs develop efficiently from both lymphoid and myeloid-committed progenitors in BM [9, 28]. PDCs are localized in peripheral and cord blood, in the thymus as well as in T cell areas of secondary lymphoid organs including peripheral lymph nodes (pLNs), spleen and Peyer's patches [11, 29]. They are identified by their cell surface phenotype as $CD11c^{lo}B220^{+}CD11b^{-}Gr1^{lo}IL3R^{lo}LY6C^{hi}$. In contrast to other DCs that show a fast turnover, pDCs in uninfected mice are long-lived and their frequency varies between different strains and organs [30].

Human DCs are subdivided into two main groups including $CD11c^{+}$ mDCs and $CD11c^{-}$ pDCs. Human pDCs phenotype is slightly different from mouse pDCs, since they are $CD4^{+}IL3\text{-}R^{hi}CD45RA^{+}(B220)HLA\text{-}DR^{+}$. In humans as in mice, mDCs constitute the majoritary of DCs and include skin DCs called Langerhans cells (LCs) and interstitial DCs (intDCs) residing in all other tissues [31]. PDCs in human and mouse are equivalent in their main function as they are both identified as the natural interferon-alpha (IFN-α) producing cells (NIPCs) and they both can be mobilized by in vivo treatment with Flt3L [32].

4. CONVENTIONAL DENDRITIC CELL (CDC) FUNCTIONS

In this text the term cDC is used to designate all mouse DCs including myeloid and lymphoid DCs, but excluding pDCs.

Various DC subsets differentially control immune responses. There is a complementarity in recognition as well as in effector functions among DC subpopulations [15]. CDCs link innate and adaptive immune responses by activating both B cells and NK cells [33]. Different patterns of cytokine production by DC subsets may occur in response to particular microbes. Also, different functions of Ag-presentation can be assigned to DC subsets depending on the conditions of stimulation [34]. Mouse lymphoid DCs ($CD8\alpha^+CD11b^-$), produce IL-12 and prime naïve $CD4^+$ T lymphocytes to secrete Th1 cytokines, whereas mDCs ($CD8\alpha^-CD11b^+$) induce both Th1 and Th2 cytokines following in vivo immunization [15, 27]. However, only lymphoid DCs possess the ability to cross-present Ag in association with MHCI [34]. While mDC induce a strong allogeneic mixed-lymphocyte reaction (MLR) response, lymphoid DCs induce a comparatively low MLR response and express Fas-ligand [35]. In fact, the limited $CD4^+$ T cell response induced by lymphoid DCs is associated with T cell death that is caused by the interaction of Fas on T cells and Fas-ligand on DCs. Because of these characteristics, lymphoid DCs seem to play a regulatory function while mDCs are stimulatory and induce a strong immune response [35, 36].

In humans, mature monocyte-derived DCs induce a Th1 response causing the differentiation of naive $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs) [10, 36]. Apart from their role in T cell priming, cDCs activate NK cells and control B cell growth and differentiation [30, 37]. They also participate in innate responses through functions such as pathogen recognition and phagocytosis [2, 3, 4]. In addition, follicular DCs (FDCs) reside in follicles of lymphoid organs and retain Ag in a native state for presentation to germinal center (GC) B cells [1]. FDCs are responsible for B cell activation and selection through the process of affinity maturation [1]. On the other hand, thymic DCs derive from an intrathymic precursor, develop and die within the organ. They show a non-migratory behavior and participate in the induction of central tolerance [20].

5. PLASMACYTOID DC (PDC) FUNCTIONS

Previously, pDCs were called plasmacytoid T cells or monocytes based on their tissue localization in T cell rich area of lymphoid organs, their round morphology with a smooth plasma membrane and an eccentric nucleus resembling plasma cells [38]. Studies showed

that non-monocytes, non-T and non-B plasmacytoid lymphocytes can activate NK cells, in vitro [39]. Later, pDCs were classified as a distinct subset of DCs because of their lack of lineage markers, the expression of MHCII and the capacity to stimulate naive T cells [40, 41]. Indeed pDCs do not express typical cell surface markers of T cells, such as CD3, B cells, such as CD19, monocytes, such as CD14 or NK cells, such as CD16 and CD56 [42]. Currently, they are recognized as IPCs, which upon microbial and especially viral infection are able to activate NK cells as well as regulate the immune responses. It is now known that IPCs are necessary for NK cell activation partly because IFN-α enhances NK cell cytotoxicity [2, 43].

PDCs are implicated in innate and adaptive immune responses to viruses [11, 31]. Upon microbial infection, pDCs accomplish their functions partly through the secretion of IFNα. PDCs produce up to 1000 times more IFN-α in response to viral stimulation than any other peripheral blood mononuclear cells (PBMCs) [32]. Apart from NK cell activation that was originally attributed to pDCs, IFN-α plays multiple immunoregulatory functions including differentiation of monocytes into dendritic-like cells, maturation of mDCs and pDCs, Th1 polarization of unpolarized Ag-experienced $CD4^+$ T cells and the differentiation of B cells into plasma cells [44, 45, 46]. Since DCs are the most potent APCs but are at low frequency in the organism, the ability of pDCs to promote monocyte-derived DCs amplifies total Ag-presenting function [7, 47]. This IFN-α-mediated effect of pDCs on monocytes and mDCs indirectly regulates T cell responses [47]. Type I IFNs are also important for pDC maturation since their maturation is drastically impaired in IFNα–receptor (IFNAR)-deficient mice [39].

In the context of viral infection, pDCs sense the presence of the virus through TLR7 and TLR-9 expressed in their endosomal compartment [48]. Upon viral recognition, pDCs control innate immune cell functions as they activate macrophages and NK cells through release of cytokines. In fact, pDCs responses to viruses include the production of IFN-α, IL12, chemokines (CCs) such as CXCL9 and CXCL10 and chemokine receptors (CCRs) such as CCR3, CCR4 and CCR5 [44]. All these factors attract and activate NK cells and T cells and modulate the function of mDCs by inducing their maturation [45, 46, 49].

The production of type I IFNs is exclusively reserved to immature pDCs. In fact, as pDCs mature, they lose the ability to produce IFN-α and acquire the ability to present Ag to T cells [42]. Thus, pDCs show a dual Ag-presenting and IFN-producing function that links innate and adaptive immunity [50]. However, pDCs possess a weaker ability to stimulate naive $CD4^+$ T cells than mDCs. PDCs are less potent APCs than mDCs because they express lower cell surface MHCII and costimulatory molecules, do not endocytose Ag as efficiently

as mDCs and lack the expression of lysosomal proteases involved in Ag processing [42, 44]. PDC and mDC differ also based on various sets of PRRs that they express (see section on TLRs). Moreover, while immature as well as mature mDCs morphology includes cellular protrusions called dendrites, immature pDCs are round and lack dendrites. PDCs can develop dendritic protrusions only upon maturation and transformation into APCs [27, 35]. It is possible that pDCs induce the differentiation of unpolarized Ag-experienced T cells that have previously been expanded by mDCs [44].

Despite the difficulty to study pDCs because of their low frequency in blood and secondary lymphoid organs of both human and mouse, different research groups have investigated the APC function of pDCs [51]. These experiments include in vivo and in vitro assays using different stimulants such as CD40 ligand (CD40L), IL-3 or viruses. CD40L is a well-known DC activator expressed on many cell types including helper T cells and seems to amplify activation of DC previously stimulated by infectious agents [52]. IL-3 is a potent maturation and survival factor for human pDCs [53]. However, it should be noted that IL-3 and CD40L are factors produced by T cells after their activation and in early responses to viruses, these signals cannot dictate DC differentiation [54].

In mouse, upon microbial, IL-3 or CD40L stimulation, pDCs differentiate into $CD8^{+}DEC205^{-}$ DCs distinct from lymphoid $CD8^{+}DEC205^{+}$ DCs [50]. Microbial infection can thus induce the generation of a new DC subtype not present in uninfected organism. Although the complete functional implication of this shift in the DC network remains to be determined, it is known that mature $CD8^{+}DEC205^{-}$ pDCs can efficiently present Ags to T cells and to a lesser extent than mDCs induce Ag-specific adaptive responses [50]. Murine pDCs generally promote Th1 responses at high and Th2 responses at low antigen dose. It has been shown that viral stimulation of pDCs induces IFN-α production and their differentiation into Th1 polarizing DCs [12, 55, 56]. Other experiments in mouse show that influenza virus stimulation of pDCs in vivo results in their differentiation into APCs. These matured pDCs induce effector/memory $CD8^{+}$ T cell responses that could be recalled 4 weeks later [43].

In human, pDCs differentiate into $CD11c^{-}$ mature DCs when cultured with IL-3 and CD40L. These CD40L and IL-3-treated pDCs have been shown to stimulate $CD4^{+}$ helper T cells, which become Th1-polarized *in vitro* due to the synergistic effect of IL-12 and IFN-α [56]. However, prior to these findings, human pDCs were called precursors of DC2 because upon stimulation with CD40L or viruses *in vitro*, they induced an IL-4-independent Th2 polarization of naive T cells [57]. In contrast to DC2s, monocyte-derived DCs were termed DC1s because they induce Th polarization and pro-inflammatory responses [57]. However,

since type I IFNs promote a Th1 response in humans, this view of pDCs as precursors of DC2s does not correlate with their ability to produce type I IFNs [42, 57]. The discrepancy between these results could reflect various conditions of *in vitro* pDCs exposure to CD40L and methods used to culture them [45, 57]. *In vivo*, DCs should receive signals with a different strength in a sequential fashion, preserving the plasticity of the DC's Th polarization function until the final stages of their maturation in the lymph node [57]. Accordingly, in order to accomplish all these diverse functions, pDCs must migrate from blood into inflamed LNs [49].

Other *in vitro* studies on human pDCs show that they influence the humoral immune responses. When activated T cells secrete IL-2 and express CD40L, these factors signal pDCs to secrete IL-6. PDC-derived IFN-α/β induces B cells to differentiate into plasma blasts while pDC-derived IL-6 is responsible for inducing their Ab secretion [58]. Thus, *in vitro*, virus-activated pDCs induce the differentiation of CD40-activated B cells into plasma cells producing virus specific Abs [43, 58].

On the other hand, a role for the induction of tolerance was attributed to pDCs. *In vitro* studies show that immature human pDCs can differentiate $CD8^+$ Tr cells that suppress Ag-specific T cell proliferation [31, 58]. These Tr cells produce IL-10, which is implicated in immuno-suppression [10, 43, 59]. IL-10 producing $CD8^+$ Tr cells inhibit Ag-specific proliferation of naive $CD8^+$ T cells in a primary MLR [59]. Another study reported, that CD40L-activated pDCs similar to immature mDCs have the ability to induce primary Tr-cell differentiation permitting peripheral tolerance [59]. One study on human pDCs demonstrates that TLR-9 stimulation of pDCs promotes the generation of $CD4^+CD25^+$ Tr cells *in vitro* [60]. Studies in mice also support that pDCs play a role in the generation of $CD4^+$ Tr cells [60].

Overall, the production of IFN-α by pDC initiates innate immune responses limiting the spread of pathogens and promotes adaptive immune responses by enhancing the function and maturation of APCs including monocytes and mDCs [47]. Also, when matured, pDCs participate directly as APCs in adaptive responses. Thus, pDCs initiate an immune response through the secretion of cytokines and link innate and adaptive immunity upon activation [42]. PDCs show great flexibility in their functions and depending on the stimuli they receive, they can induce Th1, Th2 or regulatory (Tr) responses [31, 50, 55].

Several studies have shown heterogeneity in pDC population [50, 51]. PDCs phenotype can be used to identify their subtypes since pDCs have been classified on the basis of cell surface CD4 expression. $CD4^-$ pDCs have been shown to be functionally distinct from $CD4^+$

pDCs. $CD4^+$ pDCs do not produce IFN-α although they show similar MHCII expression to $CD4^-$ cells [50]. Kinetic studies have shown that $CD4^-$ pDCs are precursors of $CD4^+$ pDCs. Moreover, blood $CD4^-$ pDCs are the precursors of splenic $CD4^-$ pDCs, which are the precursors of splenic $CD4^+$ pDCs [50].

In addition, BM pDCs from mouse could be classified based on the expression of Ly49Q [51]. Ly49Q, a type II C-lectin membrane-associated polypeptide, is a member of Ly49 NK receptor subfamily that binds to MHCI and regulates NK cell activation. However, Ly49Q is expressed on all peripheral pDCs but not on NK or NKT cells [51]. Ly49Q is not exclusively expressed by pDCs since $Gr1^+$ myeloid lineage cells and activated macrophages have been reported to express this cell surface molecule [61]. In BM, the expression of Ly49Q defines two subsets of pDC [51]. Different expression level of Ly49Q defines sequential developmental stages of pDCs derived from BM by using cytokines such as Flt3L [62]. One study showed that $Ly49Q^-$ pDCs respond less to infectious agents than $Ly49Q^+$ pDCs possibly because they have not yet acquired the full sets of cell surface and/or signaling molecules necessary for their functions against microbes [51]. Accordingly, a correlation was found between Ly49Q expression on pDCs and their maturational state [61]. In fact, $Ly49Q^-$ pDCs are mostly $CD4^-$ and upon activation they become $Ly49Q^+$ and concomitantly upregulate cell surface expression of CD4 and MHCII [61, 62].

6. PDCS AND MYELOID DCS (MDCS) MIGRATIONAL PROFILES

DC development includes distinct stages. In BM, first DC progenitors proliferate and give rise to DC precursors, which circulate in blood. Blood DCs migrate to sites of inflammation in response to chemotactic stimuli and from there they are recruited into secondary lymphoid organs to prime T cells. Thus, DCs migration is critical for optimal immune responses [56]. Migration of leukocytes is a complex process to which CCs contribute [44, 63]. Pathogens induce local production of TNF-α, which once in circulation promotes systemic inflammation and up-regulates CCL3 causing mobilization of DC precursors into the blood [64, 65]. These precursor cells develop into immature DCs that localize in various non-lymphoid tissues in order to capture Ags upon invasion of the host by foreign agents. In general, immature DCs express receptors for inflammatory CCs, which facilitate their migration to sites of inflammation [11, 15]. Local TNF-α accelerates influx of mDC precursors to sites of inflammation via CCL3 and their efflux via CCL21 [64]. Once there, they respond to inflammatory cytokines secreted by innate immune cells that promote their maturation after capturing Ags [11, 15]. Upon maturation, DCs show a CCR switch, down-regulating inflammatory CCRs (CCR5, CCR2 and CCR1) and up-regulating CCR7 [16]. The

interaction of pathogens with innate immune cells also induces the release of proinflammatory signals that cause DC migration to T cell rich zones of lymphoid organs (spleen and LNs) for Ag presentation and T cell priming [11, 63, 66]. In fact, upon maturation DCs up-regulate cell surface CCR7, a receptor for SLC/CCL21 and ELC/CCL19, which are constitutively produced in T cell areas of secondary lymphoid organs and drives DC migration to these sites [11, 15].

Serum TNF-α also up-regulates CXCL9 and E-selectin on LN high endothelial venules (HEVs), which attracts pDCs to cluster around HEVs. HEVs are specialized post-capillary blood vessels with a cuboidal endothelium, which constitute one of the entry routes to T cell zone of LNs for blood lymphocytes [1, 67]. It is now known that it is around HEVs where pDCs produce large amounts of type I IFN [56, 64, 65]. CXCR3 is required for this migration, because in CXCR3-deficient mice, trans-HEV migration of pDCs into LNs from blood is impaired, [56, 64]. In contrast to pDCs, mDC precursors as well as monocyte-derived DCs that have captured Ags migrate into the draining LNs through afferent lymphatics [11, 64, 65].

In human blood, pDC and mDC express similar sets of CCRs with the exception of CCR7 and CXCR3. Expression of CXCR3 and CCR7 is low on mDCs and high on pDCs [13]. Following maturation, both pDCs and mDCs downregulate CCR1, CCR2, CCR5, CCR6 and CXCR1 and strongly upregulate CCR7. However, in contrast to mDCs, most CCRs expressed on pDCs are not functional on circulating cells [13, 66]. In fact, only after maturation induced by stimulants, such as CD40L, are the receptors for inflammatory chemokines downregulated, and CCR7 on pDCs becomes functional and induces migration [13]. The differences between mDC and pDC migration include the expression of CCRs such as CCR7 and CXCR3, direct entry of pDCs to LNs from blood while mDCs enter LNs through afferent lymphatics and the fact that on some pDC, CCRs are not functional when they circulate in blood [13]. These differences in migration between pDCs and mDCs, suggests a distinct role for each subset in the induction and regulation of the immune responses [13]. Accordingly, PDCs could be more involved in homeostatic control of immune responses since they can induce Th1, Th2 and possibly Tr cells depending on the conditions [13]. In addition, virally stimulated pDCs have been shown to produce CCs such as CCL4 and CXCL10 that attract NK and activated T cells to pDCs [65].

7. TLR DEFINITION AND ROLES IN THE IMMUNE SYSTEM

The innate immune system is the first line of defense against pathogens and allows the initiation and regulation of adaptive immune responses for effective clearance of infectious agents [54]. To detect the presence of infection, the innate immune system first recognizes conserved molecular structures that are predominantly found in microorganisms but not in vertebrates [5, 68, 69]. Pathogen associated molecular patterns (PAMPs) are recognized by various types of PRRs expressed on innate immune cells including monocytes, macrophages, DCs, B cells and NK cells [5]. Among these cells, effector cells, such as neutrophils and macrophages, exert phagocytic and/or cytotoxic functions upon binding of PAMPs to PRRs [5, 68]. A major part of PRRs is represented by Toll-Like receptors (TLRs), which constitute a group of transmembrane signaling receptors [70]. Ten different TLRs in human and twelve in mice have been described [70]. They belong to phylogenetically conserved factors that generate selective immune responses upon ligation by various PAMPS [70, 71]. TLRs ligation activates NFκB transcriptional factor and induces the expression of many genes involved in immune responses [5, 72]. The responsiveness to a given PAMP depends on the set of TLR expressed by the cell. All TLRs permit to mount a first set of stereotyped responses such as inflammation. Individual TLRs induce distinct set of immune responses necessary for host defense. For example, intracellular pathogens require Th1 polarization of $CD4^{+}$ T cells [5, 15, 73].

It is known that TLR1 is required for the response to imiquimod, TLR2 to peptidoglycan (PG) and mycobacteria, TLR3 to double stranded (ds)-RNA (viruses), TLR4 to LPS, TLR5 to flagellin, TLR7 and TLR8 to G/U rich single-stranded (ss) RNA or imidazoquinoline, TLR9 to intracellular bacterial and viral DNA or CpG ODN [3, 68]. In humans TLR7 and TLR 9 are expressed by B cells and pDCs, while TLR2, TLR3, TLR4 and TLR8 are expressed by mDCs and monocytes [3, 68]. In the mouse, expression of TLR9 is not exclusive to pDCs and B cells and other DCs as well as macrophages express TLR9 [68].

MDC and pDC express complementary sets of TLR, providing them the capacity to respond to different PAMPs [11]. While TLR4 and TLR3-mediated mDCs activation by LPS and Poly(I:C) respectively, causes their IL-12 production, TLR9 ligation to CpG induces IFN-α production by pDCs [50, 74]. LPS is a major component of the cell wall of Gram-negative bacteria and Poly(I:C) mimics viral dsRNA that is released after virally infected cell lysis [75, 76]. The differential expression of TLRs by human DC subsets could explain selective immune responses to various pathogens. TLR9 signaling induces the maturation of pDCs among human DCs and of pDC and mDC among mouse DCs. It also activates matured

DCs to produce cytokines. Thus, TLR-signaling pathways link innate and adaptive immunity by promoting Ag-presenting function of DCs that allows in turn the generation of adaptive T cell responses [35].

In order to induce an efficient adaptive immune response, TLR signaling must also block the suppressive effect of Tr cells on T cells. Indeed, as mentioned earlier, Tr cells prevent the activation of peripheral auto-reactive T cells. It has been postulated that TLR signaling induces the concomitant secretion of IL-6 by DCs. Since IL-6 renders pathogen-specific T cells refractory to the suppressive effect of $CD4^{+}CD25^{+}$ Tr cells, TLR-signaling can overcome peripheral tolerance and induce an immune response [77].

Pathogen-derived DNA recognition as non-self by mammalian immune cells through TLR9 signaling pathway is due to its high frequency of unmethylated CpG dinucleotides. In fact, mammalian-derived DNA has much lower frequency of these dinucleotides and they are mostly methylated [63, 71, 78]. CpG ODNs are synthetic compounds that mimic microbial DNA and interact with TLR9 specifically, as shown by the absence of CpG effects in TLR9-deficient mice [71]. CpG effects include proliferation of splenocytes, inflammatory cytokine production and maturation of DCs [71]. In addition, TLR9-engaged pDC activate NK cells as indicated by CD69 upregulation and enhanced cytotoxicity. One study showed that IL-2 produced by $CD4^{+}$ helper T cells enhances NK cells activation, while Tr cells cancel this additive action by blocking $CD4^{+}$ T cell-mediated enhancement of NK cell activation [79].

In mice, CpG ODNs promotes MHCI presentation of soluble Ags to $CD8^{+}$ T cells inducing Th1-type cell-mediated responses independently of $CD4^{+}$ T cell help. This characteristic of CpG could potentially be useful in the treatment of chronic viral infections such as HIV, where there is progressive loss of Th1 immune responses along with depletion of $CD4^{+}$ helper T cells [47].

8. INTERFERON (IFN)-ALPHA DESCRIPTION AND FUNCTIONS

IFN-α and IFN-β, are classified as type I IFNs while IFNγ corresponds to type II [69]. IFN-γ is an antiviral agent as well as an activator of CTLs and is one of the main cytokines produced by Th1-polarized $CD4^{+}$ helper T cells, CTL type I and NK cells [53, 58]. Type I and II IFNs have non-redundant and complementary functions in the host response to viral infection [80]. Type I IFNs are inducible cytokines produced by different cell types in response to viral infection and exert an antiviral activity at all stages of virus life cycle [80, 81, 82]. In the mouse, 1 IFN-β and 13 IFN-α have been identified and are encoded by an

intronless multigene family [81]. Fibroblasts, T cells, macrophages, monocytes, DCs and NK cells secrete IFN-α [80]. However, pDCs identified as NIPCs produce up to 1000 times more of this cytokine than any other cell type [80]. Type I IFNs bind to a common receptor IFNAR that is expressed on a large variety of cell types [70].

Stimuli, such as viral components, endotoxin treatment of cells, dsRNA, poly (I: C) or CpG-ODNs can induce IFNα production [80, 83]. IFNα/β acts directly on most cell types and turns on biochemical pathways, which induce an antiviral state. IFNα/β antiviral effects include the restriction of viral replication and confer cellular resistance to further viral infection [58]. Various proteins are involved in IFNα/β induced antiviral state including PKR, ADAR (adenosine deaminase acting on RNA), OAS (2′,5′-oligoadenylate synthetase), RNase L and MX proteins [80, 84].

Protein kinase receptor (PKR) is a cytoplasmic serine-threonine kinase containing two conserved dsRNA-binding domains in its N-terminal region. PKR belongs to a class of more than 20 dsRNA-binding proteins, which recognize non-specifically dsRNA. Once activated, PKR phosphorylates the protein synthesis factor eIF-2α, which inhibits initiation of viral translation and consequently interferes with viral replication. Numerous viruses encode genes responsible for inhibiting PKR activity. ADAR inhibits RNA editing; OAS activates the endoribonuclease RNase-L to degrade ssRNA [80, 84] Human MxA protein and mouse Mx1 are IFN-induced GTPases that inhibit replication of certain viruses by binding target viral proteins [74]. Many viruses have developed mechanisms to escape the interferon system by inhibiting its synthesis or blocking its action as for example through suppression of PKR activation [4, 74].

Moreover, IFNα/β regulates functions of immune cells, enhancing humoral and cellular antiviral responses *in vivo* [32, 82]. These regulatory functions on immune cells include the activation of NK cells by promoting their survival and proliferation through IL-15 production. IFNα/β activates $CD8^+$ and $CD4^+$ T cells and has an effect on proliferation of B cells. Type I IFNs drive monocyte differentiation into DC-like cells, maturation of DCs as well as differentiation of pDCs into efficient APCs. IFN-α mediates its immunoregulatory functions in part through the modulation of the activity of other cytokines and CCs such as IFNγ, IL-1, IL2, IL-3, IL-8, IL-12, IL-13, IL-15, TNF-α, IP-10, etc [68, 80]. IFNα/β also affects expression of cell surface cytokine and CC receptors. For example, it causes IL-12R up-regulation on $CD4^+$ T cells promoting Th-1 polarization of helper T cells [80]. Moreover, IFN-α as well as IFN-γ enhances immunoglobulin G (IgG) production and downregulation of IgE secretion by B cells, promoting humoral immunity [80]. IFNα/β is an important cytokine in innate immune

responses since it activates innate immune cells such as NK cells to kill virally infected cells. This cytokine also causes up-regulation of MHCI and MHCII on APCs and promotes adaptive immune responses by $CD8^+$ and $CD4^+$ T cells respectively [85] Thus IFN-α bridges the innate to adaptive immune responses through the increase of total APC function [80] (See supplementary figure 11).

Studies investigating IFN-α production in response to pathogens show that this cytokine has very rapid kinetics and disappears after about 24 hours. This suggests that pDCs initiate an antiviral innate immunity early during viral infection. The capacity of pDCs to produce great amounts of IFN-α rapidly and effectively in response to viral pathogens is due at least in part to a specific genetic program [86]. IFN-α acts through multiple signaling pathways involving JAK kinases, STAT transcription regulators and IRF transcription factors [80, 84]. The induction of IFN-α secretion requires a positive feedback-loop as shown by IFNAR-deficient cells, which are inhibited in their production of IFN-α [83]. However, mouse and human pDCs rather than other cell types can bypass the positive feedback loop that is essential in the production of IFN-α. It has been reported that IRF7, which interacts with the adaptor protein MyD88 is required for type I IFN production in response to TLR7 and TLR9 through the signaling pathway involved in the positive feedback loop [39, 86]. Both human and mouse pDCs constitutively express higher levels of IRF-7 than other cell types allowing them to bypass the positive feedback in IFNα/β signaling that requires *de novo* IRF-7 synthesis [39, 86]. Moreover, the production of high-level type I IFNs in response to TLR9 necessitates the retention of MyD88-IRF-7 complex in the endosomal compartment for an adequate lapse of time and this spatiotemporal regulation is observed only in pDCs [39, 87].

9. HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION

9.1. HIV DESCRIPTION

Human immunodeficiency virus (HIV) is a retrovirus, a family member of lentiviruses that causes an infection of the immune system leading to the acquired immunodeficiency syndrome (AIDS) [67, 88]. The HIV genome contains two ssRNA molecules, each 9.2 Kb long, bound to a molecule of reverse transcriptase. Following entry into host cells, the viral reverse transcriptase copies the RNA into dsDNA. This viral DNA integrates into the host cell DNA and becomes a provirus. HIV genome is composed of nine genes flanked by non-coding long terminal repeats (LTRs) that are necessary for the integration of the provirus into the host cell genome. LTRs also contain binding sites for gene regulatory proteins that control the expression of the viral genes and are required for viral replication. HIV RNA is

within a core of viral proteins and surrounded by a nucleocapsid. The outer portion of the nucleocapsid consists of a phospholipid bilayer envelop derived from host cell containing virally encoded envelop glycoproteins, gp120 and gp41 which are required for the infection of cells [67, 88, 89].

HIV-1 possesses three typical retroviral genes: the *gag* gene encodes core structural proteins, the *env* sequences encode gp120 and gp41 of the viral envelop, mediating the membrane fusion required for the entry of the virus into host cells and the *pol* sequences encode reverse transcriptase, integrase, and viral protease enzymes necessary for viral replication. In addition, HIV-1 genome also includes six other regulatory genes, the *tat*, *rev*, *vif*, *nef*, *vpr*, and *vpu* genes [1]. Vif enhances infectivity, Vpr promotes nuclear import of viral DNA and arrest cell cycle at G2 phase, Tat is necessary for elongation of viral transcripts and markedly augments viral transcription independently of the integration site, Vpu downregulates CD4 expression and augments viral release from cells. HIV-1-protein Nef, also down-regulates MHCI. Tat and Rev are necessary for HIV replication. The Tat protein functions as a transactivator of the HIV promoter and Rev facilitates the cytoplasmic export of incompletely spliced transcripts. Vif, Vpr, Vpu and Nef are termed accessory because their inactivation does not totally block HIV replication in vitro. However, these genes are important virulence factors [89, 90].

Since the CD4 molecule functions as a receptor for the virus, HIV infects $CD4^+$ cells including $CD4^+$ T cells, monocytes and DCs [67, 88, 91]. Membrane fusion between the lipid bilayers of the viral envelope and the host cell membrane is required for viral entry to the cell. The gp120/gp41-Env complex catalyses the membrane fusion by interacting with host cell CD4 molecule and coreceptors. [89]. Host cell coreceptors for viral membrane glycoproteins are CXCR4, expressed on macrophages, and CCR5 by T cells. Certain HIV isolates, replicate efficiently in primary macrophage culture and are termed "M"–tropic or X4 for CXCR4. Other isolates replicate in T cells but not in macrophages and are called "T"-tropic or R5 for CCR5 [89].

9.2. THE CLINICAL COURSE AND IMMUNOPATHOGENESIS OF HIV INFECTION

There are two strains of HIV, called HIV-1 and HIV-2. HIV-1 is the most common cause of AIDS. However, HIV-2, which differs in genomic structure and antigenicity, induces a similar clinical syndrome [92]. The clinical course of HIV infection includes an acute infection with high level of viremia, followed by a latent stage, where the immune response controls infection but the virus persists as a provirus in infected cells [92]. Initially, HIV infection

induces specific humoral and cell-mediated anti-HIV immune responses [91]. During the latent phase, LNs and spleen are sites of ongoing HIV replication and tissue destruction. HIV infection progresses to AIDS when destruction of lymphoid tissue is complete and blood $CD4^+$ T cells drops below 200 cells / mm^3 [1]. AIDS includes various signs and symptoms such as weight loss, night sweats, fever and diarrhea. AIDS is a state of suppressed immunity mainly because of the loss of $CD4^+$ helper T cells and impairment of adaptive immune responses [93]. AIDS immunosupression underlies the development of opportunistic infections (OI) and neoplasia [93].

The latent stage can vary from less than 1 year to 15 years. The outcome of HIV-1 infection depends on the host anti-viral immune response. Long-term non-progression (LTNP) status, defined by at least 10 years of HIV infection without the development of AIDS, is associated with the development of effective and persistent T cell responses against HIV [24]. The clinical stages of HIV disease correlate with a progressive spread of HIV from the initial site of infection (i.e. blood or mucosa) to lymphoid organs. In early HIV-1 infection, gut-associated lymphatic tissue (GALT) is a principal site of virus replication and depletion of primarily lumina propria memory $CD4^+$ T cells. The immune response controls temporarily the acute infection but a chronic infection is established when virus trapped in lymphoid tissues by FDCs persists [1].

The major hallmark of the suppression of immune responses in HIV-1 infected patients is the progressive decline in the number of $CD4^+$ T cells. In fact, the consequences of HIV infection of T cells are devastating because the $CD4^+$ helper T cells play major roles in shaping immune responses [24, 93]. *In vitro* and *in vivo* studies have shown that in AIDS patients, lymphocyte proliferation in response to defined Ags is reduced. This is referred to as an anergic state of the immune system [12]. In addition to $CD4^+$ T cell depletion, HIV induces a wide range of impairments in the regulation of immune responses. These include premature atrophy of the thymus and thymocyte depletion [82], loss of LN architecture, loss of $CD4^+$ T cell function, inversion of CD4/CD8 T-cell ratio, abnormal B cell functions despite their polyclonal activation and hypergammaglobulinemia, serum auto-Abs and circulating immune complexes [92, 93, 94]. Also, since cytokines control the homeostasis of the immune system, their dysregulation in HIV infection contributes to the pathogenesis of AIDS [95].

Other defects, in the immune system of HIV infected patients, include a decrease in memory T cell responses to Ag, poor CTL responses to viral infection and lowered humoral immune responses to defined Ags [93, 94]. Some studies report a decrease in the

proportion of Th1 cytokines-secreting T-cells in HIV-infected patients while Th2 secreting T-cells are increased. MHC-restricted $CD8^+$ CTLs, found in large numbers during latent stage of HIV-1 infection, decrease with disease progression [96]. Since $CD4^+$ T cells are required at least partly for CTL activity, their depletion may explain the decline in CTLs as the disease progresses [93]. Also, since HIV is an intracellular pathogen, its clearance from the organism requires a Th1 type cell-mediated immune response in host defense [15, 73].

The ability of HIV-1 to evade the host immune responses leads to the establishment of chronic infection [55]. The viral mechanisms of immune evasion include the following strategies. The high mutation rate of HIV that allows the virus to evade detection by Ab or T cells produced in response to viral proteins before mutation. Also, HIV-Nef mediates selective down-regulation of HLA-A and HLA-B, rendering infected cells undetectable by CTLs. Concomitantly, the normal cell surface expression of HLA-C and HLA-E allows the infected cells to evade NK cell-killing as these cells are inhibited by the expression of HLA-C and –E [55]. Finally, HIV infection preferentially inhibits cell-mediated immunity due to the immune deviation from Th1 to Th2. This immune dysfunction increases the susceptibility to infection by intracellular pathogens including HIV itself [1]. In fact, since IFN-γ activates and IL-4 inhibits macrophage-mediated killing of intracellular pathogens, the shift in Th responses may explain the host susceptibility to such microbes [1, 74].

The initial adaptive immune response to HIV infection corresponds to the expansion of $CD8^+$ CTLs specific for HIV-derived peptides [12, 94]. $CD8^+$ T-cell response to HIV infection is crucial for antiviral defense through direct destruction of virally infected cells and/or through secretion of soluble antiviral molecules [91]. Thus, the partial control of HIV infection causing the transition of acute infection to the latent phase seems to be due to $CD8^+$ CTLs specific for viral peptides. On the other hand, Abs to a variety of HIV Ags are secreted by B cells within 6 to 9 weeks after infection. However, the beneficial effect of anti-HIV Ab responses in limiting the disease is questionable [12]. While the most immunogenic viral proteins in the humoral responses are gp-120 and gp-41, anti-envelope-Abs inhibits poorly viral infectivity [12]. In addition, despite the established role of humoral immune response in many viral infections, Abs in sera against HIV-1 have only weak neutralizing activity and are mostly directed against virion debris [92]. However, high level of Abs to HIV in infected patients suggests an interaction between B cells and activated T cells in the development of immune activity [92]. Moreover, neutralizing Abs able to block the infectivity of virus may limit the replication of HIV during the asymptomatic stage of infection [93].

HIV infection disrupts cell-mediated and humoral immunity and renders the host susceptible to OI and malignancies [12, 93]. Highly active anti-retroviral therapy (HAART) suppresses viral replication and partially restores CD4$^+$ T cell counts, reducing AIDS-related mortality [12]. Some reports concluded that adaptive immunity is the most critical component of the immune system for control of HIV infection and that HIV-specific CD4$^+$ helper T cells and CTL responses may decide the rate of disease progression [93, 97].

9.3. DC DEFECTS AND IMPLICATION IN HIV

DCs are the first mucosal cells infected by the virus during oral and sexual transmission and participate in viral dissemination by transmitting the virus to CD4$^+$ T cells [98]. Mucosal DCs that have been infected process HIV-1 proteins and carry live HIV into the LNs where they present the viral Ags to T cells. In LNs, HIV-1 replication is the most active and thus DCs contribute to the efficient propagation of HIV by their migration that is mediated by CCR7 upregulation upon activation [98].

DCs send signals to T cells that promote their ability to replicate the virus [99]. The DC-T cell microenvironment is an explosive site for HIV propagation because activated T cells become highly susceptible to HIV-1 and enhance viral replication. HIV-1 preferentially infects CD4$^+$ T cells specific for HIV-1 Ags [97]. Viral replication is fast and efficient in activated T cells and macrophages, partly because cellular factors that positively regulate viral transcription are abundant in these cells [99]. HIV-infected DCs initiate rounds of T cell infection by transmitting HIV to resting T cells in LNs [92]. Some DCs show latent infection and become productively infected after maturation. This shift of non-productive to productive infection is a way for DCs to facilitate the persistence of the virus in the organism [100]. This is because a productively infected cell produces many virions each capable of infecting cells and amplifying the infectious cycle [1]. Moreover, in the acute infection, virus particles are trapped in the FDC network and this is another important mechanism for DCs to maintain HIV infection over time [94].

DCs are not only central to the pathogenesis of HIV-1 by serving as initial and continuous source of the virus, but paradoxically are also essential for the induction of effective anti-HIV immune responses involving both CD4$^+$ and CD8$^+$ T cells [92, 97, 101]. Carriage of virus by DC into LNs allows clustering and priming of T cells and production of antiviral immune responses [100]. Therefore, DCs depletion during HIV infection is implicated in HIV-1 pathogenesis by reducing immune system capacity to prime T cells and to generate antiviral responses. However, the loss of DCs when they become overtly infected imitates HIV

dissemination [91]. Depletion of HIV infected DCs and T-cells, is due to either direct lysis by the virus or by HIV-specific CTLs. Also, in AIDS, DC development from $CD34^+$ stem cells is impaired [101]. Thus, DCs disappearance from blood of HIV-1 infected patients could be caused by various mechanisms. These events include failure of DC precursors to differentiate, their death due to infection or CTLs and their relocalization into the secondary lymphoid tissues after maturation [55].

Since HIV-1-induced maturation of DCs may contribute to their decrease in the blood of patients with high viral loads, this process may have important consequences for HIV-1 cellular transmission and HIV-1-specific T-cell responses [102]. MDC maturation depends on the stimuli produced in the local environment by HIV-1 activated pDCs [45]. PDCs' IFN-α secretion in response to HIV-1 induces the maturation of bystander $CD11c^+$ DCs (mDCs in human). MDCs maturation is characterized by the induction of CD83 and CCR7 expression and the upregulation of CD80 and CD86 allowing their migration to LNs and Ag-presentation to T cells [45]. However, it has been reported that the T-cell stimulatory function of DCs is impaired in HIV infection possibly due to defective co-stimulatory molecules expression [103]. Accordingly, viral proteins such as Nef and Tat selectively induce immature DC biology that allows DC-T cell interaction without up-regulation of co-stimulatory molecules for effective priming of T cells [104]. Defects in DCs maturation can be beneficial to HIV-1 pathogenesis because immature DCs promote the induction of peripheral tolerance to self-peptides, and HIV may use this function of DCs to inhibit immune responses [50]. In addition, IL-12 is a cytokine involved in the initiation of Th1 type of immune responses and its production depends on DCs maturational state. The altered production of IL-12 by DCs in HIV-1 infection could be involved in the loss of Th1 type immune responses in HIV [15, 73].

Overall, DC abnormalities are important in the induction of immunosuppression caused by HIV [96]. The infection of DCs, their depletion and impaired signaling to T cells participates in shaping the pattern of immune responses in HIV-1 infected individuals. Treatment that reverses the defects caused by HIV in the DC population may improve cell-mediated immunity [92].

9.4. PDC DEFECTS AND IMPLICATION IN HIV INFECTION

The preservation of pDCs and mDCs is crucial for shaping anti-HIV immunity [45]. PDCs expressing CD4, CXCR4 and CCR5 (the major coreceptors of HIV-1) are susceptible to infection by the virus [32]. It is known that HIV infects pDCs as well as mDCs. But, it is not

clear if HIV-1 stimulates pDC through TLRs or after direct entry into the cytoplasm [41, 105]. PDCs respond to HIV-1 infection by producing cytokines such as IFN-α, TNF-α and chemokines, up-regulating cell surface maturational markers, such as MHCII, CCR7 and CD83. Thus, HIV-activated pDCs acquire the ability to migrate in response to CCL19, which functions as the ligand for CCR7 and is expressed in lymphoid organs [60].

Several differences have been reported between pDCs and mDCs in HIV infection. In the case of intravenous HIV infection, pDCs may be one of the first cells to transport virus into LNs. This is due to pDCs direct access from blood to pLNs by trans-HEV migration [56, 64]. Thus, pDCs are involved in rapid trafficking of the virus to LNs where viral spread and replication are most efficient [45, 60]. In addition, HIV-activated pDC migration to lymphoid organs seems necessary for mDC maturation since HIV does not directly activate mDCs, but seem to be activated by cytokines produced by pDCs [45]. Another difference between pDC and mDC in HIV infection is that viral replication in pDC requires their activation by CD40L, while mDCs replication is independent of CD40L mediated maturation [106]. Moreover, HIV-activated pDCs acquire the ability to stimulate naïve $CD4^+$ T cells, but less efficiently than mDCs probably due to the lower upregulation of costimulatory molecules [45]. However, both DC subsets can efficiently transmit HIV to T cells [45, 97].

Clinical findings support pDCs implication in HIV-1 infection. In fact, the decrease of pDCs number in the blood of HIV-1-infected individuals correlates with disease severity as for the reduction in $CD4^+$ T cell counts [32]. This depletion of blood IPC in AIDS patients is not due to a generalized hematopoietic failure. It has been reported that elevated levels of IL-10 as observed in HIV infection could decrease IFN-α production and the frequency of pDCs because IFN-α is a survival factor for pDCs [74].

PDCs seem to play a particular role in the resistance to OI during the course of HIV infection. This is based on many findings that show an increase in pDC number in long-term non-progressor individuals compared to AIDS-developing patients [107, 108]. Moreover, during HAART, an increase in pDC frequency is associated with normalization of $CD4^+$ T cell count, OI resistance and overall clinically apparent immune reconstitution [108, 109]. Accordingly, one study has reported the existence of a correlation between pDC number after one month of HAART and viremia after interruption of HAART. In fact, HAART must be discontinued because of various complications that it induces in treated patients. This correlation suggests that pDC count can be used as a tool to identify individuals in whom HAART interruption can be beneficial [110]. Thus, IPC count is used as a new parameter to monitor the status of the immune system in HIV-infected subjects [11].

An important role for IPCs in immune responses against HIV was first suggested, because of the known inhibitory effects of IFN-α on HIV replication [109]. The level of IFN-α is undetectable in the serum of healthy individuals while this cytokine is increased in acute HIV-infection. However, IFN-α production by total PBMCs has been reported to be decreased during the course of HIV-1 infection [74, 107, 109]. PBMC IFN-α production correlates with pDC frequency in HIV infection as well as in other diseases [107, 109]. Thus, defects in IFN-α production are caused by both pDCs depletion and lower production per cell [32]. The levels of IFN-α in blood during the acute HIV infection correlate with HIV-1 viremia. The progressive decrease of the initially elevated levels of this cytokine coincides with the reduction of viral load and $CD4^+$ T cells. Paradoxically, IFN-α augmentation at late stages of AIDS has been associated with poor clinical prognosis [111, 112].

The role of IFN-α in the control of HIV-1 infection is based on diverse antiviral effects of this cytokine (supplementary figure 10) [69, 80]. The mechanisms of antiviral action of type-I IFNs in HIV infection are either direct or indirect due to the induction of infected cell lysis by NK cells, specific CTLs, or Th1 cells. As previously mentioned in this text, the direct effects of IFN-α are multiple and include the induction of IFN-response genes such as MxA, which decreases HIV-1 replication [69, 80]. HIV-1 induces multiple mechanisms to counteract these effects. For example, HIV-1 Tat protein inhibits IFN-α-induced PKR activity in infected cells [80].

The indirect effects of type-I IFNs include the enhancement of pDCs survival, the induction of mDCs maturation and monocyte differentiation into efficient APCs [113]. In addition, NK cell defects in HIV-infection are partly due to IFN-α responsiveness and/or production [112]. In fact, an important part of the innate immune response to HIV-1 is due to NK cells [112]. NK cells produce IFN-γ that helps eliminating HIV-infected cells through antigen-dependent cytotoxicity. It has been reported that the percentage and the activity of NK cells are decreased in HIV infection [112, 114]. The reduced NK cell cytotoxicity has been found at all stages of HIV infection and this defect is increased during disease progression [12]. Apart from NK cells activation, another indirect effect of IFN-α in HIV is due to the promotion of antiviral Th1 immune responses. HIV-infected pDCs have been shown to induce a Th1 polarization of allogeneic $CD4^+$ T cell responses partly due to IFN-α mediated IL-12R upregulation on $CD4^+$ helper T cells [41, 60]. Thus, since IFN-α is involved in Th1 immune responses, pDCs depletion during HIV infection and their repletion post-HAART could reflect the progressive loss and reconstitution of Th1 pathway, respectively [108] (supplementary figure 12).

On the other hand, pDCs production of IFN-α in thymus may lead to upregulation of MHCI on thymocytes, [55]. Such upregulation may limit HIV replication in the thymus by enhancing the antiviral immunity [74]. However, this response seems not sufficient or optimal for the control of virus replication in this central lymphoid organ [74]. Paradoxically, some studies suggest that the enhanced IFN-α production in HIV-infected thymus participates in the pathogenesis of HIV because it correlates with thymocyte depletion decreasing T cell generation [55]. Despite many theoretical reports and experimental findings, the role of IFN-α in HIV-1 infection is not yet well known. In fact, controversy exists concerning the beneficial effects of this cytokine because treatment of HIV-1 infected patients with IFN-α generated mixed result [74].

9.5. THE USE OF CPG ODNS ON HIV INFECTION

Synthetic CpG ODNs induce IFN-α production by pDCs and favor Th1 response and cell-mediated immunity by $CD8^+$ T cells independently of $CD4^+$ T cell help. Since these parameters are impaired in HIV-1 infection, therapy using CpG appear useful to reverse these defects [47, 74].

As mentioned in the section on pDCs function, the effect of IFN-α produced by pDCs on monocytes has important consequences for the generation of immune responses. In fact, since DCs are at low frequency in the organism, the maturation of monocytes into DCs amplifies total Ag-presenting function of the immune system [7, 47]. Exposure of PBMCs to CpG ODN leads to decreased expression of monocyte marker CD14 and their maturation. Monocytes derived from HIV-1 infected patients, show an impaired maturation in response to CpG. It has been demonstrated that reduced IFN-α production and responsiveness contribute at least in part to defective monocyte maturation in response to CpG [47]. Thus, the reduced IFN-α production, which correlates with HIV replication and viremia, may interfere with the efficacy of CpG in HIV-1 infection [47].

10. THE BIOLOGICAL SYSTEM USED FOR THIS STUDY

The model used for this study is the CD4C/HIV transgenic (Tg) mouse on a C3H background. Either only *nef*-expressing (CD4C/HIV^{mutG}) or, expressing *nef, env* and *rev*-expressing (CD4C/HIV^{mutA}) Tg mice, which show an identical phenotype were used for experimental investigations [115].

10.1. CD4C/HIV TRANSGENIC (TG) MOUSE DESCRIPTION

In order to obtain a small animal model of HIV-1 infection, our laboratory generated a Tg mouse expressing HIV-1 genes (see the section on materials and methods and supplementary figure 13). In these Tg mice, HIV-1 genes were expressed in the same cell subsets as those normally infected by HIV-1 in human. These cells compromise $CD4^+$ T cells and cells of the DC/macrophages lineage [115, 116].

For constructing CD4C/HIV Tg mice, human CD4 gene promoter sequences fused to mouse CD4 gene enhancer was used to express whole HIV-1 coding sequences [74]. Previous studies showed that the regulatory sequences contained in the CD4C promoter can direct the specific expression of a gene in $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes, as well as peripheral $CD4^+CD8^-$ T cells, and macrophages [116, 117]. In this Tg mouse model, HIV-1 gene(s) is expressed in cell populations that are natural targets of HIV-1 infection in humans [116]. This pattern of HIV genes expression is pathogenic in mouse and induces a disease with major hallmarks of AIDS [116]. One study in CD4C/HIV Tg mice revealed that a relatively high level of viral RNA is required to cause the AIDS-like disease and the severity of the disease correlates with the level of transgene expression [116]. In order to examine the implication of individual HIV-genes in the pathogenesis of this disease, several Tg mice expressing selected HIV-1 genes were generated and the expression of *nef* was shown to be required and sufficient for disease induction [115].

In the transgene of $CD4C/HIV^{mutG}$ mice, all HIV-1 genes were rendered non-functional by mutation except for the *nef* gene. The transgene in $CD4C/HIV^{mutA}$ is essentially similar to $CD4C/HIV^{mutG}$ with the exception that besides HIV-1 *nef* gene, it also retains the *rev* and *env* genes of HIV-1 [115, 116].

10.2. AIDS-LIKE DISEASE AND IMMUNE SYSTEM DYSFUNCTIONS IN TG MICE

CD4C/HIV Tg mice develop an AIDS-like syndrome that includes organ diseases; thymus and LN atrophy together with wasting and premature death [116]. The organ diseases compromise interstitial nephritis and segmental glomerulosclerosis in kidney, lymphocytic interstitial pneumonitis in lung and myocytolysis together with myocarditis in heart [116]. The signs and symptoms of AIDS-like disease resemble more closely those observed in pediatric than adult AIDS. This is in accordance with the fact that HIV genes in Tg mice are expressed early in life as it is the case for children with vertically transmitted HIV infection. The immune system dysfunctions in Tg mice include cell surface CD4 down-regulation, loss

of thymocytes, depletion of peripheral $CD4^+$ T cells, an increase in B cells and their activation accompanied by autoantibodies [116, 118, 119]. Tg mice similarly to what is reported in HIV-1 and SIV diseases, show impaired capacity to generate germinal centers (GCs) following primary immunization with OVA. GCs are central regions of primary follicles in lymphoid tissues, which are required for the generation and maintenance of immunological memory and for B cell affinity maturation [1, 118]. One of the main reasons of the impaired GC formation in Tg mice is the decrease in CD40L due to $CD4^+$ T cell depletion and their defective CD40L production [118].

Previously DCs were studied in CD4C/HIV Tg mice but the technique used for their purification excluded pDCs [119]. This study reported a decrease in lymphoid DCs and an accumulation of mDCs in LN. Moreover, splenic DC's Ag-presenting function was found to be reduced in accordance with their immature phenotype. Also, it is known that immature DCs are not able to prime T cells but are permissive for HIV replication [120]. Therefore, DCs characteristics in CD4C/HIV^{mutG} mice suggest a role for Nef in favoring HIV replication while preventing the production of efficient antiviral immune responses. [119].

10.3. HIV-1 PROTEIN NEF

Nef is a 27-35 kilo-dalton (Kda) protein that is common to HIV and SIV [93]. The *nef* gene is unique to the primate lentiviruses [physical and functional]. The HIV-1 *nef* gene encodes a phosphoprotein, detected abundantly in the cytoplasm soon after HIV-1 infects a cell [93]. Nef anchors to the membrane of infected cells by its myristic acid moiety covalently bound to the N terminus. Despite the initial findings suggesting that Nef suppresses viral gene expression (hence the name "negative factor"), it is now clear that Nef is important for HIV pathogenesis [89]. The presence of Nef deletion in some long terminal non-progressor patients implies that Nef may play a role in the persistence of high viral loads in HIV-1 infected patients [89]. In fact, Nef augments viral replication and infectivity through a combination of multiple effects. Nef functions are described in the following paragraphs and include inhibition of apoptosis, CD4 and MHCI downregulation, modulation of cellular signal transduction pathways and stimulation of virus infectivity [93, 116].

The anti-apoptotic effect of Nef contributes to the viral persistence by inhibiting the death of infected macrophages and DCs that serve as a continuous reservoir for the virus [119]. Nef as well as Env and Vpu down-regulate CD4; Nef accomplishes this function by increasing the rate of CD4 internalization from the membrane [121, 122]. CD4 is a type I integral membrane gp that is required for T cell maturation and is involved in the activation of

mature T helper lymphocytes [1]. CD4 allows the stabilization of the interaction between the TCR on T cells and MHCII complexes on APCs and recruits Lck for efficient transduction of the TCR signal [1]. Nef down-regulates CD4 from the cell surface in order to prevent the inhibitory effect of CD4 in viral release from infected cells. In fact, because of its high affinity for the glycoproteins of viral membrane, CD4 traps viral envelop interfering with viral release from infected host cells. Other beneficial effects for HIV caused by CD4 down-regulation include the liberation of Lck that can promote T cell activation, thus favoring viral gene expression [121, 123]. Nef interacts with Lck tyrosine kinase and impairs both proximal and distal Lck-mediated signaling events, thus inducing TCR signaling defect, which is involved in AIDS pathogenesis [124].

A viral strategy to inhibit antiviral T cell responses is due to Nef, which causes MHCI and II downregulation on infected cells including APCs altering their ability to prime T cells [120, 121]. Nef-induced MHCI down-regulation is dependent on a tyrosine found in the cytoplasmic tails of HLA-A and B, by delaying transport of MHC from the endoplasmic reticulum (ER) to the plasma membrane [80, 122]. Nef does not inhibit the expression of HLA-C and –E, necessary to protect from NK-cell mediated lysis [122]. As MHCI is responsible for presenting Ag to CTL, Nef-mediated MHCI down-regulation is part of HIV evasion mechanisms from host immune responses [123]. However, Nef-mediated removal of MHC molecules from APC's surface cannot prevent T cell priming. In fact, peptide-loaded MHC can remain on the cell surface for a short period before its downregulation by Nef, allowing T cell priming [121]. Recently, it has been reported that Nef also mediates loss of costimulatory molecules CD80 and CD86 from the APC surface [119]. This effect was observed *in vitro* in macrophages and DCs from both human and mouse. Nef promotes *in vivo* amplification of infection by inducing cellular pathways of T cell activation [125]. Moreover, HIV-1 Nef can possibly induce conditions required for the entry of the virus into non-activated cells. For example, in infected macrophages, Nef stimulates the production of soluble factors that cause T cell susceptibility to HIV entry [99].

11. RATIONALE AND OBJECTIVES OF THIS STUDY

The aim of this study is to describe the characteristics of pDCs from CD4C/HIV Tg mice, which develops an AIDS-like disease. The CD4C/HIV-Tg mouse model expressing Nef is a useful tool to study Nef-mediated immune dysfunctions in vivo and Nef contribution in HIV pathogenesis. The findings in this mouse model can contribute knowledge about the pathogenesis of human HIV infection. Moreover, since murine pDCs are considered the equivalent of the human pDCs due to many common features, mouse models can be used

to examine pDCs in contexts of microbial infections, tumors, and autoimmune disease [1, 42]. This study focuses on aspects of pDCs functions, which are altered or undetermined in HIV-infected subjects. An important role for IPCs in immune responses against HIV has been suggested [109]. In HIV infection, pDCs are reduced in blood and production of IFN-α is impaired [105, 111]. Defects in IFN-α production seem to be caused by both pDCs depletion and lower production per cell [41]. Thus, delineation of the role of IPCs and IFN-α in HIV-1 infection is important for better understanding of the pathogenesis of AIDS [105].

The rationale of the present study on pDC is based on previous findings showing immune dysfunctions including DC impairments in AIDS-like disease [119]. This study showed that the distribution of lymphoid DCs is different from mDCs in Tg mice [119]. Indeed, lymphoid $CD8\alpha^{+}$ DCs are decreased in LN of Tg mice while myeloid $CD8\alpha^{-}$ DCs are increased in the same organ [119]. This difference suggests that DC subpopulations are differentially affected by HIV-1 gene(s) expression in Tg mice [119]. Accordingly, several findings support the differential effect of HIV-1 on pDCs and mDCs in human [126]. For example, based on in vitro studies, HIV-1 replication in human immature pDC has been shown to require CD40L, while HIV-1 replication in mDC can proceed without CD40L-induced maturation [106]. However, DCs studied in this model did not include pDCs, since DCs identified as $CD11c^{hi}MHCII^{hi}$ cells were negatively selected and purified from lymphoid organs using anti-B220 Ab in order to eliminate B cells. This purification technique excludes pDCs, because they express B220 and low amount of CD11c and MHCII [119].

In addition, HIV-1 infection affects the innate immune system [107, 112]. IFN-α produced by pDCs contributes to innate immune responses in HIV infection because it directly inhibits viral replication in infected cells and stimulates APCs as well as NK cells as described previously. Thus, the decrease in IFN-α during the course of HIV infection participates in the reduction of an innate immune response against the virus. Parallely, since NK cells play a major role in innate immune responses, the quantitative defect of NK cells and their diminished function during HIV-1 infection, which are partly due to the decrease in IFN-α, are parameters involved in the suppression of innate immunity in HIV-1 infection. However, the clinical significance of innate immunity suppression is not well known [107, 112]. In this model, since the viral protein is present in the organism from the embryo stage, the innate immune system could be dysfunctional and may participate in the pathogenesis of AIDS-like disease. The study of pDC as a cell type with major roles in innate immunity can help to reveal this potential impairment of innate immunity in Tg mice.

The objectives of this study is based on previous findings on cDCs alterations in CD4C/HIV Tg mice as well as on various defects of pDCs observed in human HIV-infection. Thus, the expression of the transgene in HIV-Tg mice derived pDCs as well as their frequency, distribution, maturational state, localization, *in vitro* survival and response to immuno-stimulants were examined. In addition, preliminary results were obtained on pDC IFN-α content and their expansion and maturation in the presence of Flt3L.

The frequency and number of pDCs in blood and lymphoid organs were measured because of the clinical reports on AIDS patients correlating these parameters with disease progression [32]. Alterations in distribution and/or localization of pDCs could result from impaired migrational behavior. PDC homing is crucial for their appropriate function and affects the outcome of the host immune response. CD4C/HIV Tg mouse model is useful for examining pDC homing in the presence of HIV protein Nef because the study of pDC localization in HIV-infected humans is not feasible. In addition, since pDC phenotype and response to stimulants reflect their state of differentiation and their functions, the study of these features provides information about pDCs potential implication in the immune dysfunction of CD4C/HIV Tg mice. For example, since IFN-α is crucial for mDCs maturation, the alteration in the production of IFN-α may contributes to mDCs immature phenotype observed in Tg mice [119].

The future work should address questions concerning the effect of pDCs stimulation, expansion and/or depletion on AIDS-like disease by injecting CpG-ODN, Flt3L and/or depleting Abs to pDC into Tg and control nTg mice, respectively. To study pDCs responses to in vivo CpG stimulation, pDCs IFN-α production and morphology changes upon maturation could be measured. Furthermore, in order to directly evaluate the implication of IFN-α in the development of AIDS-like disease, IFNAR-knockout X CD4C/HIV-double Tg mice can be generated and examined for the development of the disease.

Finally, the exploration of molecular mechanisms will be useful in explaining pDCs characteristics in CD4C/HIV Tg mouse model. Indeed, although the ability of pDCs to secrete type I IFNs in response to viruses is known, the signaling events that lead to type I IFN secretion have not yet been elucidated [85]. Thus, the level of expression of molecules such as IRF7, IRF3, STAT3 and Mx proteins could be determined because these molecules are involved in IFN-α signaling pathway and DC maturation [6].

CHAPTER 2: MATERIALS AND METHODS

1. MICE

CD4C/HIV^{mutG} transgene was constructed by fusing 14.4 kbp CD4C promoter (Hanna et al., 1994) to 8.8 kbp BssHII-SacI fragment of HIV-1 NL4-3,I which was mutated in all genes except the *nef* gene, and the simian virus 40 polyadenylation sequences. The CD4C promoter consists of 12.5 kbp upstream sequences of human CD4 gene containing 2.6 kbp of 5' upstream sequences, exon 1, intron 1 and few bases at the beginning of exon 2, as well as the 1.9 kbp mouse CD4 enhancer at the 5' end (Hanna et al., 1998a; Hanna et al., 1998b). CD4C/HIV^{mutA} transgene is essentially similar to CD4C/HIV^{mutG} with the exception that besides HIV-1 *nef* gene it also retains the *rev* and *env* genes of HIV-1 [115] (supplementary figure 12). Sex-matched littermates were used between 6 and 10 weeks of age, because the complete DC system required for adaptive immunity in the mouse is not fully developed until 5 weeks of age [127].

The presence of the transgene was confirmed by Southern blot hybridization of tail DNA with ^{32}P-labeled total HIV-1 sequences as a probe, as described previously [115]. The Tg mice and their non-Tg littermates were housed in the same cages.

2. ANTIBODIES AND REAGENTS

The following antibodies have been used: Rat monoclonal (MC) anti-mouse IFN-α which reacts with and neutralizes IFN-α 1, 2, 4, 5(p), 6(T) and A isotypes, goat anti-mouse IgM-FITC, rat MC anti-mouse CD4-PE and CD4-APC, rat MC anti-mouse CD8-PE, mouse anti-mouse thy1.2-FITC (CD90), rat antimouse CD11b-PE, rat anti-mouse CD86-FITC (B7-2), mouse anti-mouse MC I-A^k (MHC Class II)-PE, rat MC anti-mouse CD25-biotin, hamster MC anti-mouse TCRα/β-FITC and anti-mouse Ly49Q-FITC were purchased from Cederlane Technical. The antibodies 30H12 (anti-thy1.2) was obtained from Ledbetter and Herzenberg (Immunol. Rev. 47: 63-90, 1979), purified rat anti-mouse CD19 (MC), rat MC anti-mouse CD45R/B220-pe-CY5, rat MC anti CD45R/B220-APC, strepavidin PE-CY7, hamster MC anti-mouse CD11c (integrin α_x chain)-biotin-conjugated, hamster MC anti-mouse CD3-FITC and hamster MC anti-mouse CD69-FITC were purchased from BD Pharmingen. Recombinant mouse IL-3 was from R and D Systems. Goat IgM-Texas Red was from Southern Biotechnology Associates (Birmingham, Ala.). Irrelevant rat IgG2a, and rat IgG2b

monoclonal antibodies were used for isotype controls. The generation of the soluble CD8α-CD40L (ligand) has been reported previously [128]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was from X63-mGM-CSF-transfected plasmacytomas [129].

Oligonucleotides containing a fully phosphorothioated CpG motif, called 20620, is a C-Class oligo and was a gift from Dr Arthur M.Kreig (Coley Pharmaceutical Group) and used at 5 µg/ml after titration and as suggested by the sender.

Mouse Flt3L cDNA was previously cloned in our laboratory in pcDNA3 vector containing puromycin selection marker (supplementay figure 14.B). 293T cells were transiently transfected with 10 µg of Flt3L cDNA using the calcium phosphate precipitation method [130]. Supernatant was collected 48h later, aliquoted and frozen until use. The same 293T cells were also transfected with an empty plasmid (containing no insert) as control. The supernatant obtained from culture of these cells in the same conditions were used as a negative control and pDC generation was measured by FACS (see supplementary figure 13.B)

3. PURIFICATION OF PDC FROM SPLEEN AND LYMPH NODES

To purify pDCs, we employed isolation and enrichment procedures similar to those used to purify mouse DC [119]. Briefly, spleens were meshed and single cell suspension was prepared. This was followed by rad blood cell (RBC) lysis by resuspending pelleted cells in 5 ml of Gey's solution for 5 minutes. Cell suspensions from subcutaneous LN were prepared followed by mild enzymatic digestion with collagenase type 4 (Worthington 4188) at 8mg/ml and DNase I (Sigma DN25) at 10mg/ml. Cell suspensions from 3 or 6 mice were pooled from either spleen or LN and light-density cells were selected using a percoll gradient. Contaminant cells were removed by either immunomagnetic bead depletion or during FACS analysis by gating out of $CD19^+$ and $Thy1.2^+$ or IgM^+ cells [50, 119] (figure 1.A). All these steps of purification were done under sterile conditions if cells were to be put in culture.

4. GENERATION OF BONE MARROW-DERIVED PDC

DCs were derived from BM culture with the use of advanced method of BM-derived DC (BMDDC) enrichment [131]. Precisely, BM-derived cell suspension was obtained by flushing the femurs and tibias of mice sterilely. Total cells were seeded at 2 X 10^6 per 100 mm Petri

dish (Falcon) in 10 ml of medium. The medium used was RPMI-1640 (Gibco-BRL, Life Technologies) supplemented with 10% inactivated fetal bovine serum (CanSera, Rexdale, Ontario), L-glutamine (Gibco-BRL), 2-mercaptoethanol, and antibiotics. Supernatant from X63-mGM-CSF cells was added at final dilution of 10% (=20ng/ml). At day 3, 6 and 8 half of the culture supernatant was collected, centrifuged and cell pellets were resuspended in fresh media (the same as described above), and added to the culture (see supplementary figure 13.A for FACS results on non-adherent cells at day 10). At day 10 post-enrichment, non-adherent cells were collected (refered to post-enrichment in Table-II) by gentle pipetting, centrifuged and resuspended in the same type of media but in different conditions for further maturation. Stimulated conditions (See results table-II) contained 5% GM-CSF, 5% CD8α-CD40L and 0.1ng/ml of rIL-3, whereas in medium alone (MA) no cytokine was added [50]. Following over-night O.N. culture, non-adherent fraction of cells was obtained and determined to be DCs (CD11c^{+}) including pDCs by FACS.

For Flt3L enrichment of BM-derived cells, 1 X 10^{6} cells per ml were seeded in a 60 mm petri plates in a total volume of 5 ml medium. The medium was the same as described above supplemented with Hepes buffer. Supernatant containing Flt3L was added at 20% concentration ratio to the media. Titration of this supernatant compared to commercial Flt3L reveald that the concentration used in BM cultures correspond to 35 to 40 ng/ml [46]. Every 3–4 days, 2.5 ml of medium was replaced with fresh medium and Flt3L, as in advanced method mentioned above. At day 9, further maturation was done O.N.; half of the culture was further matured with CpG (at 5 µg/ml) and the other half was used as MA [50]. A second petri was prepared at day 0 and collected at day 9 and examined as post-Flt3L enrichment.

5. IMMUNOFLUORESCENCE

Mouse organs were embedded in OCT compound, frozen in isopentane and stored at -80°C. Serial cryosections were prepared, fixed in 95% acetone at -20°C for 20 min, dried at room temperature, and stored at -20°C until further analysis. For immunolabeling, sections were rehydrated in PBS (Life Technology), blocked with PBS + 20% FCS$_{I}$ and then sequentially incubated with primary and secondary antibodies in a humidified dark chamber. Slides were mounted with vectashield aqueous solution (Vector Laboratories, Inc. Burlingame, CA), and analysis was performed by either confocal microscopy (Zeiss LSM 510) or with a Zeiss Axiovert S100 TV microscope [119].

Intracellular IHC was performed on cytospins of freshly sorted pDCs from spleen of 6 pooled Tg and 6 pooled non-Tg mice as described previously. The cells were permeabilized by 0.05% saponin in PBS solution prior to blocking and staining. All steps of IHC were performed using 0.05% saponin in PBS solution.

6. QUANTITATION OF PDCS BY IMAGE ANALYSIS

Serial cryosections of LNs and spleen from non-Tg (n = 6) and Tg (n = 6) mice 2 to 5 months of age were prepared and labeled with biotinylated anti-CD11c MC Ab (Pharmingen) and B220-FITC followed by streptavidin-Alexafluor 488-conjugate (Molecular Probes). Slides were mounted with Vectashield solution. Sections were scanned and color images were captured with a Zeiss Axiovert S100TV or confocal microscope (Zeiss LSM510). Analysis was done with Northern Eclipse 6.0 software and LSM browser respectively. PDCs were visualized as yellow cells by superimposing images taken in red with the same image in green ($CD11c^{+}B220^{+}$). For study of lymph nodes, images of two LNs per animal were taken in the cortical and paracortical zones (mean of ten images by 40x objective per animal). For studies of the spleen, images were taken in the white pulp (mean of ten images by 40x objective per animal). In the spleen, one T zone was visualized per field. The nonspecific background labeling was subtracted prior to data acquisition. Quantitation of the area taken up by yellow fluorescent cells (square micrometers) was calculated and compared between non-Tg and Tg mice [119].

7. FLOW CYTOMETRY

Immunolabeling was performed on ice in FACS buffer (1x PBS, 1% bovine serum albumin, and 0.01% sodium azide), and fluorescence intensity was analyzed with a FACScan apparatus (Becton Dickinson, San Jose, Calif.). Blood sample for FACS analysis was obtained from retro-orbital vein using a heparinized capillary. Blood was collected in a ependorff tube containing 30 µL of EDTA (5mM) in PBS for each capillary added. This was followed with RBC lysis and stained as other samples.

8. IN SITU HIBRIDIZATION (ISH)

ISH is the cellular localization of specific nucleic acid sequences (DNA or RNA), using a labeled complementay strand. This technique was performed on cytospins of freshly isolated sorted cells, using ^{35}S-UTP-labeled antisense and control sense RNA probes as

described previously [116]. Cells from non-Tg control animals hybridized with antisense probes, as well as Tg animal tissues hybridized with sense probes, failed to exhibit any specific hybridization signal.

9. GIEMSA STAINING OF PURIFIED PDC

For cytological analysis, cytospins of freshly isolated FACS-purified $CD11c^{lo}B220^{+}$ cells were prepared (500 *g*, 5 min), air dried, fixed in methanol and stained first with May Grunwald and subsequently with Giemsa solution [32, 50].

10. CYTOKINES AND STIMULANTS OF PERIPHERAL PDCS

Cell suspensions from 3 or 6 mice were pooled from either spleen or LN and light-density cells were selected using a percoll gradient under sterile conditions. The recovered cells from pLN and spleen were matured for 24 hours following the same conditions as for BM described above [11, 50]. Stimulated conditions contained 5% GM-CSF, 5% CD8α-CD40L and 0.1ng/ml of rIL-3, whereas in medium alone (MA) no cytokine was added [50].

11. FETAL LIVER CELL TRANSPLANTATION: CHIMERA MOUSE MODEL

Congeic strains of mice that differ at the CD45 (common leukocyte Ag) locus were used to construct transplantation chimera mouse model. MC Abs specific for the two allelic variants of the CD45 molecule can identify cells derived from different donors. Since this Ag is expressed on all nucleated blood cells, CD45 is a genetic marker for following the progeny of transplanted fetal liver cells. Transplantation of fetal liver cells, similarly to BM cells, establishes hematopoiesis driven cells by genetically marked cells in the irradiated recipient mouse [132].

This Table shows the breedings for the generation of embryos used as donors

Breeding	**$CD4C/HIV^{mutG}$ Tg, $CD45.2^{+}$**		Beeding	**C3H, nTg, $CD45.1^{+}$**	
C3H, nTg, $CD45.2^{+}$	nTg, $CD45.2^{+}$	Tg, $CD45.2^{+}$	**C3H, nTg, CD45.2+**	nTg, $CD45.1^{+}$, $CD45.2^{+}$	nTg, $CD45.1^{+}$, $CD45.2^{+}$

Donors: Fetal livers from 14.5 day old embryos (E14.5) coming from the following breedings [(nTg (C3H, CD45.1^{+}) x nTg (C3H, CD45.2^{+}), and (Tg (CD4C/HIVMutG, C3H CD45.2^{+}) x nTg (C3H, CD45.2^{+})] were harvested. Single cell suspension was made in HBSS supplemented with 10% FBS, under sterile conditions, with a syringe plunger. Cell suspension was filtered through a Nytex mesh (BSH Thompson, Montreal). Remaining fetal tissue was typed for transgene expression by PCR. When typing was known, the following mixes were prepared [A: HIV Tg (C3H, CD45.2^{+},50%) mixed with nTg (C3H, CD45.1^{+} CD45.2^{+}, 50%) and B: nTg (C3H, CD45.2^{+}, 50%) plus nTg (C3H, CD45.2^{+} CD45.1^{+}, 50%)]. After one wash, cells were counted (RBC cell lysis was performed in the counting aliquot). Cells were then resuspended in HBSS solution supplemented with 2% FBS at a concentration of 20x106 cells per mL.

Hosts: C3H/HeN (CD45.2^{+}) hosts (8 to 12 week-old) were lethally irradiated (950 Rads) using Mark I-68A1 Irradiator (Cs-137, J.L. Shepherds & Associates). Hosts were injected, via the tail vein, with $4x10^{6}$ fetal liver cells in 0.2 mL of HBSS solution supplemented with 2% FBS 4-6 hours after irradiation. Hosts were assayed 2 to 3 months after transplantation [133].

12. PCR TECHNIQUE FOR TRANSGENE DETECTION

A piece of fetal tissu was placed in 200 µL of lysis buffer (0.1M NaCl, 0.01M EDTA, 0.05M Tris pH 7.5, 0.5% Nonidet 40 and 0.05% of Tween 20) supplemented with 10 L of proteinase K (10 mg/mL) and digested at 55°C for 20 min. to 2 hours. After digestion, lysates were centrifuged to pellet non-digested tissue and 100 µL of supernatant was taken. This aliquot was heated at 100°C for 8 min, further diluted (10 µL in 200 µL of water) and further heated at 55°C for 30 minutes. PCR reaction was performed with nef specific primers (sense primer: CATGGAGCAATCACAAGTAG, antisense primer:

GGTACTAGCTTGAAGCACCA). Detection of the mouse myb gene using sense primer: CCAGTCACGTTCCCTATCCT and an anti-sense primer:GCCTGCTGTCCCTTCAGCTC was done as a control. The PCR was performed under the following conditions (40 seconds at 94°C, 1 minute at 54°C, 30 seconds at 72°C for 1 cycle, followed by 40 seconds at 94°C, 45 seconds at 54°C, by 1 minute at 72°C for a total of 24 cycles was performed.

12. STATISTICS

Statistical analysis of results was done with two-tailed Student's t test. P values of <0.05 were considered significant. Data are means ± standard deviation (SD).

CHAPTER 3: RESULTS

1. IDENTIFICATION AND MORPHOLOGY OF PDC FROM LYMPHOID ORGANS: TG MICE DERIVED PLN PDC EXPRESS HIGHER LEVEL OF CD11C ON THEIR SURFACE AND DISPLAY UNUSUAL LIGHT SCATTER FACS PARAMETERS COMPARED TO THEIR EQUIVALENTS FROM NTG MICE. THESE CHARACTERISTICS ARE SIMILAR BETWEEN TG AND NTG MICE-DERIVED SPLENIC PDC.

In order to identify pDC in secondary lymphoid organs, total pLN-derived cells as well as splenocytes have been used for DC enrichment as mentioned in the section on materials and methods. Mice were used between the ages of 6 to 10 weeks because the complete DC system required for adaptive immunity in the mouse is not fully developed until 5 weeks of age [127]. DC-enriched cell-suspensions were stained with multiple Abs against cell surface markers of pDCs allowing their identification by the use of multicolor fluorescence-activated cell sorter (FACS). As shown in figure 1A.b PDCs were defined as $CD11c^{lo/int}B220^{+}IgM^{-}$ cells among live cells. An Ab against IgM was used in order to exclude contaminant B cells that have not been eliminated by DC enrichment technique (Figure 1A.a). Also, the exclusion of contaminant B cells, which share with pDCs the expression of B220 with other markers such as CD19, was less efficient for pDC population identification by FACS (figure 1A.c).

In terms of light scatter characteristics that are indicators of cell size (FSC) and cell granularity (SSC), pDC show the expected FACS profiles consistent with their reported morphology resembling plasma cells [38]. As observed in figure 1B, pDCs correspond to intermediate to large size cells with low granularity that is representative of their round shape and absence of dendrites. Accordingly, Giemsa staining of freshly sorted splenic pDCs shows no difference in the morphology of Tg-derived and nTg-derived pDCs (figure 1C).

In contrast to splenic pDCs shown in figure 1B, pLN-derived pDCs from Tg mice express higher levels of CD11c than their equivalents from nTg mice (figure 1D). Moreover, $CD11c^{hi}$ pDCs (green dots) observed among Tg pLNs-derived cells show an unusual light-scatter pattern with a wide range of SSC, reaching higher levels than $CD11c^{lo}$ pDCs (purple dots). This could be indicative of the presence of dendrites on $CD11c^{hi}$ pDCs, which correspond to cells at a more mature stage of development than $CD11c^{lo}$ pDCs. Since the stage of maturation of pDCs is associated with their function [50], the shift in CD11c expression of

pLN-derived pDCs may be due to a functional change in pDC population in Tg mice pLNs, which is the site where the immune response is induced.

FIGURE 1: PDC IDENTIFICATION AND MORPHOLOGY

Figure 1.A: PDC identification by FACS

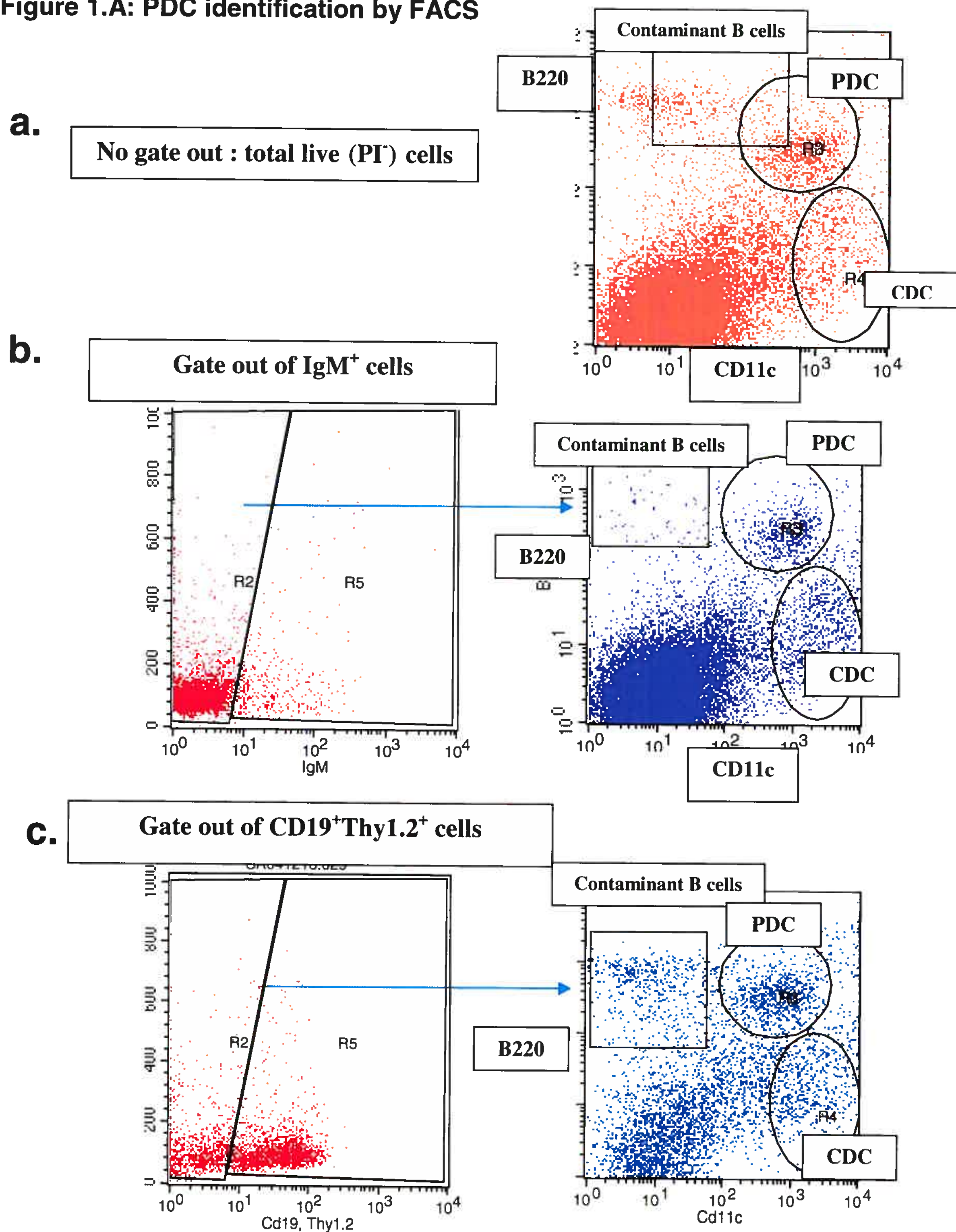
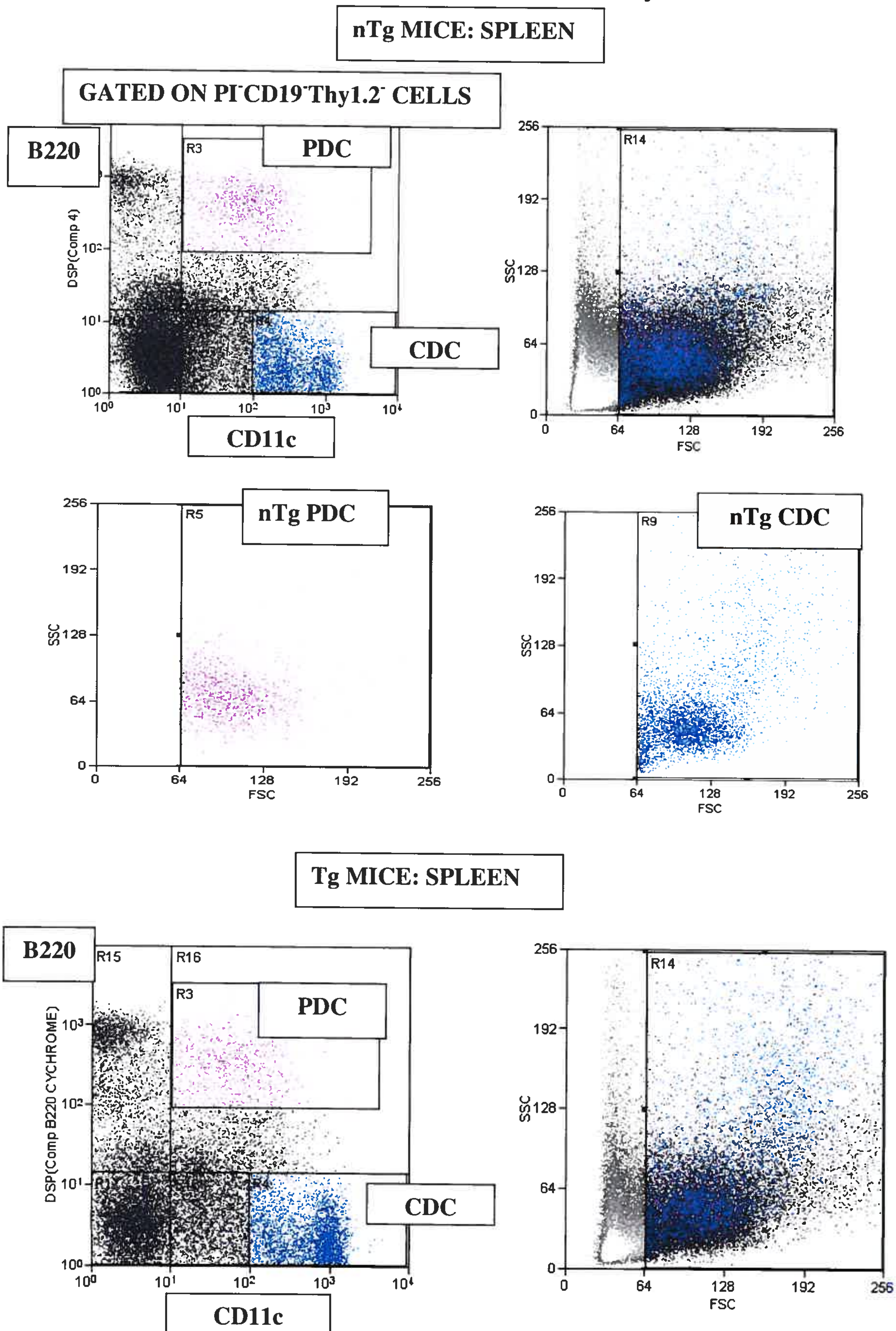


Figure 1.B: Splenic pDCs light scatter characteristics by FACS



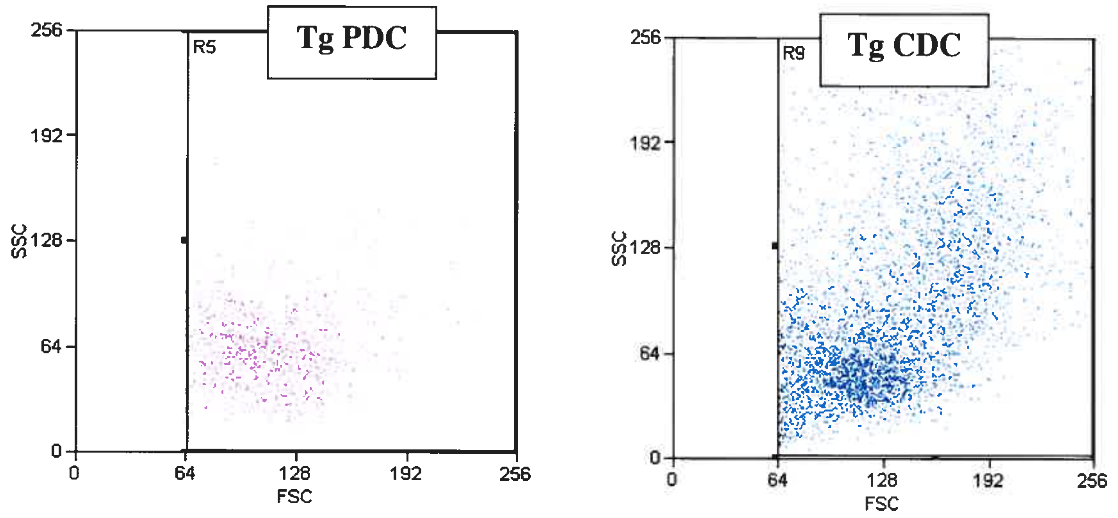


Figure 1.C: Giemsa staining: PDC morphology

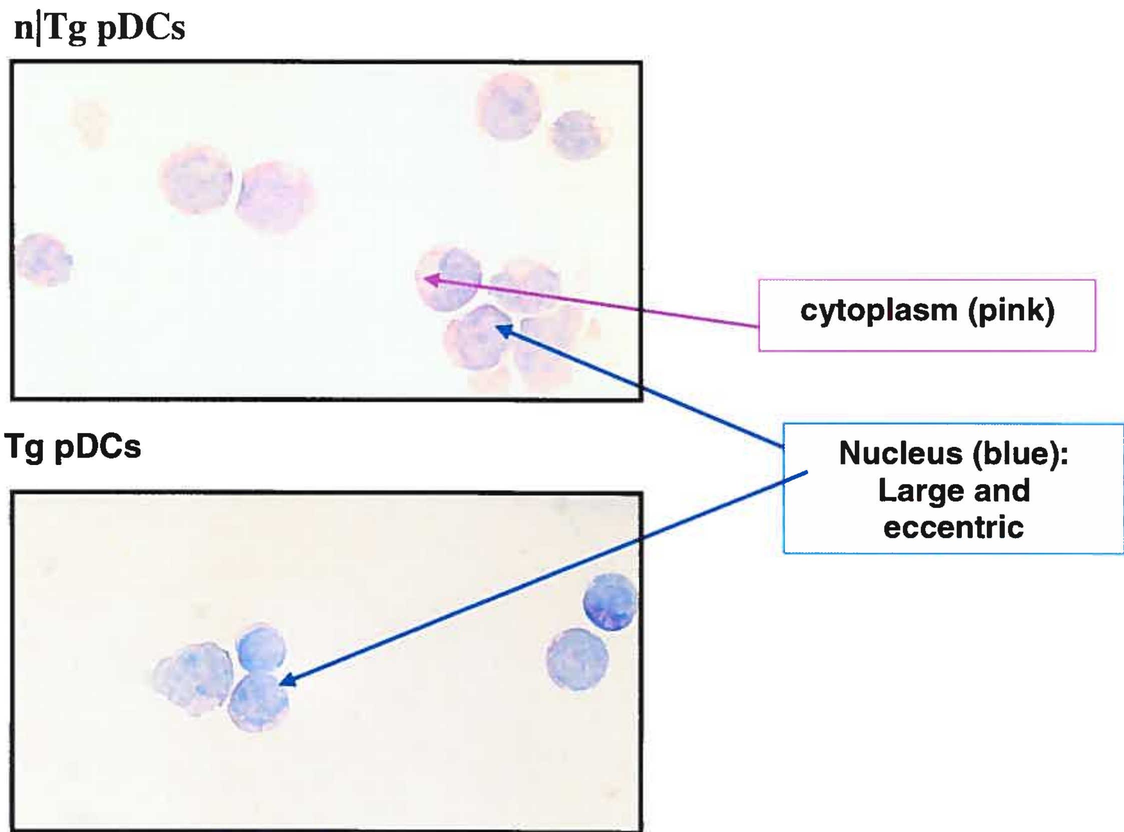


Figure 1.D: PLNs pDC light scatter characteristics by FACS

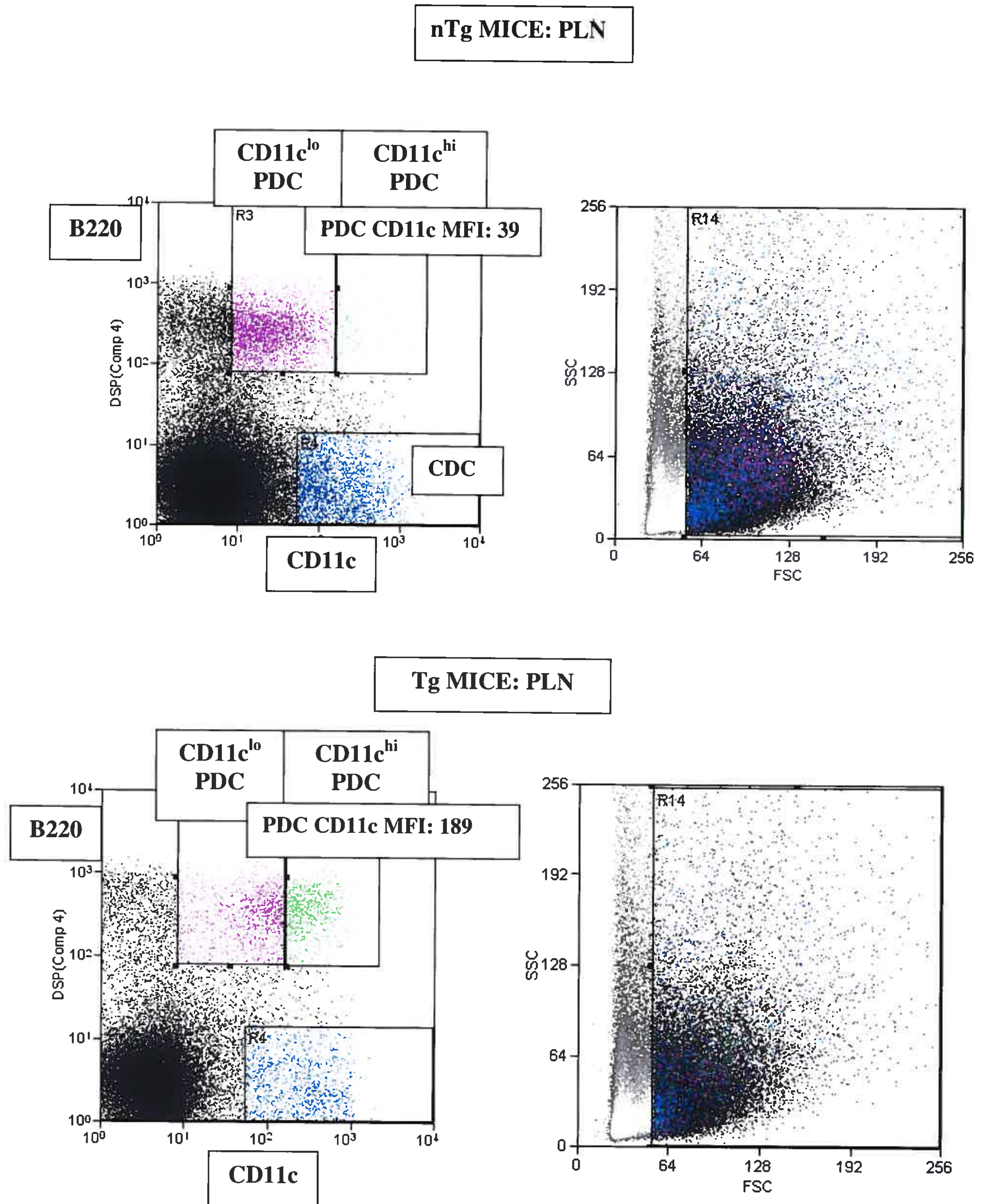


FIGURE 1. PDC identification and morphology

A) In all experiments in this study examining pDCs from peripheral lymphoid organs, cell suspensions were prepared from spleen or pLNs of a pool of 3 to 6 Tg and non-Tg, sex- and age-matched littermate mice and enriched for DCs as described in materials and methods. Contaminant B cells that have remained in DC-enriched cell suspensions after percoll separation are represented by $CD11c^{-}B220^{+}$ cells shown in figure 1.A.a. These cells were eliminated during FACS analysis since they were identified by the use of different B cell makers apart from B220 expressed on pDCs [50]. After gating on live cells, which do not stain with propidium iodide (PI), a marker of dead cells, IgM^{+} cells were eliminated by gating and among IgM^{-} cells pDCs were identified as $CD11c^{lo}B220^{+}$ while cDCs were defined as $CD11c^{hi}B220^{-}$ cells (figure 1.A.b.). It should be noted that cDCs include $CD11b^{-}CD8\alpha^{+}$ lymphoid and $CD11b^{+}CD8\alpha^{-}$ myeloid DCs. The FACS panels in a, b and c in figure 1.A. correspond to FACS analysis in which B cells were not gated out, IgM^{+} cells were gated out and $CD19^{+}thy1.2^{+}$ cells were gated out respectively. Comparing the presence of contaminant B cells in figures 1.A. a, b and c allows visualizing that the least contaminant B cells is obtained when IgM co-staining is used to distinguish pDCs from B cells.

B) This figure shows FACS panels on splenic pDCs derived from both Tg and nTg mice in order to reveal their light scatter characteristics. While blue dots correspond to cDCs with a wide range and reaching higher levels of SSC and FSC values, purple dots correspond to pDCs with lower SSC values and more homogeneous FSC values than blue dots (cDCs).

C) Freshly sorted splenic pDCs identified as $CD11c^{lo/int}$ $B220^{+}IgM^{-}$ cells (as shown in figure 1.A.b.) were cytospined and stained with Giemsa in order to visualize their morphology. Images from Tg and nTg derived pDCs show their round morphology lacking dendrites and an eccentric and large nucleus.

D) This figure shows FACS panels on pLNs pDCs derived from both Tg and nTg mice and reveals their light scatter characteristics. In Tg mice pDCs show a shift to higher expression of CD11c compared to nTg as reported by mean fluorescence intensity (MFI) of CD11c expression. These $CD11c^{hi}$ pDCs (green dots) possess higher SSC

values than CD11c^{lo} pDCs (purple dots), mimicking cDCs (blue dots) light scatter pattern since SSC mean values of CD11chi pDCs is closer to cDCs than to CD11clo pDCs.

2. THE BIOLOGICAL SYSTEM

CD4C/HIV Tg mice develop a severe AIDS-like disease characterized by the progressive loss of CD4^{+} T cells [116]. In figure 2, some of the clinical features of these Tg mice can be observed. The clinical observations of CD4C/HIV Tg mice include hypoactivity, lower body size and weight, ruffled hair and curved back when compared to normal mice. At autopsy, macroscopically observed signs compromise spenomegaly with a dark color and irregular surface spleen as well as shrunk and pale kidney with granular surface [116]. CD4C/HIV^{mutA} Tg mice express *rev, env* and *nef* genes of HIV-1 and their phenotype is the same as CD4C/HIV^{mutG}, which express *nef* only. Both of these Tg mice develop the same AIDS-like disease with similar course as described in the introduction.

FIGURE 2: THE BIOLOGICAL SYSTEM

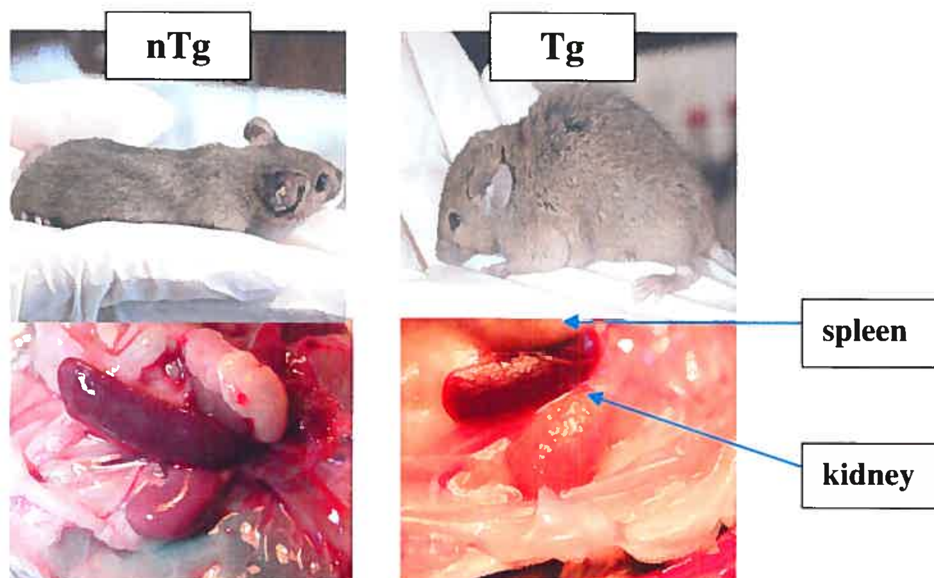


FIGURE 2.

CD4C/HIV^{mutG} Tg mice appearance is compared to nTg mice. In the upper panels, some of their clinical abnormalities such as the lower body size, ruffled hair and curved back can be observed. The lower panels show macroscopic signs of the AIDS-like disease

including dark color and irregular surface spleen as well as shrunk and pale kidney with granular surface.

3. TRANSGENE EXPRESSION IN PDC: A MAJOR PROPORTION OF PDCS IN CD4C/HIV TRANSGENIC MICE EXPRESS NEF.

It has been reported that DCs from CD4C/HIV^{mutA} express the Tg but the technique used for DC purification in this model excluded pDCs [119], and therefore no information is available on pDCs transgene expression. In order to be consistent with this previous study on DCs, CD4C/HIV^{mutA} mice were used to assess Tg expression on pDCs. The transgene expression on pDCs was examined by three different techniques. The transgene was detected by *in situ* hybridization (ISH) (Figure 3A) as well as immunohistochemistry (IHC) (Figure 3C) on sorted splenic pDCs. Transgene positive cells were counted on slides from ISH and the compiled results show that 50 to 60% of splenic pDCs express the Tg (Figure 3B). Moreover, on a per-cell basis, moderate expression of the transgene was detected on pDCs compared to high expressing thymocytes by counting the number of silver grain deposits on positive cells.

In addition, transgene expression on pDCs was measured by a direct technique using CD4C/GFP-Nef X CD4C/HIV^{mutG} double Tg mice were used. Since green fluorescent protein (GFP)-Nef Tg mice express low levels of Nef and do not develop an AIDS-like disease, this mouse model cannot be used for retracing Nef-expressing cells in a diseased organism. In order to obtain a mouse model developing the AIDS-like disease and concomitantly allowing the detection of Nef expression by FACS based on GFP co-expression, CD4C/GFP-Nef mice were bred with CD4C/HIV^{mutG} mice and double Tg CD4C/GFP-Nef X CD4C/HIV^{mutG} mice were produced in our laboratory. These double Tg mice develop an AIDS-like disease and allow the delineation of Nef expressing cells. FACS analysis on pDCs derived from these mice show that 40% of splenic and 25% of pLNs pDCs expressed GFP (figure 3D). Based on these findings the level of transgene expression by pDCs in pLN is lower compared to pDCs in spleen.

FIGURE 3: TRANSGENE EXPRESSION IN PDC

Figure 3.A: PDC transgene expression by ISH

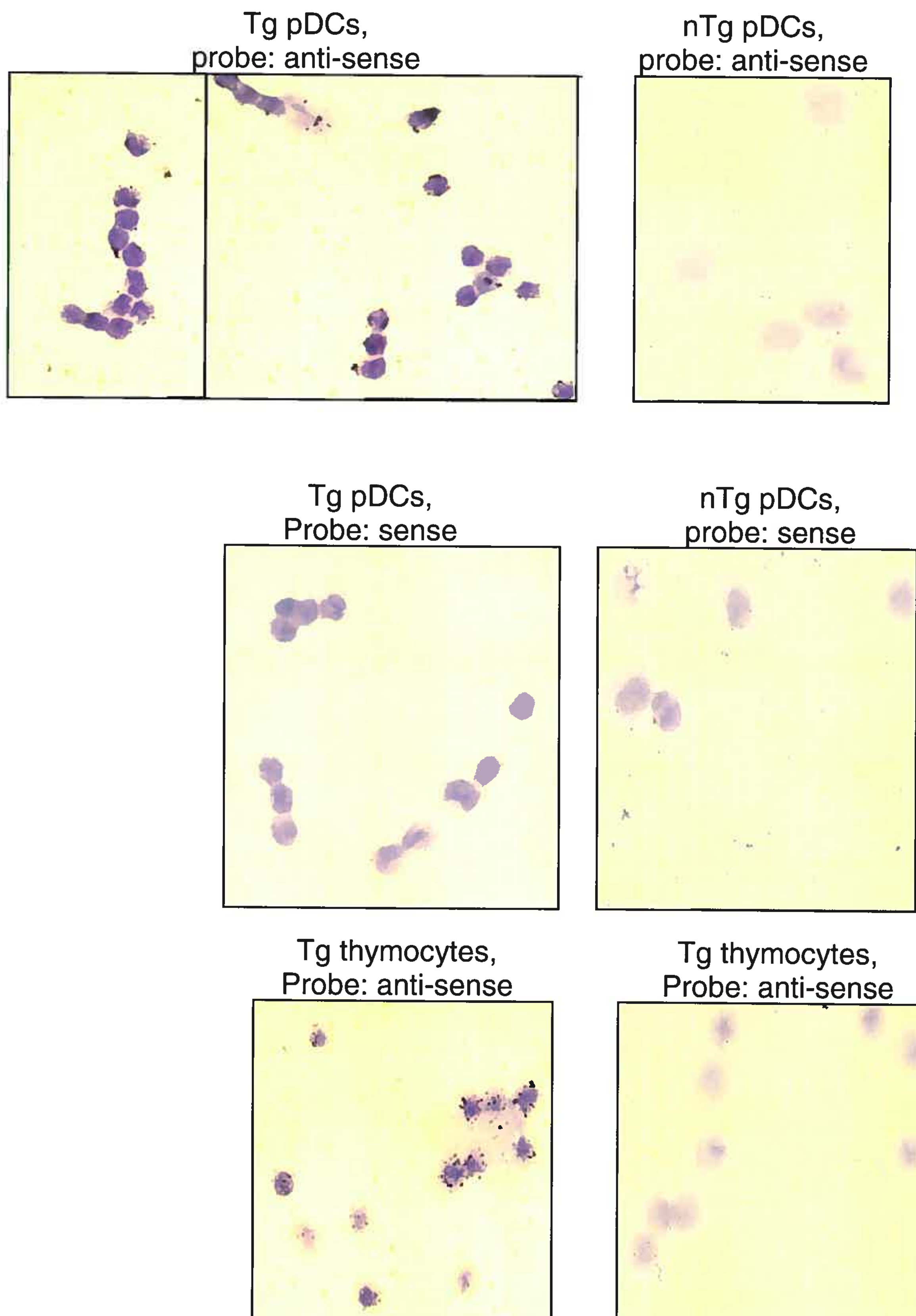


Figure 3.B: Transgene expressing pDCs in Spleen (by ISH)

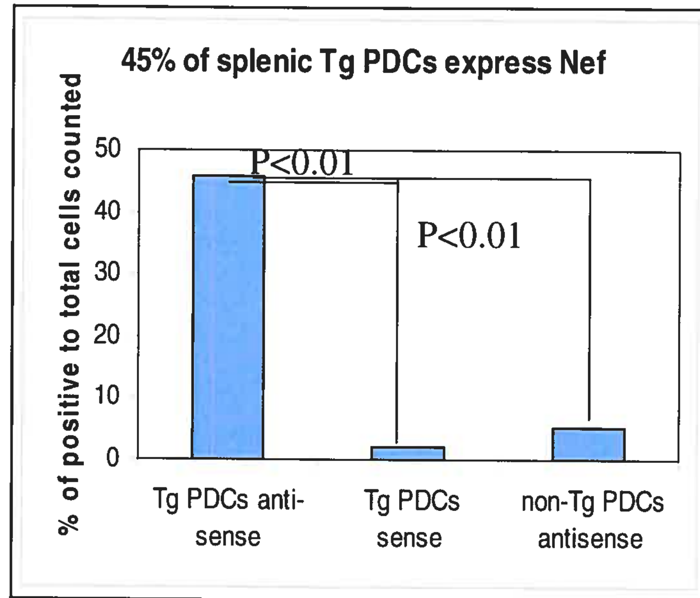
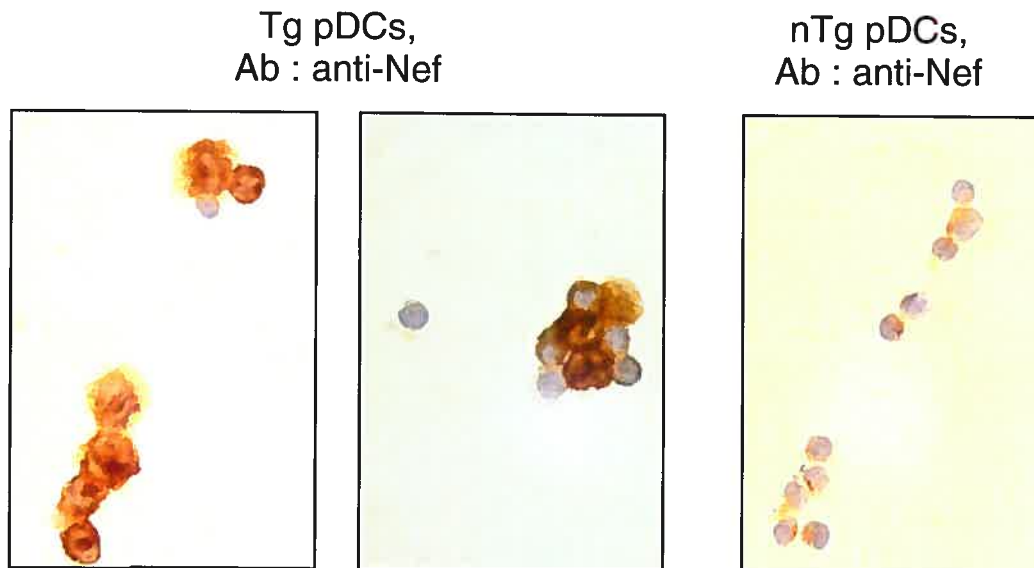


Figure 3.C: PDCS transgene expression by IHC



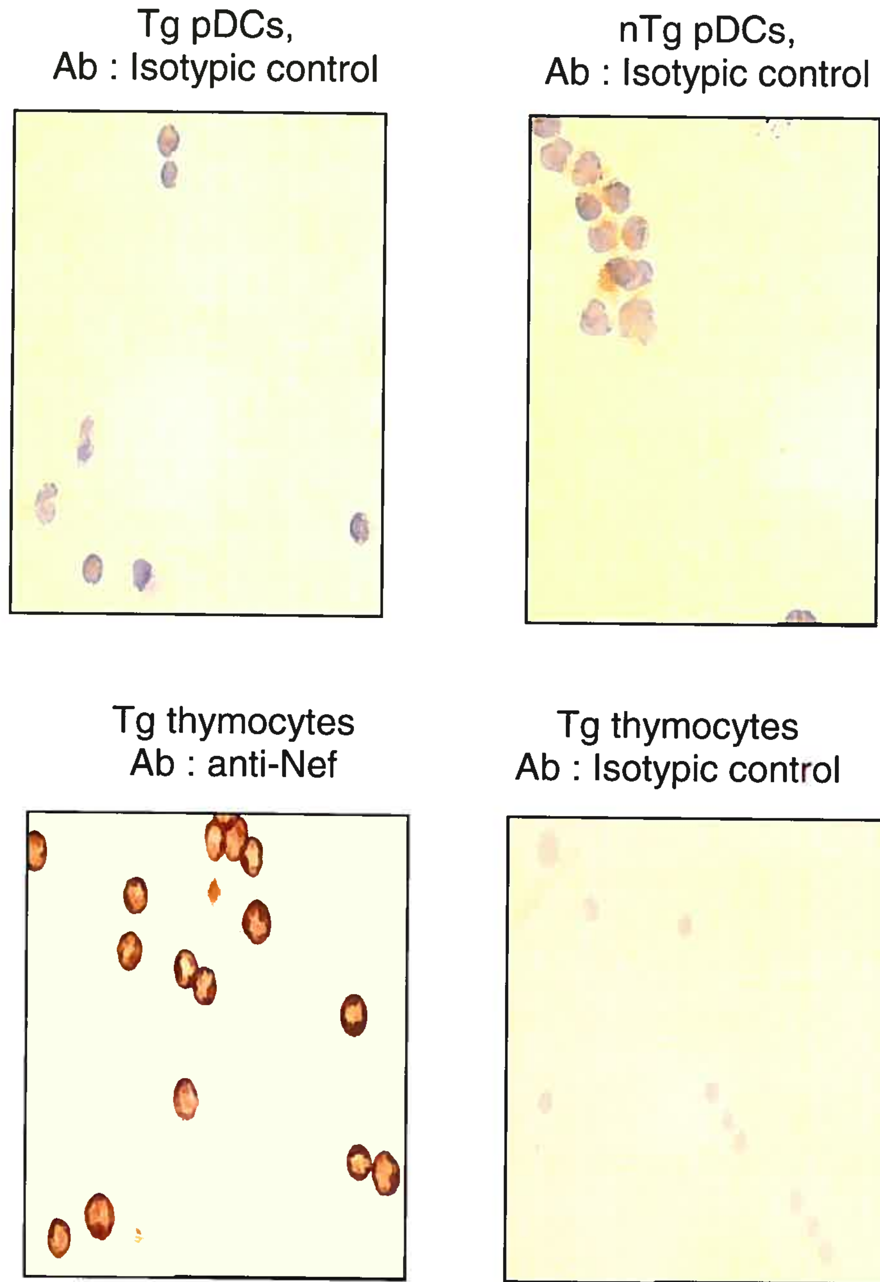


Figure 3.D. PDCs transgene expression by FACS

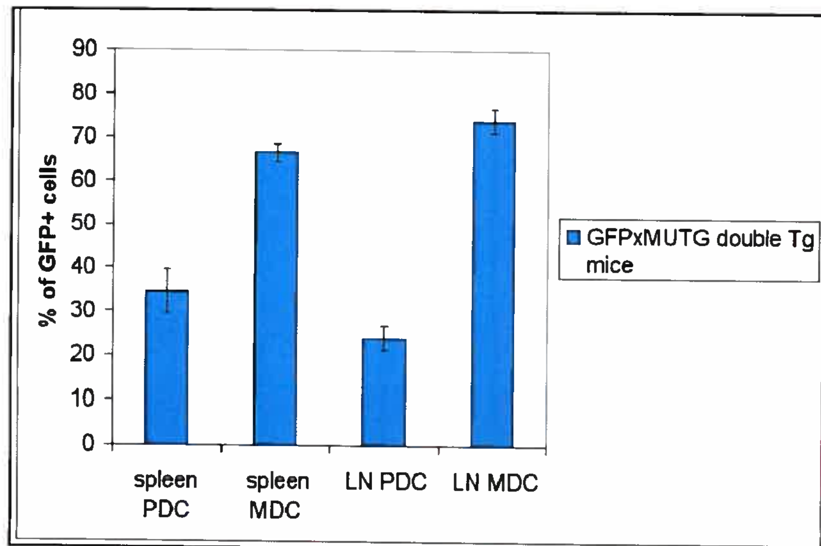


FIGURE 3. Tg expression in pDC

A) ISH technique was performed on freshly sorted splenic pDCs based on their identification as described in figure 1.B. After cytospin, sorted pDCs were examined for transgene expression by ISH with 35S-labeled HIV-1-specific anti-sense and control sense riboprobes [116]. Sorted thymocytes were used as a positive control as they are known to express high levels of the transgene [116]. PDCs from nTg animals hybridized with antisense probes, as well as Tg mice-derived pDCs hybridized with sense probes, failed to exhibit any specific hybridization signal. The number of experiment for ISH as well as for IHC was 1. The experiment was done on splenic pDCs derived from a pool of 3 sex- and age-matched Tg (CD4C/HIVmutA) and non-Tg littermates.

B) ISH results were compiled by a cell count done on 600 cells and the results are shown in the graph. Cells with 2 or more silver grains were considered as transgene positive cells since based on previous experiments with this technique unspecific labeling can be excluded in this way.

C) IHC were performed on freshly sorted cytospined splenic pDCs stained with an anti-Nef Ab. Thymocytes were stained as a positive control as they are known to express high levels of the transgene [116]. Staining with isotypic control Ab. was done on both Tg and nTg pDCs to show non-specific binding.

D) FACS analysis was done on DC-enriched cell suspension derived from lymphoid organs of CD4C/GFP-Nef X CD4C/HIVmutG double transgenic mice. Nef-expressing pDCs correspond to GFP^+ cells among total pDC population identified as described in figure 1.B. The graph shows the compilation of results obtained from 2 independent experiments. The variation in the percentage values of GFP^+ pDCs between the two experiments is shown as SD represented by y-axis error bars.

4. PDCS FREQUENCY, DISTRIBUTION, PHENOTYPE AND IN VITRO SURVIVAL: IN CD4C/HIV TG MICE, PDC FREQUENCY IS INCREASED IN THE SPLEEN AND REDUCED IN PLN AND THEIR IN VITRO SURVIVAL AS WELL AS THEIR PHENOTYPE IS ABNORMAL

4.1. FREQUENCY AND DISTRIBUTION

Splenic pDCs absolute cell number as well as percentage is significantly increased while pLNs-derived pDCs are diminished in Tg mice (Table I). Therefore, pDCs distribution is abnormal in Tg mice. PDCs higher frequency in spleen in this model is similar to the increase observed in mice soon after viral challenge (MCMV) corresponding to the time of maximal IFN-α production [34]. For data obtained on pLNs-derived pDCs, it should be taken into consideration that pLNs in Tg mice are atrophic and the total cell number is significantly reduced compared to pLNs from normal mice [116].

Blood pDCs are different from pDCs derived from all lymphoid tissues since they are principally composed of $CD4^{-}CD8^{-}$ subpopulation [50]. As mentioned in the introduction, blood pDCs are immature precursors of pDCs residing in lymphoid tissues and based on FACS results, the percentage of blood pDCs is about 2 folds higher in Tg mice (Figure 4). This result could partly explain pDCs augmentation in spleen as they may be increased in the red pulp. However, experiments visualizing pDCs in the red pulp are necessary to confirm this hypothesis.

4.2. *IN VITRO* SURVIVAL

It is known that DCs die rapidly in culture in MA while granulocyte-macrophage colony stimulating factor (GM-CSF), and/or rIL-3 enhance their survival [1, 50]. CD40L is a maturational stimulus for pDCs [57]. The response of splenic pDCs to a combination of immuno-stimulants was examined in terms of their survival. For this, the absolute cell number as well as the frequency of pDCs was compared between two culture conditions, stimulated and MA (table II). While nTg mice-derived splenic pDCs show a slightly higher survival in terms of absolute cell number in stimulated condition compared to MA, Tg pDCs survive less in response to in vitro stimulation than to MA. Based on these data, Tg pDCs show a defective survival response to immunostimulants *in vitro*.

4.3. PHENOTYPE

CD4 and CD8 expression on pDCs is used to distinguish four functionally distinct subpopulations [50]. The expression of these markers were measured on pDCs derived from peripheral lymphoid organs of both Tg and nTg mice. As shown in figure 5A and 5B, the expression of CD4 is drastically decreased on Tg pDCs compared to nTg pDCs derived from both pLNs and spleen. This decrease is observed as well in the percentage of pDCs expressing CD4 as in their level of cell surface expression of CD4 as reflected by MFI values. The expression of CD8 (percentage and MFI) is also decreased in Tg-derived pDCs compared to nTg-derived pDCs, but to a lesser extent than CD4.

TABLE I: PDCS FREQUENCY AND DISTRIBUTION IN LYMPHOID ORGANS

EX VIVO	PDC	
SPLEEN	%	# X 10^5
nTg	3.08 ± 0.26	1.2 ± 0.05
Tg	5.23 ± 0.38	4.8 ± 0.04
PLN	%	# X 10^4
nTg	1.38 ± 0.06	3.03 ± 0.63
Tg	0.68 ± 0.09	0.24 ± 0.09

TABLE-I: PDCs were identified as described in figure 1.B. and recovered from peripheral lymphoid organs. The purification technique included DC enrichment and sort by FACS as described in materials and methods. The percentage and absolute number of cells were assessed by FACS analysis of sorted cells and cell count by the technique of blue trypan exclusion of dead cells, respectively. The number of experiments was at least 3. In each experiment, pDCs were derived from a pool of 3 to 6 Tg (CD4C/HIV^{mutA}) and nTg sex- and age-matched littermate mice between 6 to 10 weeks old. Data are shown as mean values ± SD between different experiments.

TABLE II: PDC IN VITRO SURVIVAL

Spleen: post-culture	**PDC**	
Medium alone (MA)	**%**	**# X 10 ^{5}**
nTg	6.6 ± 0.5	0.5 ± 0.007
Tg	5.1 ± 0.2	1 ± 0.01
Stimulated	**%**	**# X 10 ^{5}**
nTg	5.75 ± 1.3	1.2 ± 0.4
Tg	4.2 ± 0.2	0.4 ± 0.3
Stimulated / MA	**%**	**# X 10 ^{5}**
nTg	1.17 ± 0.18	2.4 ± 0.8
Tg	0.82 ± 0.07	0.38 ±0.26

TABLE-II: For survival assays, cell suspensions from spleen enriched for DCs were prepared as described in Table I but cells were not sorted. NTg and Tg (CD4C/HIV^{mutA}) mice-derived cell preparations were seeded at a concentration of 0.5 to 1 million cells per ml in Falcon Petri dishes. Cells were recuperated after 24 hours, counted and prepared for FACS analysis. Cell culture in MA was used as a base-line control for stimulated condition. Stimulation refers to the addition of GM-CSF, CD40L and rIL3 to the same media used in MA. PDCs and cDCs absolute cell number and percentage were parameters measured to assess their survival. The response to immunostimulants is represented by the ratio of percentage or absolute cell number in stimulated condition to MA.

FIGURE 4: PDC FREQUENCY IN BLOOD

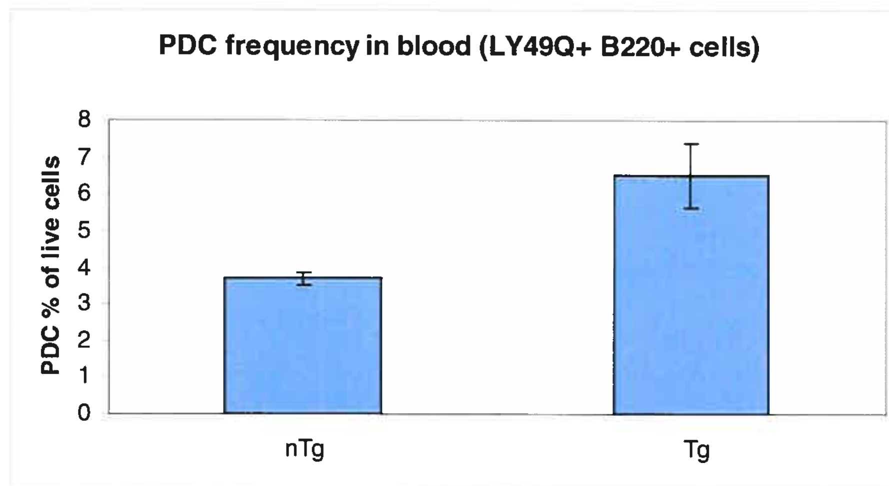
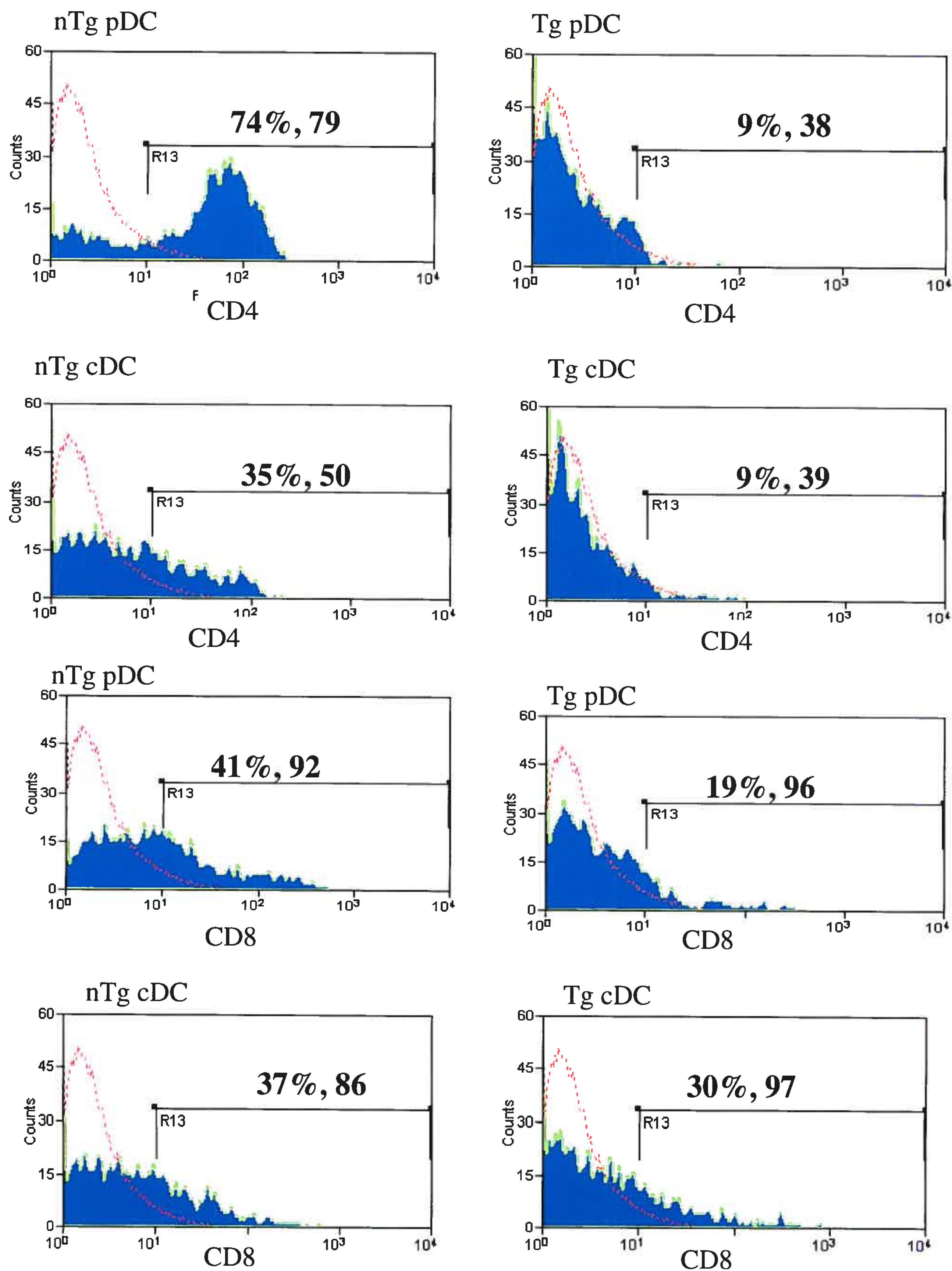


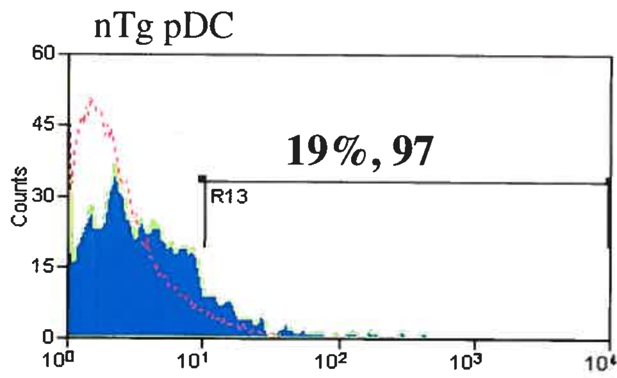
FIGURE 4.

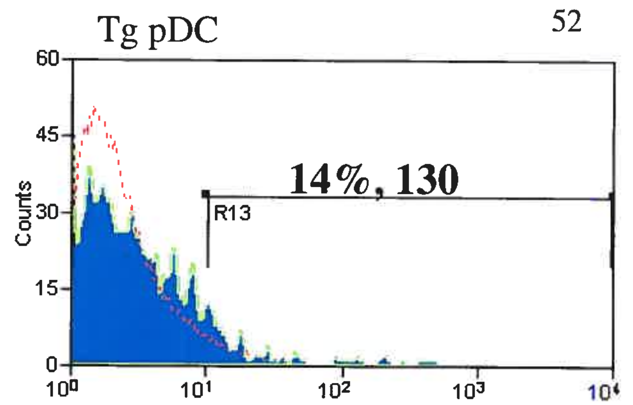
FACS analysis was performed on blood extracted from intraocular vein after the removal of red blood cells as described in materials and methods. LY49Q and B220 co-staining were used to identify pDCs, since in the periphery Ly49Q is expressed on pDCs but not on NK cells [51]. Also, $LY49Q^{+}B220^{+}$ cells correspond to pDCs because $Gr1^{+}LY49Q^{+}$ cells other than pDCs do not express B220 in mouse peripheral blood [51]. Results shown in figure 4 are the compilation of data obtained from 2 independent experiments and in each experiment 2 Tg ($CD4C/HIV^{mutG}$) and 2 nTg sex- and age-matched littermate mice have been used separately. In this graph mean values of the 2 Tg mice and 2 nTg mice are reported and y-axis error bars represent the SD between the 2 experiments.

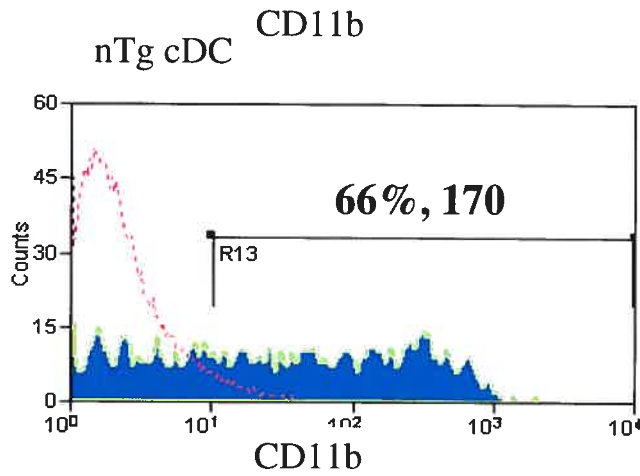
FIGURE 5: PHENOTYPE

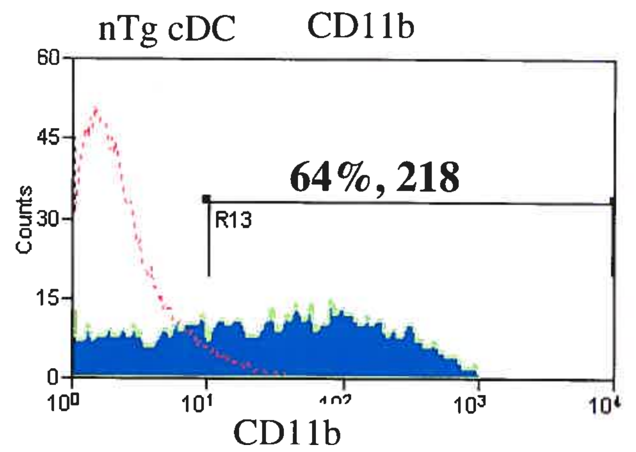
5.A: pLN pDC phenotype

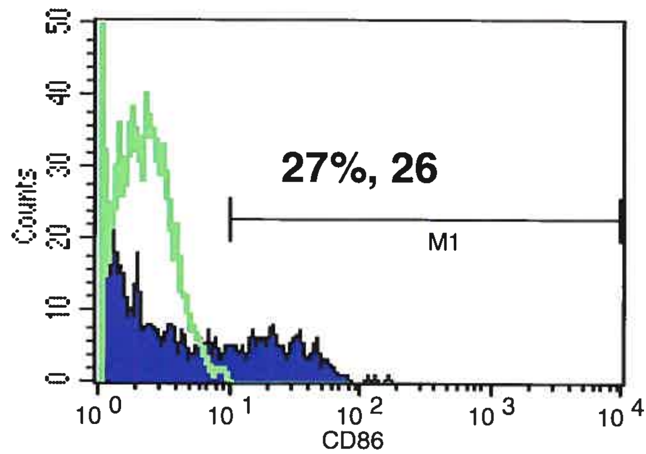


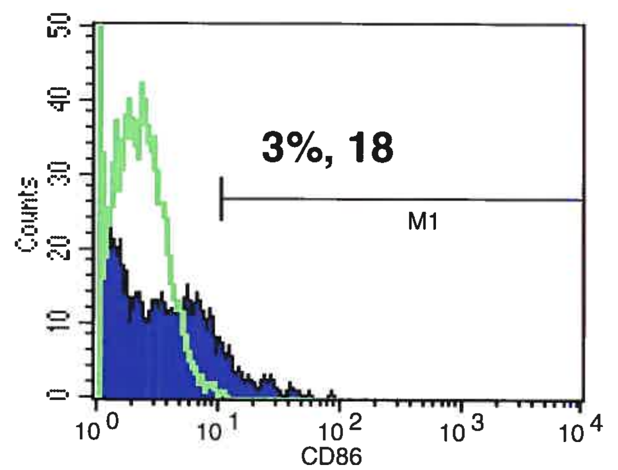

nTg pDC
19%, 97
R13
Counts
CD11b


Tg pDC
14%, 130
R13
Counts
CD11b


nTg cDC
66%, 170
R13
Counts
CD11b


nTg cDC
CD11b
64%, 218
R13
Counts
CD11b


27%, 26
M1
Counts
CD86


3%, 18
M1
Counts
CD86

5.B: Splenic pDC phenotype

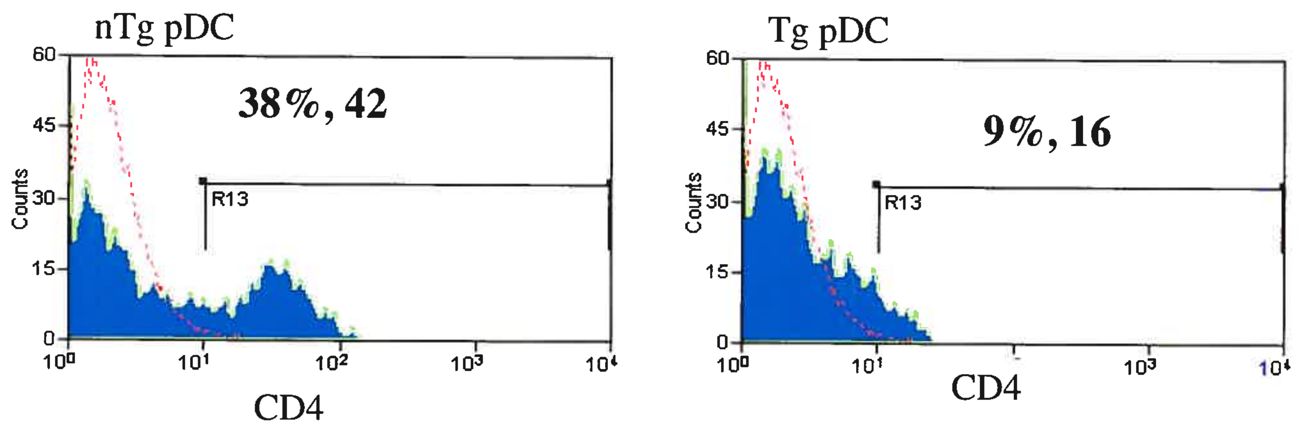


FIGURE 5: PHENOTYPE

FACS analysis was done on DC-enriched cell suspensions from lymphoid organs prepared as described in table II. Two lymphoid organs, pLNs **(A)** and spleen **(B)** were examined in this experiment. PDCs and cDCs were defined as $CD11c^{lo}B220^{+}IgM^{-}$ and $CD11c^{hi}B220^{-}IgM^{-}$ cells as described in figure 1.B. Histograms show the expression of each marker on gated pDCs and cDCs. The doted lines correspond to the isotypic control for the marker and define the unspecific binding of the Ab used. For both pLN and spleen the expression for each marker on pDCs and cDC is indicated in the corresponding histogram as percentage and MFI values.

5. PDCS MATURATIONAL STATE AND RESPONSE TO *IN VITRO* STIMULATION: TG MICE-DERIVED PLN AND SPLENIC PDCS SHOW A LOWER MATURATIONAL STATE EX-VIVO. TG MICE-DERIVED PLN AND BM PDCS MATURATIONAL RESPONSE TO IN VITRO STIMULATION IS ALTERED WHEN COMPARED TO NTG MICE.

Both cDC and pDC up-regulate their cell surface MHC class II expression upon maturation [19]. In fact, MHCII–mediated Ag presentation by DC subtypes is dependent on a precisely regulated cell type specific and quantitatively controlled pattern of MHC class II expression [50]. In order to examine the state of maturation of Tg pDCs compared to nTg pDCs, FACS analysis were done on *ex-vivo* DC-enriched cell suspensions from peripheral lymphoid organs. In addition, cell suspensions were prepared from BM and then enriched for DCs by an advanced culture method that allows the elimination of granulocytes as described in the

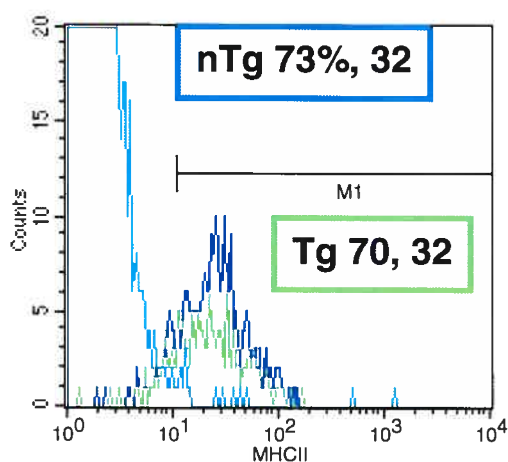
section on materials and methods and in the supplementary figure 14.A. BM-derived pDCs MHCII expression was assessed *ex-vivo* and after DC-enrichment.

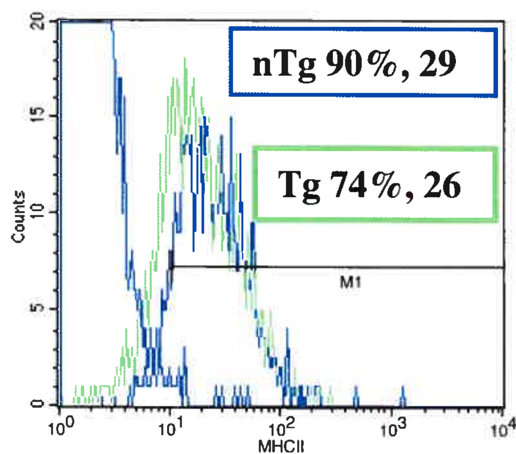
Results in Table-III.A show that BM-derived pDCs express the same levels of MHCII (percentage and MFI) in Tg and nTg mice. However, MHCII expression (MFI) on pDCs from both spleen (Table III.B) and pLNs (Table III.C) is lower in Tg mice compared to nTg. This indicates a defect in the maturational state of Tg pDCs in the periphery but not in BM, which is a central lymphoid organ. In contrast to *ex-vivo* results, BM pDCs after 9 days of culture for DC enrichment show lower expression of MHCII (% and MFI) when derived from Tg compared to nTg mice.

Combinations of DC stimulants, which are known to induce maturation i.e., GM-CSF, CD40L and rIL-3 have been used for *in vitro* maturational assays [50]. Although, murine pDCs unlike human pDCs do not express high levels of IL-3R, they are expected to mature moderately upon GM-CSF and rIL-3 stimulation [50, 35]. FACS analysis was performed on DC-enriched cell suspensions from BM, spleen and pLNs that have been cultured with immunostimulants. Post-stimulation, MHCII expression is lower on pDCs derived from BM and pLNs of Tg mice when compared to their nTg equivalents (Table-III.A and -III.C). However, Tg mice-derived splenic pDCs express similar levels of MHCII than nTg splenic pDCs after *in vitro* stimulation (Table-III.B). This later data suggests that Tg mice derived splenic pDCs response to maturational stimuli must be higher compared to the response of their nTg equivalents because stimulation rescued their *ex-vivo* phenotype of lower MHCII expression. For pLNs, the ratios of MHCII expression on pDC to cDC are reported in Table-III.C. PDCs remain less mature than cDCs ex-vivo and post stimulation in Tg and nTg mice.

TABLE-III. PDCS MATURATIONAL STATE AND RESPONSE TO *IN VITRO* STIMULATION

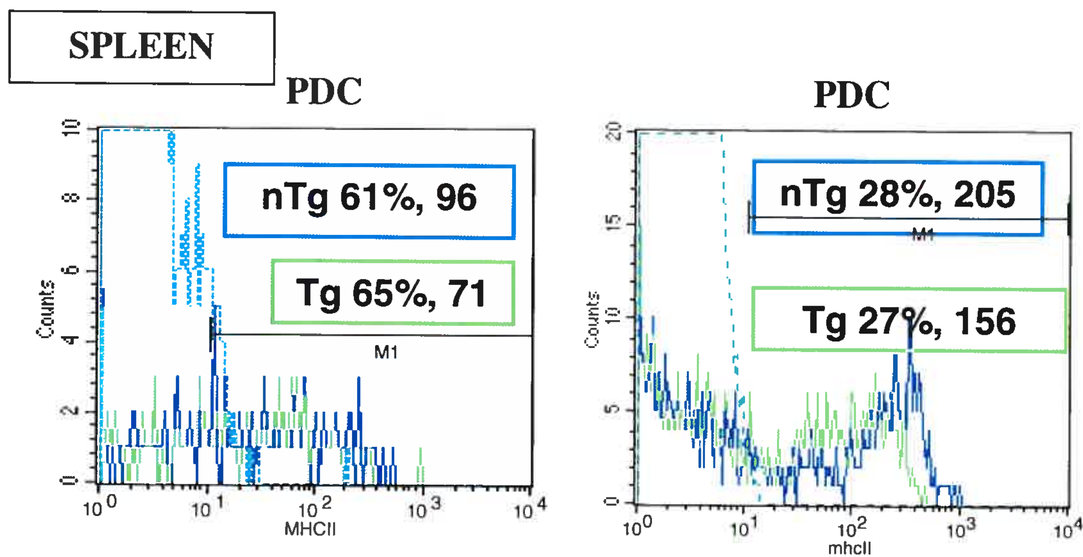
A. BM	PDC	
EX VIVO	MHCII %	MHCII MFI
TG/NTG	1 ± 0	0.97 ± 0.10
POST-ENRICHMENT		
TG/NTG	0.70 ± 0.05	0.83 ± 0.07
POST-STIMULATION		
TG/NTG	0.72 ± 0.1	0.57 ± 0.06





B.SPLEEN	PDC	
EX VIVO	MHCII %	MHCII MFI
Tg/nTg	1.15 ± 0.19	0.75 ± 0.07*
POST-STIMULATION		
TG/NTG	1.17 ± 0.12	1.17 ± 0.29

* $p<0.05$



C. pLN	PDC	
EX-VIVO	MHCII%	MHCII MFI
TG/NTG	0.85 ± 0.04*	0.77 ± 0.05*
PDC/CDC		
NTG	0.42 ± 0.23	0.36 ± 0.12
TG	0.41 ± 0.13	0.35 ± 0.23
POST-STIMULATION	GM-CSF + CD40L + rIL3	
TG/NTG	0.96 ± 0.10	0.66 ± 0.
PDC/CDC		
NTG	0.42 ± 0.17	0.22 ± 0.09
TG	0.42 ± 0.14	0.17 ± 0.03

* $p<0.05$

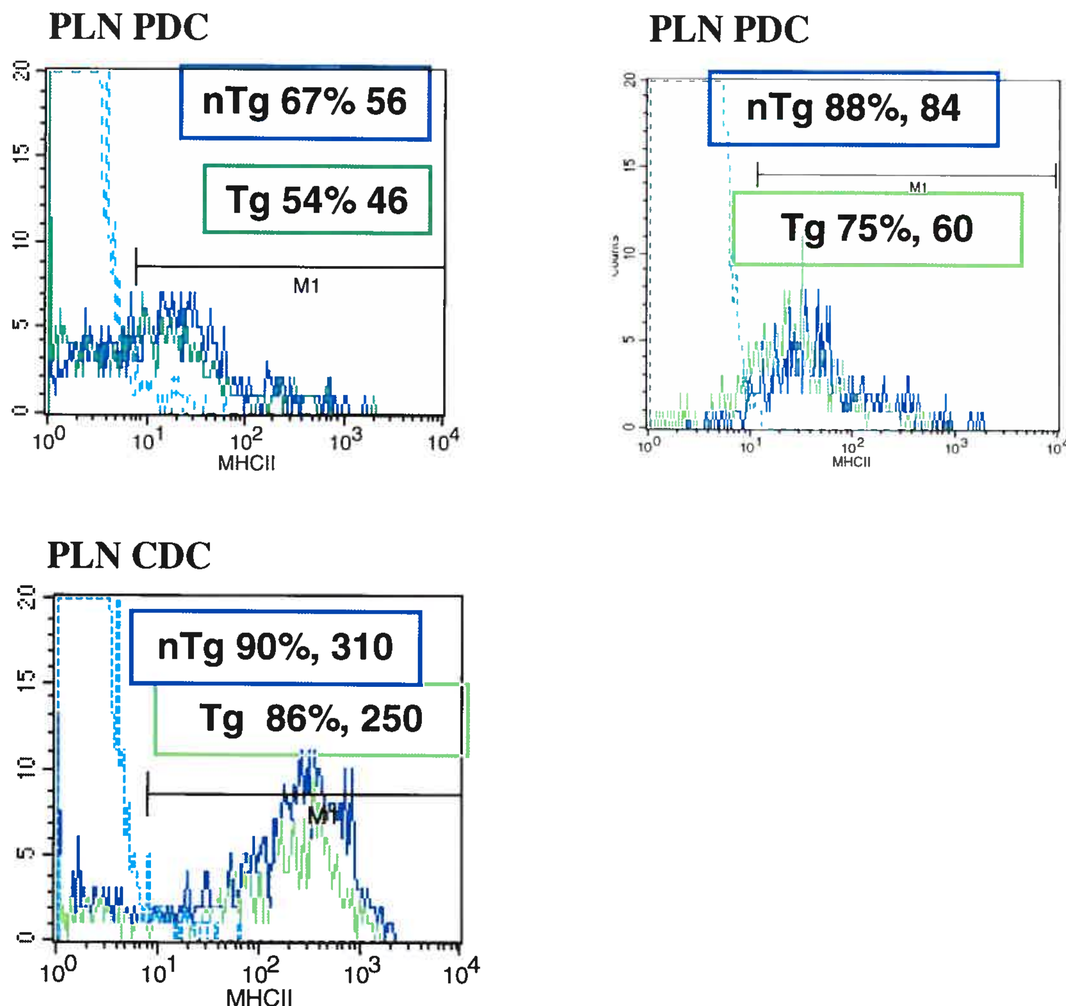


Table III. PDCs maturational state and response to immunostimulants

A) BM-derived pDCs MHCII expression was assessed *ex-vivo* by FACS analysis on cell suspensions obtained by BM flushing. Post-enrichment designate the use of the advanced method of DC enrichment for BM as described in the section on materials and methods and in supplementary figure 13.A [131]. Further stimulation was performed on DC-enriched cell suspensions from BM by adding immuno-stimulants to the same media used for the enrichment and allowing cells to stay in culture for another 24 hours. This later condition corresponds to post-stimulation in Table III-A. MHCII expression is reported as the percentage of gated pDCs expressing this molecule and as MFI, which corresponds to total cell surface expression of MHCII. Results are reported as ratios of Tg/nTg for each parameter examined and SD (±) corresponds to variation between mice (n=4).

B), C) DC enriched cell suspension was prepared from a pool of 3 Tg and 3 nTg mice as described in the section on materials and methods. FACS analysis was done on DC-enriched cell suspensions *ex-vivo* from peripheral lymphoid organs. PDCs were identified as described in figure 1.B. and MHCII expression on gated pDCs is reported in Table **III.B** for spleen and **III.C** for pLNs. Post-stimulation corresponds to culture for 24 hours of DC enriched cell suspension in the presence of a combination of immuno-stimulants including GM-CSF, CD40L and rIL-3, which are known to induce DC (including pDC) maturation [1, 50, 57]. Results are reported as ratios of Tg/nTg for each parameter examined. Two experiments were done for spleen and pLNs maturational assay and SD (±) corresponds to variation in the values of Tg/nTg ratios between experiments. The number of independent experiments for pDCs MHCII expression ex-vivo was 4.

N.B. In pLNs, *ex-vivo* and post-stimulation, MHCII expression (% and MFI) was compared between pDCs and cDCs and this is reported as ratios of PDC/CDC. In addition examples of actual values of MHCII expression (% and MFI) are reported in the corresponding FACS histograms following Table III-A, -B and C for BM, spleen and pLN pDCs, respectively.

6. PDCS DERIVED FROM FLT3L-ENRICHED BM PRECURSOR CELLS AND THEIR RESPONSE TO CPG: THE MATURATIONAL STATE OF FLT3L-INDUCED PDCS FROM BM IS SIGNIFICANTLY LOWER WHEN CELLS ARE DERIVED FROM TG COMPARED TO NTG MICE. BM-DERIVED FLT3L-INDUCED PDCS MATURATIONAL RESPONSE TO CPG IS SIMILAR WHEN CELLS ARE DERIVED FROM TG AND NTG MICE.

Flt3L is a growth factor and the ligand for the receptor fms-like tyrosine kinase (Flt)-3, which is a member of platelet-derived growth factor (PDGF)-receptor superfamily [28], expressed by a restricted subset of early multipotent hemopoietic progenitor cells [134]. This factor is known to increase the number of DCs, B cells and NK cells in adult mice [135]. Flt3L expands PDC population both in vivo and in vitro, and is also crucial for their development in physiological conditions. In fact, Flt3L-deficient mice completely lack this specific DC subset [43].

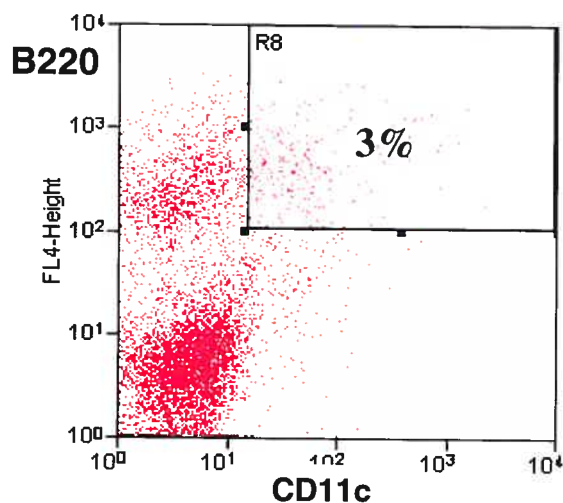
PDCs generation from BM precursor cells enriched with Flt3L was assessed comparing CD4C/HIV^{mutA} Tg to nTg mice. The purpose of this experiment was to verify if the expression of Nef early in life at the embryonic stage, could affect BM residing pDC precursor cells response to Flt3L. This question was addressed because in HIV-1 infection, pDCs are depleted and among other mechanisms, their regeneration from BM precursor cells might be inefficient. Moreover, Flt3L can be a useful tool to expand pDC population since pDCs depletion correlates with immunosuppression and the augmentation of their number during HAART is associated with immune reconstitution [107, 108, 109].

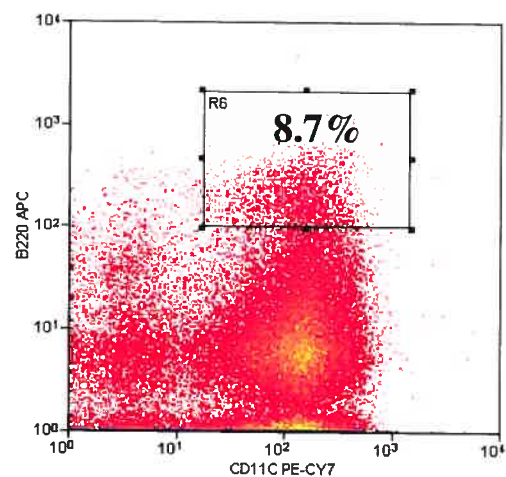
FACS panels in figure 6.A. show the expansion of BM pDCs in response to Flt3L by comparing to ex-vivo BM-derived cells. As shown in figure 6.B., Flt3L-mediated pDC expansion from BM precursor cells is slightly but not significantly decreased in Tg mice. The maturational state of these pDCs, assessed by their cell surface expression of MHCII and CD86 is significantly lower when they are derived from Tg compared to nTg mice (figure 6.C and 6.D). Moreover, pDCs expression of Ly49Q was measured because of its association with functional features of BM-derived pDCs [51]. A major proportion (about 80%) of Flt3L-induced BM-derived pDCs from nTg mice express Ly49Q and this expression is slightly decreased when pDCs are derived from Tg mice (figure 6.E).

CpG stimulation is known to induce pDCs proliferation and maturation *in vivo* as well as *in vitro* [47]. CpG is a specific ligand for TLR-9 and since its effects on pDCs are mediated through TLR-9 signaling pathway, pDCs response to CpG can provide information regarding TLR-9 expression and/or signaling in pDCs [63, 71]. Further in vitro maturation of Flt3L-enriched BM derived cells was used to evaluate pDCs expansion and maturation in response to CpG stimulation. Based on values of CD11c MFI reported in FACS panels of figure 6F, the expression of CD11c is up-regulated on CpG-stimulated pDCs-derived from Tg mice and not from nTg mice. FACS results obtained after this maturational assay were compiled and shown in figures 6G and 6H. In contrary to pDCs obtained post-Flt3L enrichment of Tg mice BM cells showing a defect in their MHCII expression, maturational response of pDCs induced by CpG is comparable between Tg and nTg mice.

FIGURE 6: *IN VITRO* FLT3L ENRICHED BM DERIVED PDCS AND CPG STIMULATION

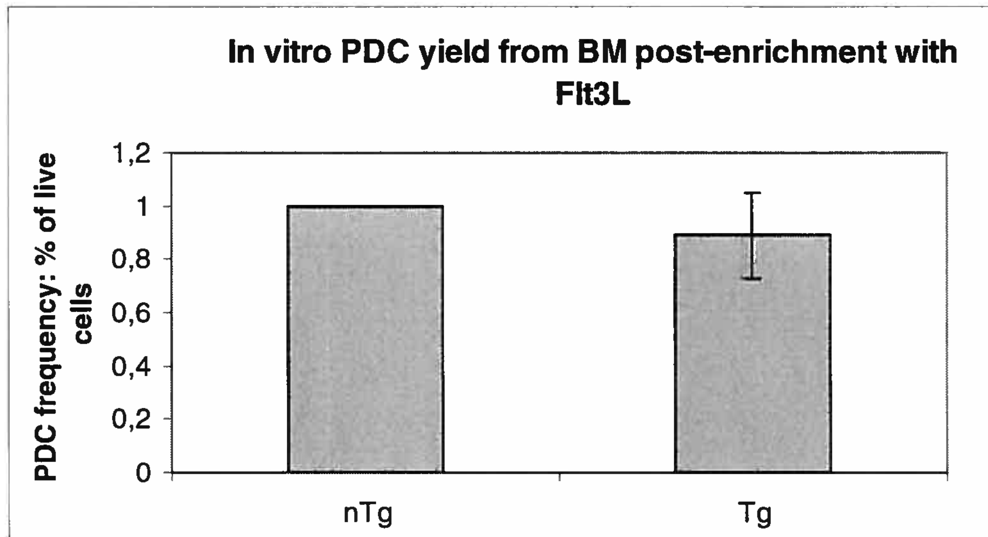
6.A. FACS analysis



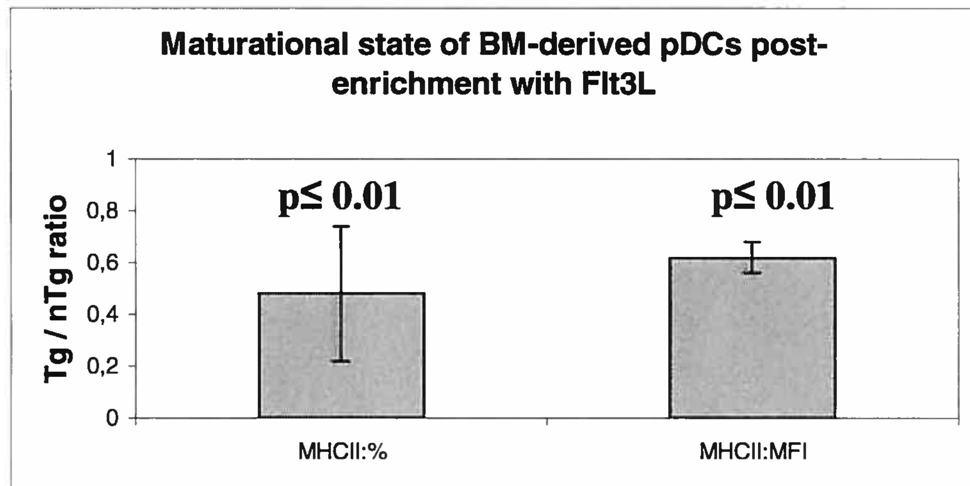


BM: ***ex-vivo*** **BM: Post-Flt3L enrichment**

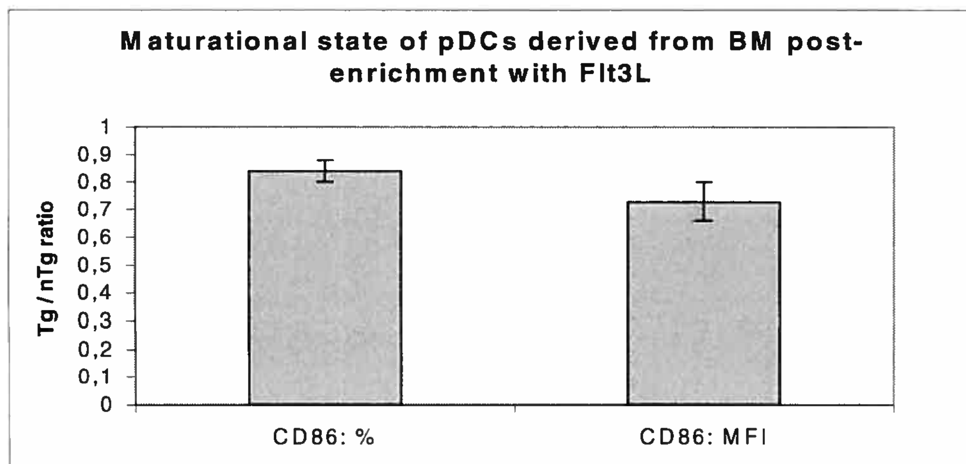
6.B.



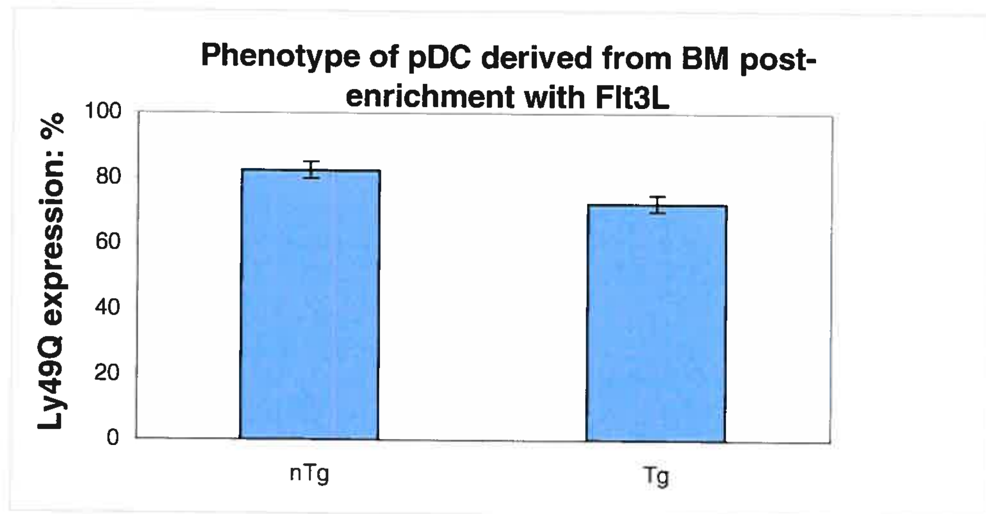
6.C.



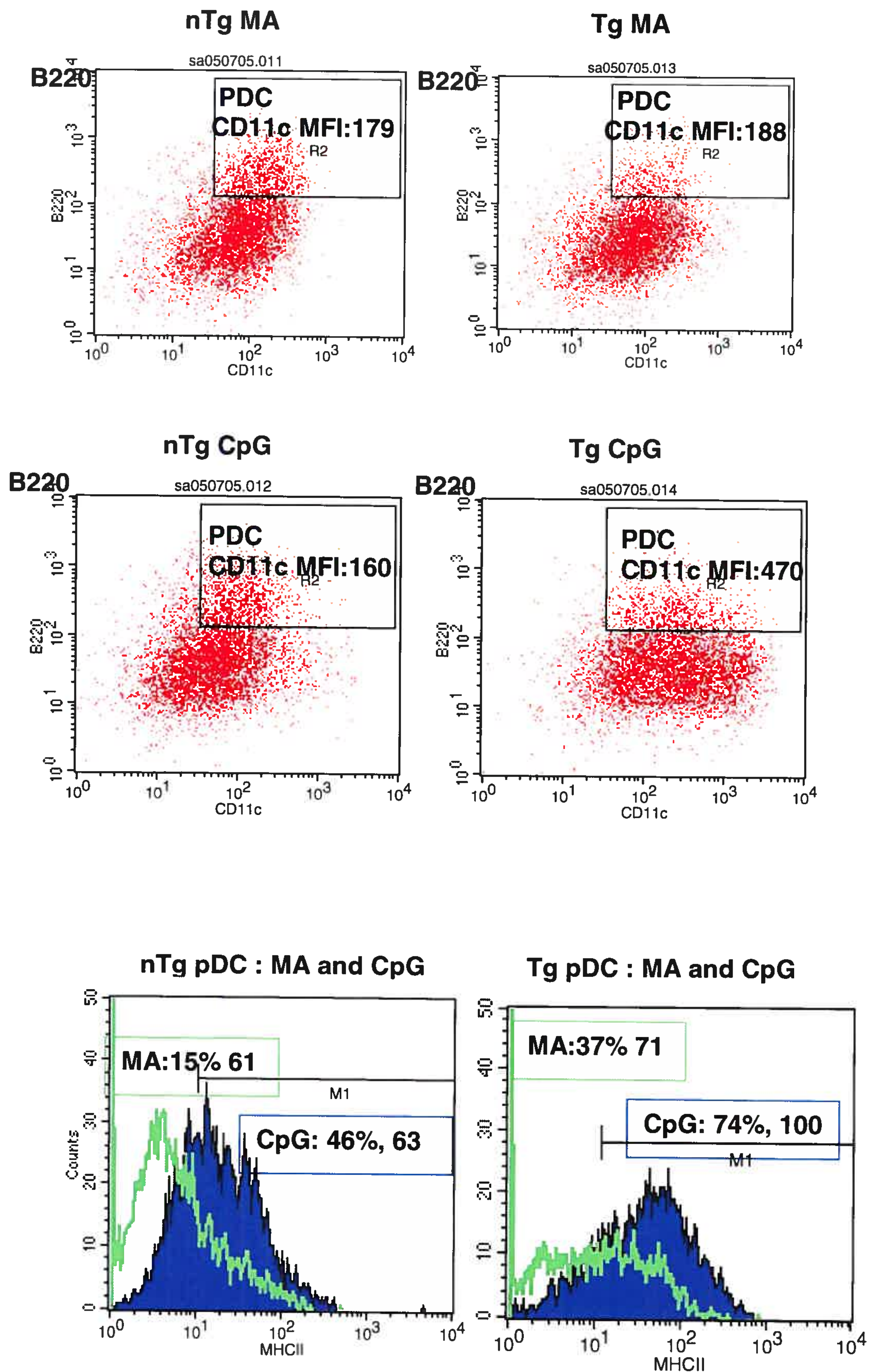
6.D.



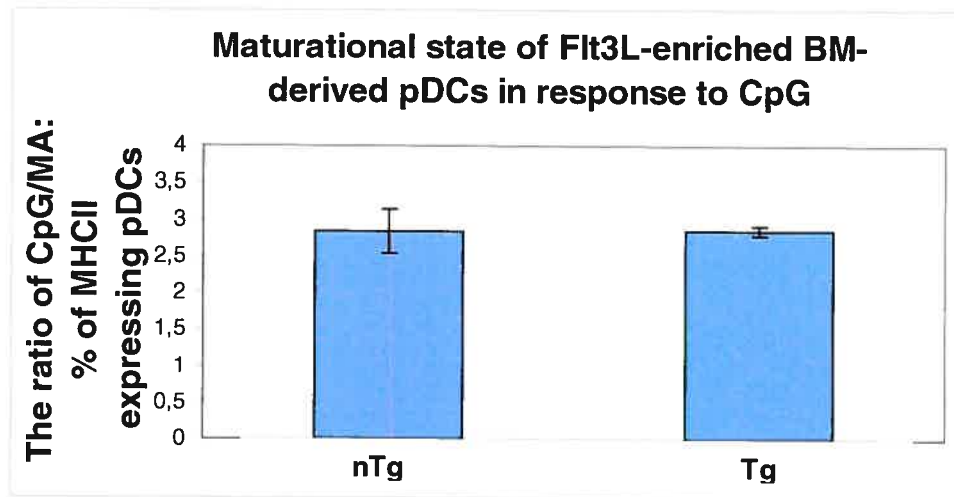
6.E.



6.F. CpG stimulation : FACS profiles



6.G.



6.H.

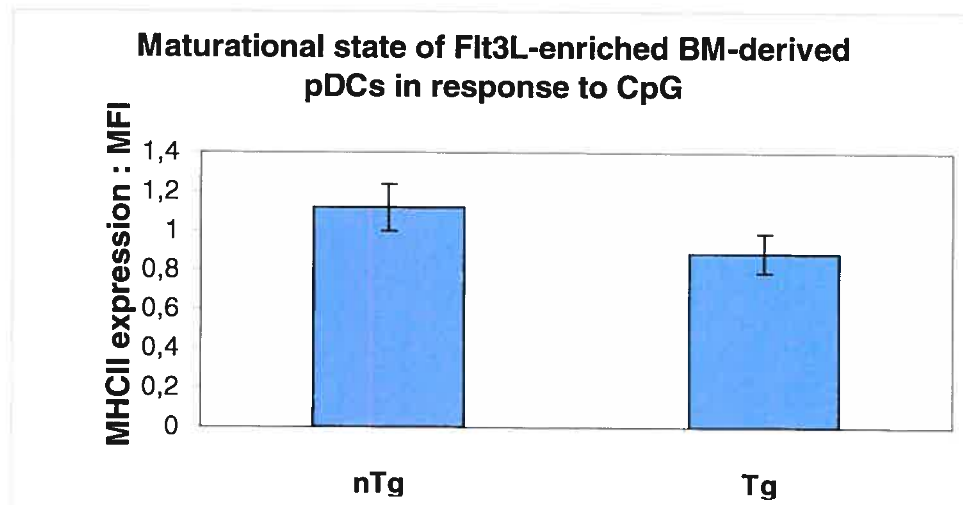


FIGURE 6: *In vitro* Flt3L-enriched BM derived pDCs and their further maturation by CpG

A) FACS analysis was done on total BM-derived cells cultured in the presence of Flt3L, in the form of supernatant from cells that have been transfected by a plasmid containing mouse Flt3L gene (supplementary figure 13.B). The negative control for Flt3L enrichment corresponds to BM-derived cells cultured with the supernatant from transfected cells with an empty plasmid lacking Flt3L gene as described in material and methods (supplementary figure 13.B). PDCs were defined as $CD11c^{+}B220^{+}$ cells. Flt3L enrichment assays were done three times with BM cells from 2 mice in each experiment (n=6).

B) Results obtained from FACS analysis on Flt3L enriched BM cells were compiled. This graph shows pDCs yield defined as their percentage of live (PI^{-}) cells. In each experiment and for each mouse, the value obtained for pDC yield was fixed to 1 for nTg and the ratios of Tg/nTg are reported with a y-error bar corresponding to SD between all results.

C) The maturational state of Flt3L-enriched BM derived pDCs was assessed by their expression of MHCII. In this graph the percentage of MHCII expressing pDCs as well as MFI of MHCII are reported as Tg/nTg ratios. The SD corresponds to variation in these ratios between different mice and experiments ($P \leq 0.01$).

D) This graph shows the expression of CD86 on Flt3L-enriched BM derived pDCs. Results are shown similarly to graph C.

E) Ly49Q expression by Flt3L-induced pDCs is shown in this graph. However, this graph shows preliminary results since data are obtained from only 2 mice in one experiment.

F) On day 9 of culture post-Flt3L enrichment, half of the recovered cells were cultured in two different conditions: MA and CpG stimulation following the same conditions as for the enrichment (See section on further maturation in materials and methods). FACS plots showing pDC populations after culture in MA and CpG are shown here. FACS histograms corresponding to MHCII expression on gated pDCs for both MA and CpG conditions are shown. Examples of values obtained for MHCII expression (%, MFI) are reported in these panels.

G) , H) The graph shows compiled FACS results of MHCII expression on pDCs (% in graph G and MFI in graph H), after further CpG stimulation. The results are reported as ratios

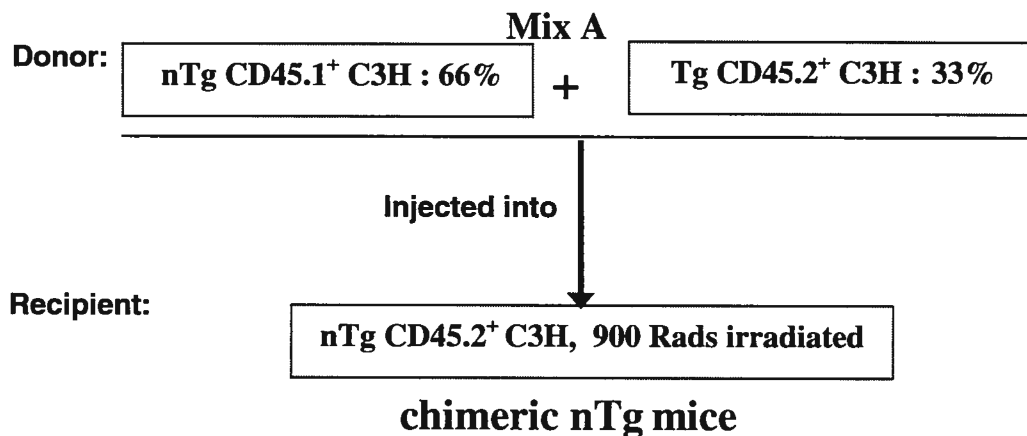
of CpG/MA of MHCII expression (%) in order to examine pDCs response to CpG compared to MA used as a control.

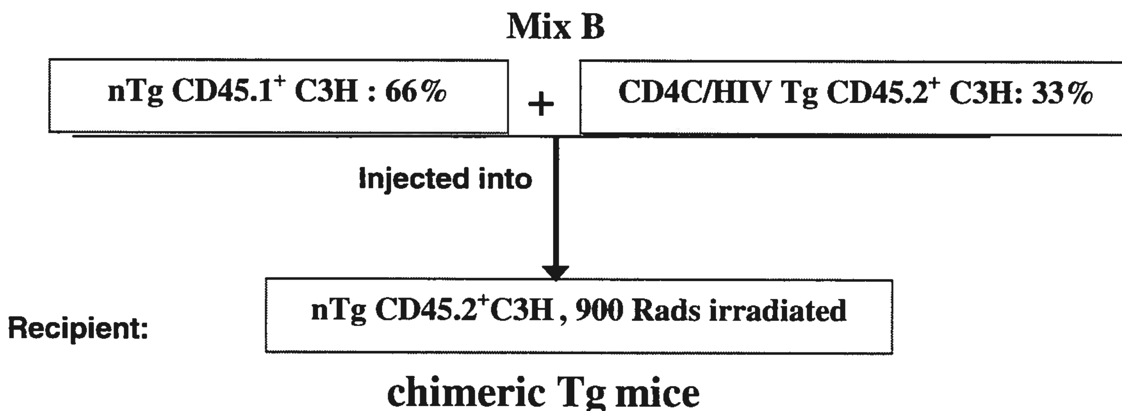
7. TRANSPLANTATION CHIMERA MOUSE MODEL USED FOR THE STUDY OF PDCS

A transplantation chimera mouse model has been designed in our laboratory in order to study thymic T cell generation in CD4C/HIV^{mutG} mice (Pavel Chrobak et al., unpublished data). As described in the section on materials and methods, chimeric mice were obtained by injection of fetal liver cells into irradiated syngeneic mice. The following diagram shows the construction of these chimeras and the use of chimeric Tg mice for tracing Tg versus nTg-derived cells based on the expression of different isoforms of the CD45 cell surface marker.

1.Breeding for generation of embryos to use as donors (see section on materials and methods)

2.Fetal liver preparation and injection into recipient mice





The above diagram shows that the same proportion of CD45.1^{+} (66%) and CD45.2^{+} (33%) cells were injected into the recipient mice to produce both chimeric Tg and chimeric nTg mice. Figure 7.A shows the percentage of chimerism, which corresponds to the relative proportion of CD45.1^{+} and CD45.1^{-} cells, in different splenic immune cell populations. Values of the percentage of chimerism must be compared between chimeric Tg and chimeric nTg mice in order to evaluate potential differences in the reconstitution of peripheral immune cells between CD45.1^{+} and CD45.1^{-} cells.

As shown in figure 7.A, for NK cells, macrophages and B cells, the proportion of CD45.1^{+} relative to CD45.1^{-} cells is similar or slightly different in chimeric Tg compared to chimeric nTg mice. In contrast, for CD4^{+} and CD8^{+} T cells, the ratio of CD45.1^{+} to CD45.1^{-} cells is significantly higher in chimeric Tg compared to chimeric nTg mice. Same experiments in pLN show similar results for CD4^{+} and CD8^{+} T cells as in spleen suggesting a defect in T cell generation from Tg mice-derived cells (Pavel Chrobak, unpublished data).

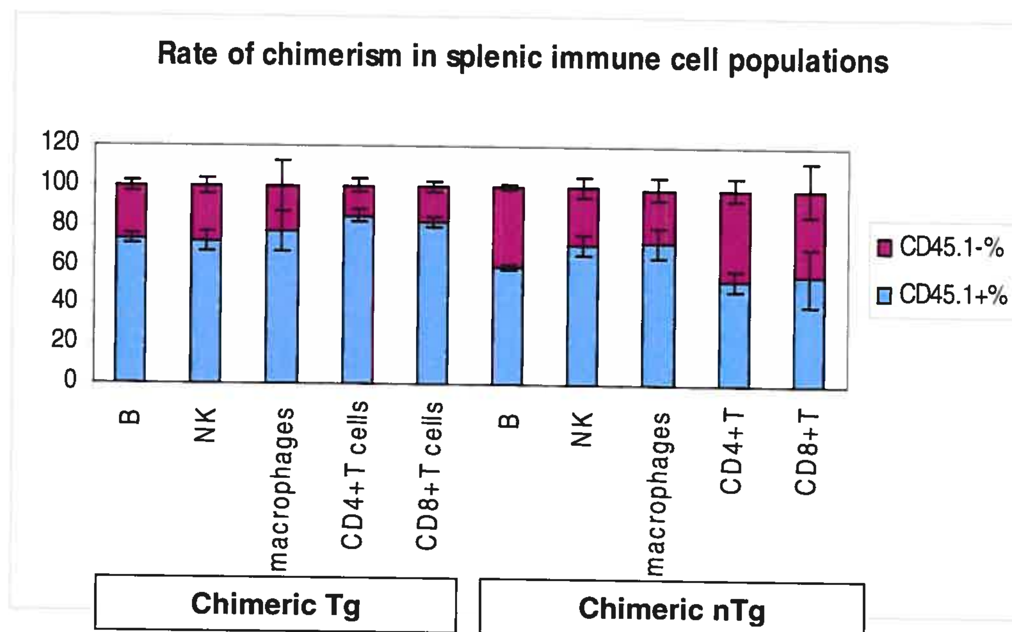
Chimera mouse model was used to determine the *in vivo* effect of Nef expression by transgene expressing cells derived from CD4C/HIV^{mutG} Tg CD45.2^{+} donor on nTg cells from nTg CD45.1^{+} donor since they are both present in chimeric Tg mice. This effect corresponds to the indirect effect of Nef as opposed to the direct effect of Nef, which refers to its effect on cells expressing the transgene. The indirect effect of Nef can be evaluated by comparing, between chimeric Tg and chimeric nTg mice, the characteristics of cells derived from nTg progenitors, which are CD45.1^{+} cells. In fact, CD45.1^{+} cells in both nTg and Tg chimeras are derived from nTg mice progenitors, but in chimeric Tg mice, in contrast to chimeric nTg mice, this population is in the presence of Tg-derived cells (CD45.1^{-}). It should be noted that CD45.1^{-} cells in chimera mouse model may include a minority of residual host cells from the recipient mouse, which have not been eradicated by irradiation [133]. Thus CD45.1^{-} cells are not comparable in chimera mouse model.

We have used this model to study the indirect effect of Nef on various features of pDCs generated from Flt3L-enriched BM cells. As shown in figure 7.B.1, the relative frequency of CD45.1^{+} and CD45.1^{-} cells among live pDCs is equal between chimeric Tg and chimeric nTg mice. This suggests that in Tg mice, pDC precursors among BM cells display no defect in their potency to generate pDCs in response to Flt3L.

The examination of the maturational state of Flt3L-enriched BM derived pDCs revealed that CD45.1$^+$ cells in chimeric Tg mice show slightly higher level of MHCII e xpression (MFI)compared to their equivalent cell population in chimeric nTg mice (figure 7.C). This difference may be due to an indirect effect of Tg mice-derived cells on nTg mice-derived cells in chimeric Tg mice. The slight increase in MHCII expression of nTg pDCs (CD45.1$^+$ in chimeric Tg mice) could reflect a compensatory mechanism in the presence of Tg cells, which show a maturational defect (figure 6C and 6D). In fact, maturational factors such as IFN-α may be produced in excess in cultures from chimeric Tg mice in order to compensate the maturational defect of Tg mice derived pDCs.

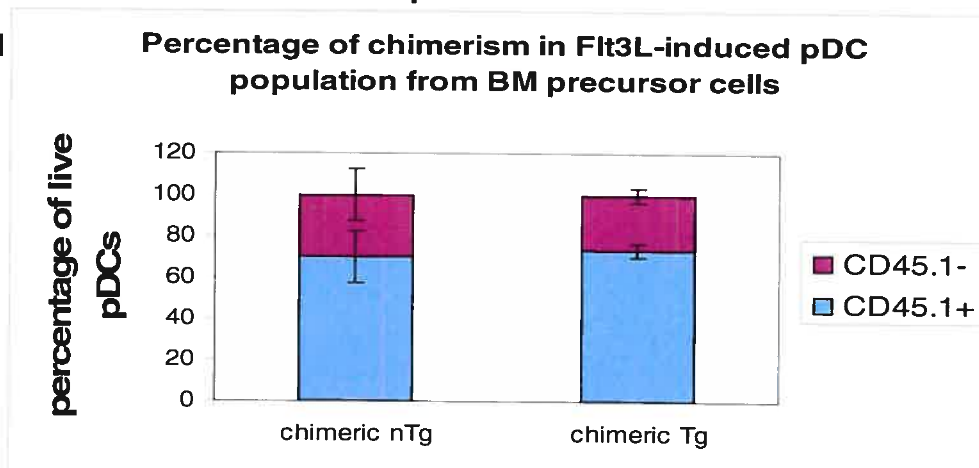
FIGURE 7: CHIMERIC MOUSE MODEL

7.A. The percentage of chimerism in spleen



7.B. Flt3L-enriched BM-derived pDC

7.B.1



7.B.2.

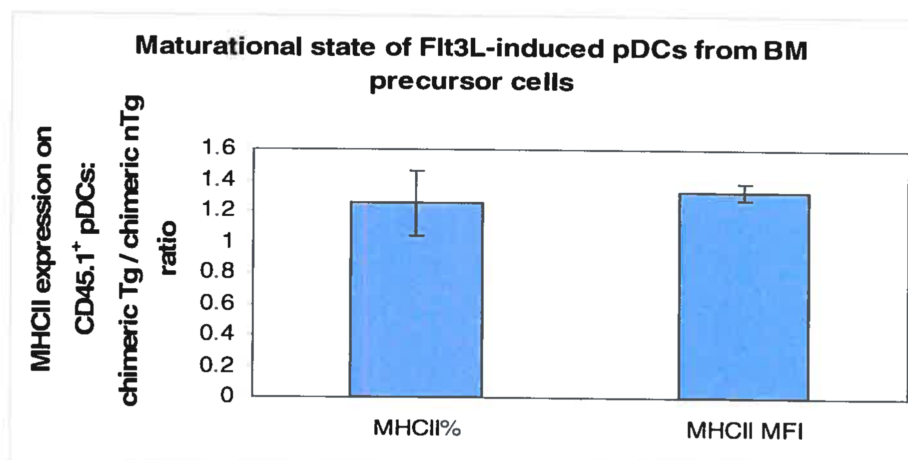


FIGURE 7: Transplantation chimera mouse model

Total of 5 chimeric Tg and 4 chimeric nTg mice have been prepared by fetal liver transplantation (Material and methods). FACS analysis was done on cell suspensions prepared from spleen and Flt3L-enriched BM cells. Figure 7.A and 7.B show the compilation of FACS results obtained from four experiments for the spleen and three experiments for Flt3L-enriched BM cells, respectively.

In each experiment, one C3H Tg mouse was used as a positive control for the expression of CD45.2 and one $CD45.1^+$ congeneic mouse (C3H), was used as a positive control for the

expression of CD45.1 (supplementary figure 15). In all experiments, cells were stained for markers of immune cell populations and either CD45.1 or CD45.2 in independent wells. CD45.2 staining was used as a control for staining all cells (CD45.1$^+$ and CD45.1$^-$ cells) originating from both parents in chimeric mice.

A) The percentage of chimerism was obtained by FACS analysis on total splenocytes after RBCs were removed. Each immune cell population was identified according to their specific markers. B cells were defined as B220$^+$IgM$^+$ cells, CD4$^+$ T cells as TCRα/β$^+$CD4$^+$, CD8$^+$ T cells as TCRα/β$^+$CD8$^+$ cells, macrophages as CD11b$^+$CD11c$^-$ cells and NK cells as CD3$^-$DX5$^+$ cells. The proportions of CD45.1$^+$ to CD45.1$^-$ cells were determined in each population.

B) BM cells were enriched with Flt3L as described in the section on materials and methods and in the legend of figure 6.A. Figure 7.B.1. shows data obtained from FACS analysis on recovered cultured cells from both chimeric Tg and chimeric nTg mice reported as the percentage of CD45.1$^+$ and CD45.1$^-$ cells among live (PI$^-$) pDCs that have been yielded in vitro post-Flt3L enrichment of BM cells. In figure 7.B.2 pDCs maturational state is reported as MHCII expression (percentage and MFI). MHCII expression on CD45.1$^+$ pDCs is compared between chimeric Tg and chimeric nTg mice in order to evaluate the indirect effect of Nef.

8. PDCS LOCALIZATION: PDC SHOW AN UNUSUAL HOMING IN VARIOUS ZONES OF PERIPHERAL LYMPHOID ORGANS IN CD4C/HIV TRANSGENIC MICE.

Previously, histological examination revealed abnormalities in the spleens of CD4C/HIV Tg mice including partial to extensive loss of splenic architecture, frequent hypocellularity, and occasionally fibrosis. Such extensive destruction of the lymphoid organs is likely to severely affect the function of the immune system [74]. In addition, an unusual mDC homing has been reported in peripheral lymphoid organs of Tg mice [119], and since pDCs trafficking is known to be implicated in mDCs migration to LNs [45], pDCs localization in LN and spleen of Tg mice have been examined.

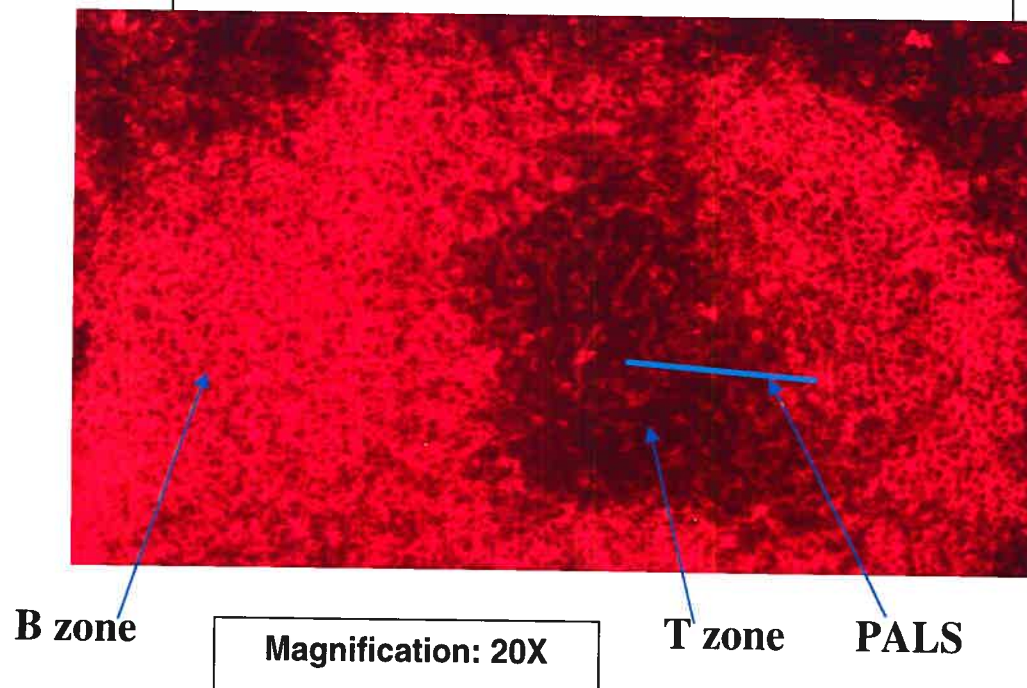
As shown in figures 8.A.2 and graph 8.C., contrary to nTg mice, pDCs are absent or very few in the splenic T zone of Tg mice. Figure 8.B and graph 8.D show a lowered number of pDC in B-T cell transitional zone of Tg compared to nTg mice. PDCs are few but similar in both Tg and nTg marginal zone (MZ) (graph 8.E.) In pLNs, pDCs number in T zone is similar in Tg and nTg mice, but their number is lower in B-T transitional zone and enhanced in SC zone of Tg mice compared to nTg animals (graphs 8.F, 8.G and 8.H). Thus, pDCs in the lymphoid organs of Tg mice display a homing defect. These unusual localizations could reflect a migrational defect of pDCs in Tg mice.

FIGURE 8: PDCS LOCALIZATION

Figure 8.A. nTg mice splenic white pulp

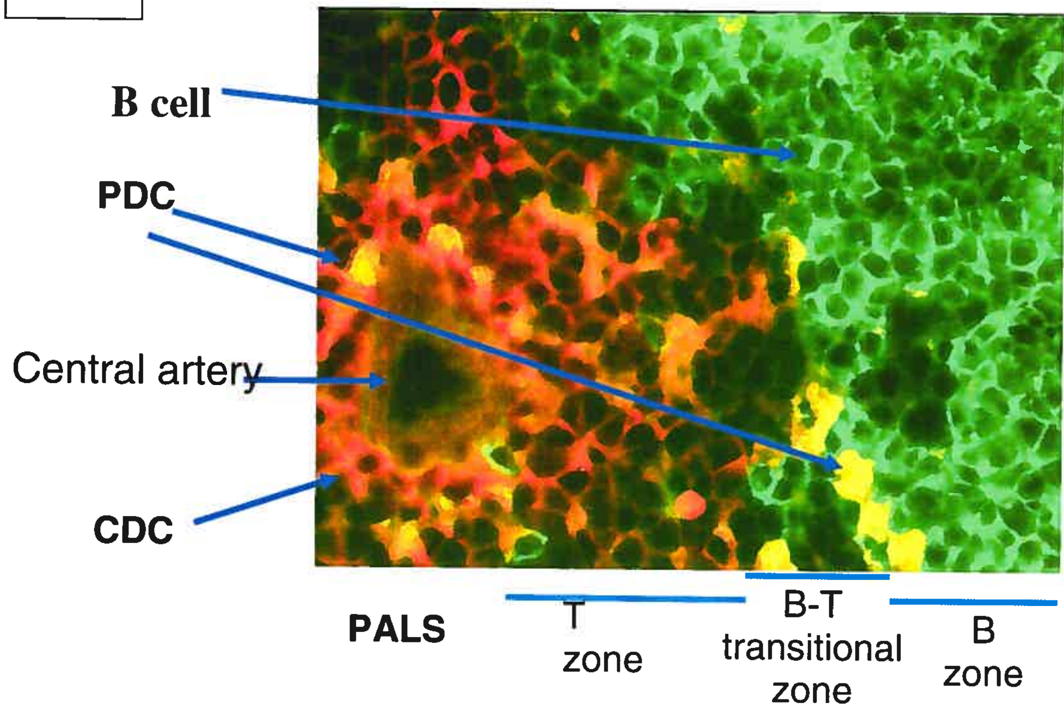
8.A.1 **Structure of PALS in nTg mice**

IHC staining with IgM-Texas Red; B cell: Red



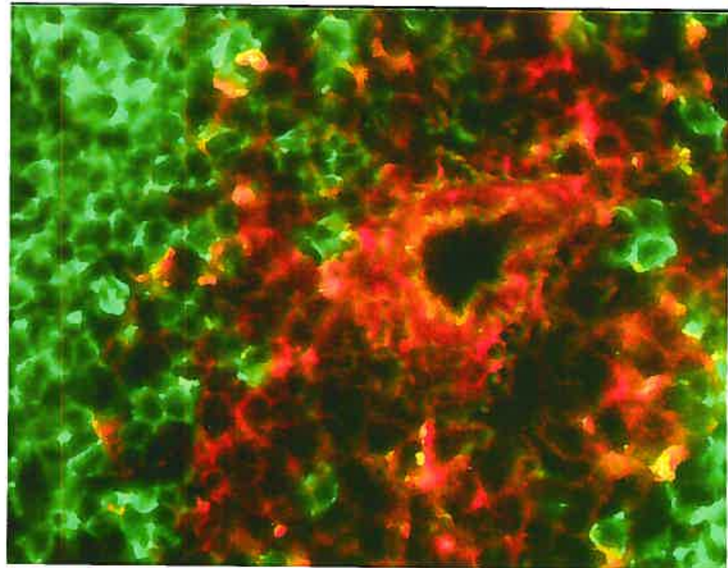
8.A.2

nTg splenic white pulp



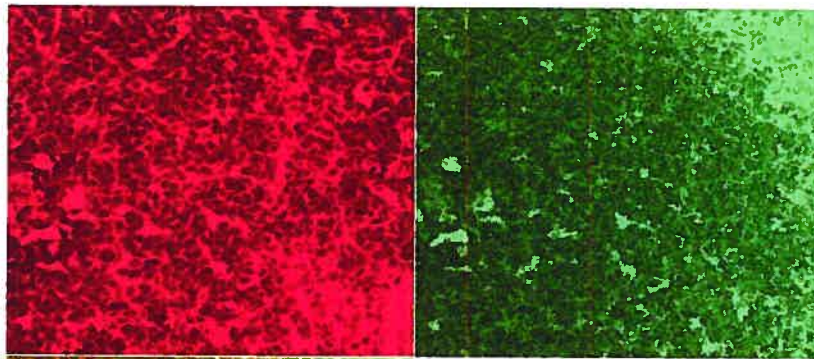
IHC staining with CD11c-Alexa Red and B220-FITC
PDCs: yellow, CDCs: red, B cells: green

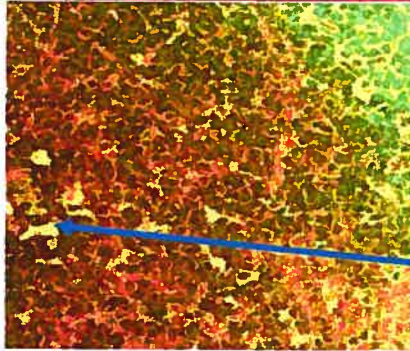
Tg splenic white pulp



Zeiss Axiovert S100TV microscope
Magnification: 40X

8.B. Spleen T and T-B cell transitional zone

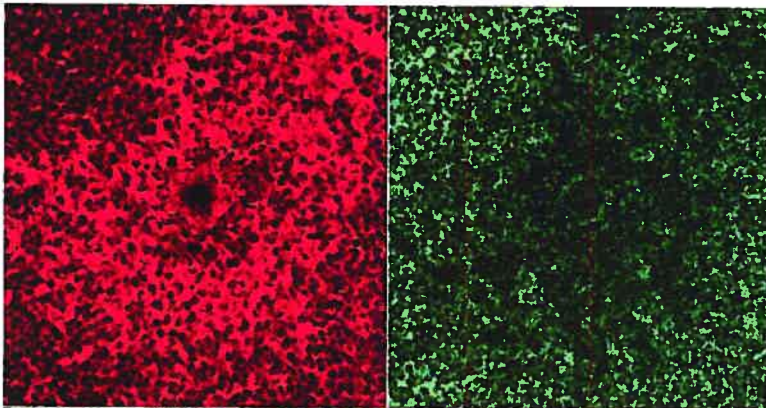


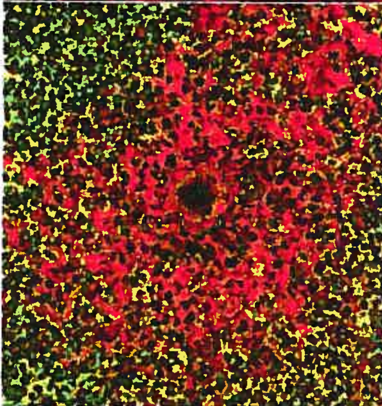


nTg

PDC

IHC staining with CD11c-Alexa red and B220-FITC:
PDCs: yellow
CDCs: red
B cells: green

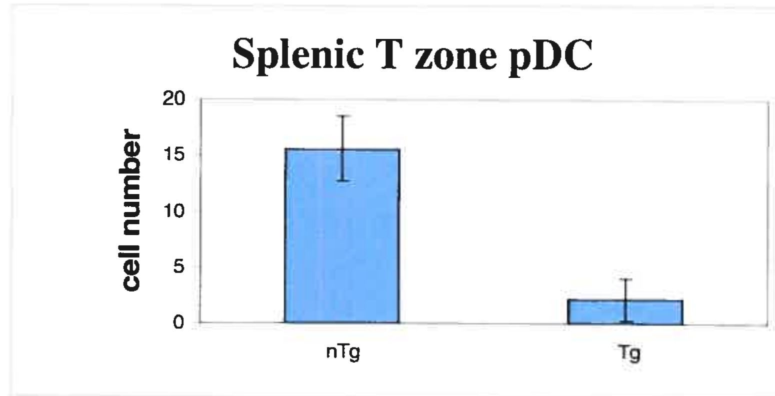




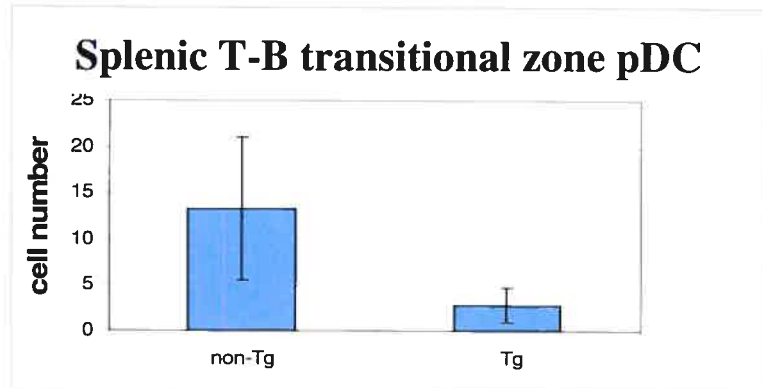
Tg

confocal microscope(Zeiss LSM510): 40X magnification

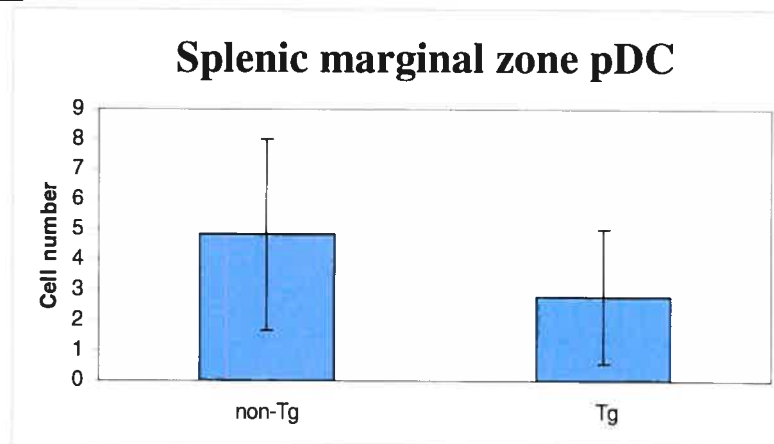
8.C.



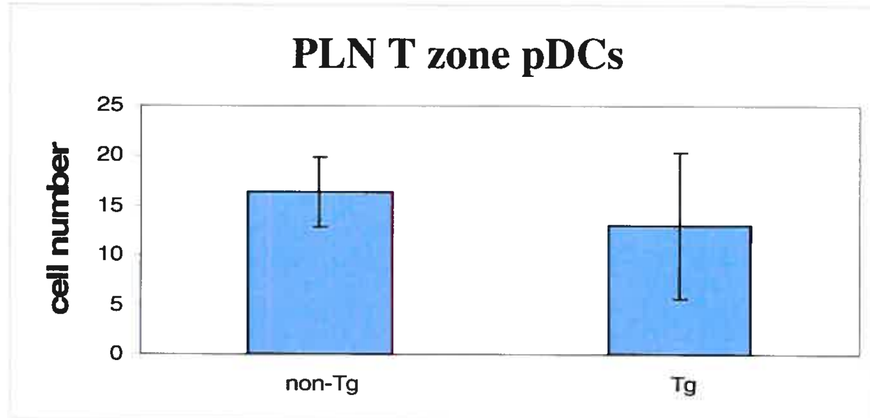
8.D.



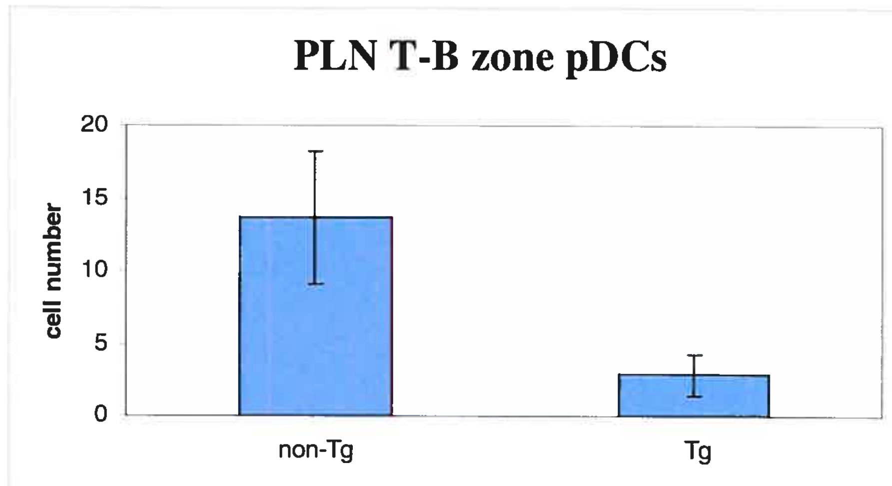
8.E.



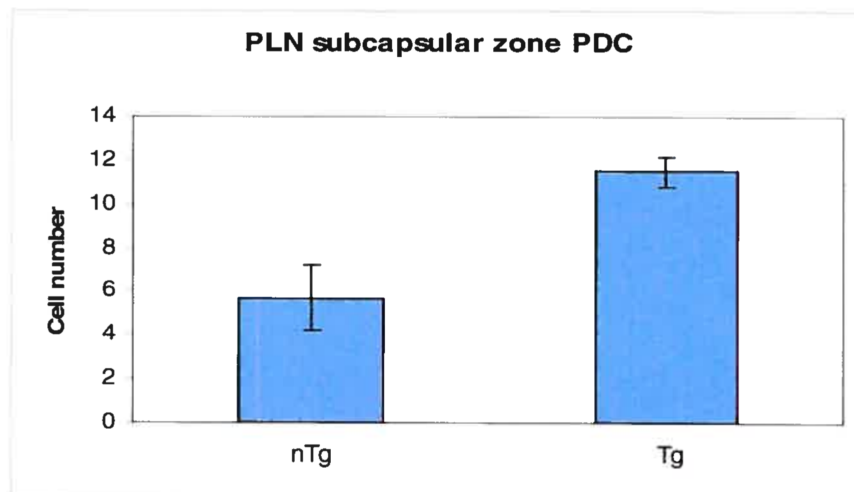
8.F.



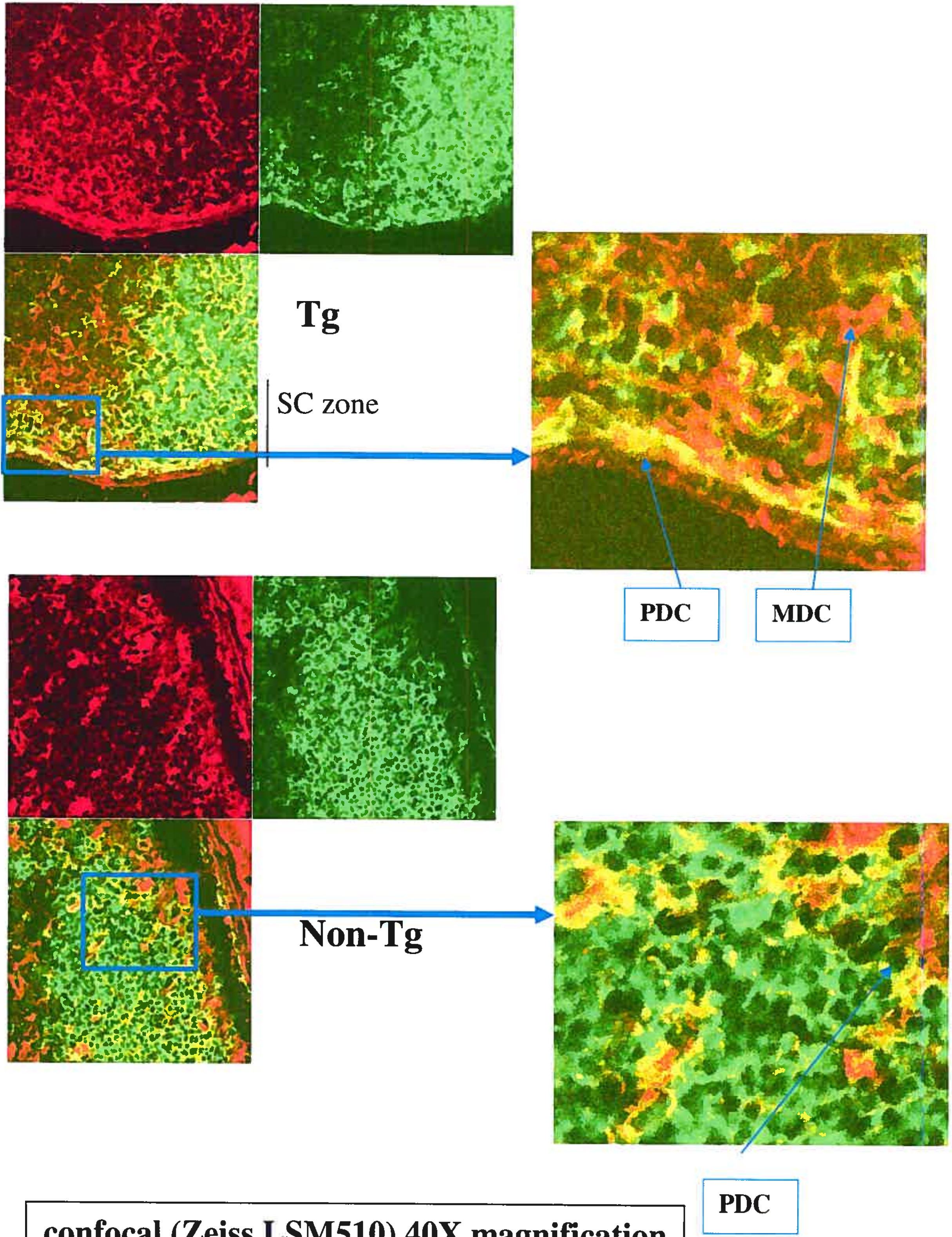
8.G.



8.H.



8.I. PLN subcapsular (SC) zone



confocal (Zeiss LSM510) 40X magnification

FIGURE 8: PDCs localization

IHC was done on spleen and LN of CD4C/HIV^{mutA} Tg mice and nTg littermates. Results are shown in figure 8.A. for spleen and 8.B. for pLN. IHC technique was used on frozen tissue sections (see materials and methods). Pictures were taken by either confocal or Axiovert microscopy at 40X level of magnification. The number of experiments was 3. In each experiment 3 Tg and 3 nTg littermate mice were used. Average number of fields counted per spleen was 10. One peri-arteriolar lymphoid sheath (PALS) was visualized per field as shown in figure 8.A.1. In the picture of figure 8.A.1, T-cell and B-cell zones are visualized at 20X magnification and B cells are seen in red by IgM-Texas Red staining. In figures 8.A.2, 8.B. and 8.H, pDCs are visualized as yellow by the superimposition of CD11c Alexa-red and B220 FITC-green staining.

Results obtained for pDCs number visualized by IHC in different regions of peripheral lymphoid organs are compiled and reported in graphs 8.C, D and 8.E for spleen and 8.F, 8.G and 8.H for pLN. SDs (Y-axis error bar) refers to intra and inter-experiments variations in pDCs number counted on all slides.

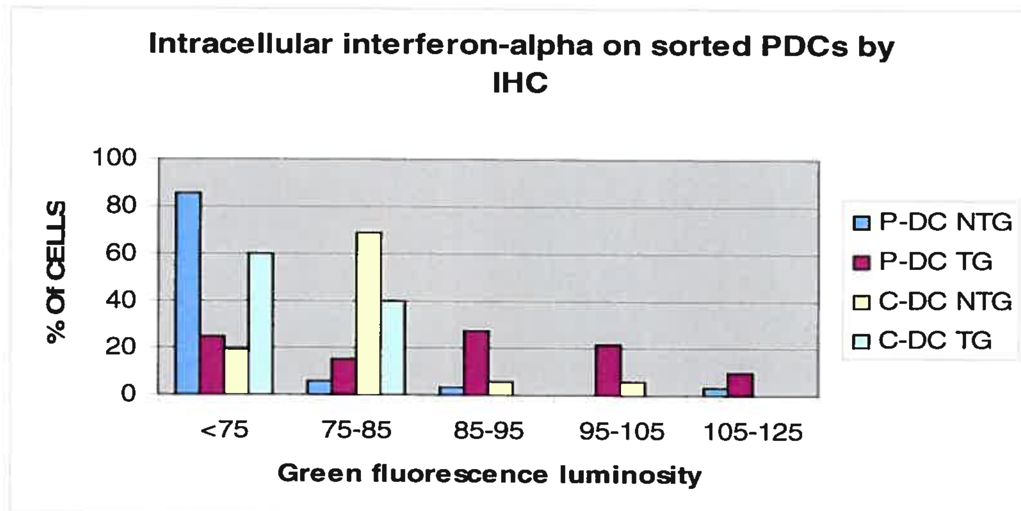
9. PDC INTRACELLULAR CONTENT IN IFN-ALPHA: SPLENIC PDCS DERIVED FROM THE CD4C/HIV TG MICE CONTAIN HIGHER LEVEL OF INTRACELLULAR IFN-ALPHA AS COMPARED TO THEIR EQUIVALENTS FROM NTG MICE.

In order to measure pDCs content in IFN-α, the technique of intracellular staining by IHC on permeabilized pDCs was used as described in the section on materials and methods. The major subtype of IFN-α produced by pDCs is IFN-A1 [86]. The Ab used in this experiment specifically detects 5 isoforms of IFN-α including A1. Preliminary results obtained from this experiment revealed a significant augmentation of IFN-α in Tg compared to nTg mice derived PDCs. As shown in figure 9, cells containing high level of IFN-α are more abundant in the Tg mice derived splenic pDC population.

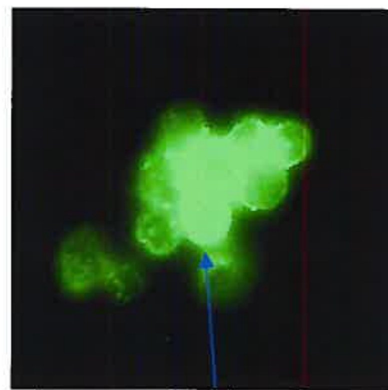
This result may reflect a higher production of the cytokine by pDCs. More experiments using ELISA along with intracellular FACS techniques are necessary to document pDCs IFN-α production *ex-vivo* and under different *in vivo* and *in vitro* conditions.

FIGURE 9: PDCS INTRACELLULAR CONTENT IN IFN-ALPHA

9.A.



9.B.

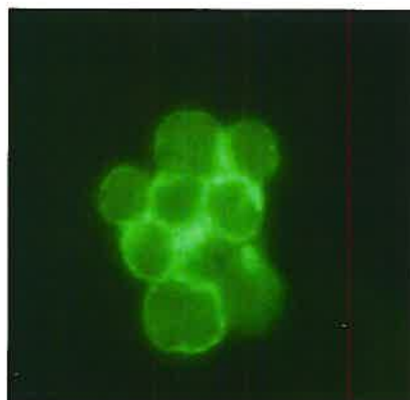


Tg PDCs

Non-Tg PDCs

Intracellular IFN-α: (FITC)

8.C.



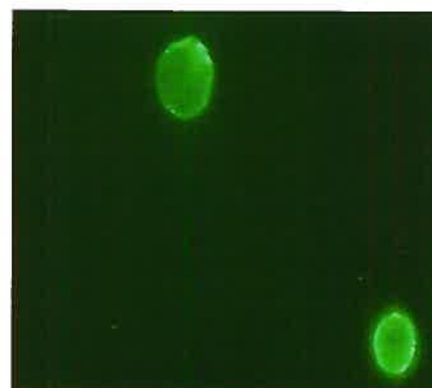


FIGURE 9: PDCs intracellular IFN-α

Isotypic control

FITC alone

FIGURE 9: PDCs intracellular IFN-α

Splenic pDCs were sorted from a pool of 6 Tg and 6 nTg mice. Purified IFN-α specific antibody (Ab.) was used in IHC reactions on cytospins of freshly sorted and permeabilized pDCs as described in materials and methods (See section on immunofluorescence). The level of Ab. staining was measured by Northern Eclipse software as green fluorescence luminosity. About 20 fields of each Tg and non-Tg derived pDCs were examined (figure 9.B). The quantification of IFN-α staining was reported in the graph 9.A comparing all populations of pDCs and CDCs from Tg and non-Tg mice. FITC alone representing the staining of cells with the secondary antibody alone as well as isotypic controls for the primary Ab against IFN-α have been used to subtract nonspecific binding (figure 9.B.). The non-specific background staining corresponds to green fluorescence luminosity <75 and is subtracted prior to data compilation (graph 9.A, x axis). The experiment has been done one time.

10. THE EFFECT OF *IN VIVO* CPG ADMINISTRATION ON IMMUNE CELL POPULATIONS: PRELIMINARY RESULTS SHOW THAT PDC MOBILIZATION INTO BLOOD AND MATURATIONAL RESPONSE TO CPG ARE NOT ALTERED IN TG MICE. IFN-ALPHA-MEDIATED EFFECTS OF CPG CAUSING NK CELL ACTIVATION AND MDC MATURATION ARE PRESERVED IN TG MICE.

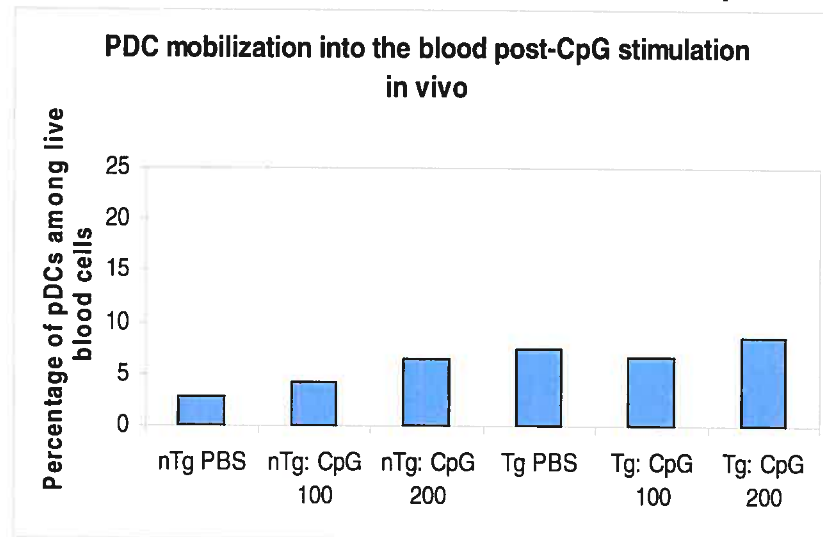
Bacterial DNA contains a high frequency of CpG motifs, which stimulate immune cells through TLR-9 [63]. Synthetic CpG mimic bacterial DNA and its injection into mice and humans has been shown to cause pDC mobilization into blood, maturation and production of IFN-α [35, 71]. This cytokine induces mDC maturation and augments mDCs Ag-presenting function [73]. In addition, CpG induce NK cell activation, including enhanced cytotoxicity and cell surface CD69 up-regulation [136, 137]. Both, pDC and mDC are required for NK cell activation in response to CpG and IFN-α is a strong inducer of CD69 expression on NK cells [23, 137]. Moreover, TLR-9 signaling pathway blocks the suppressive effect of $CD4^{+}CD25^{+}$ Tr cells allowing T cell stimulation by activated APCs [72]. It has been reported that pDC are defective in the induction of monocyte maturation in response to CpG in HIV^{+} patients [47]. In mouse, contrary to human, B cells and pDCs are not the only immune cell populations expressing TLR-9, which is also expressed by mDCs [68]. In order to evaluate the effect of Nef expression on pDC and mDC response to TLR-9 stimulation in vivo, we administered CpG to above mentioned $CD4C/HIV^{mutG}$ Tg mice.

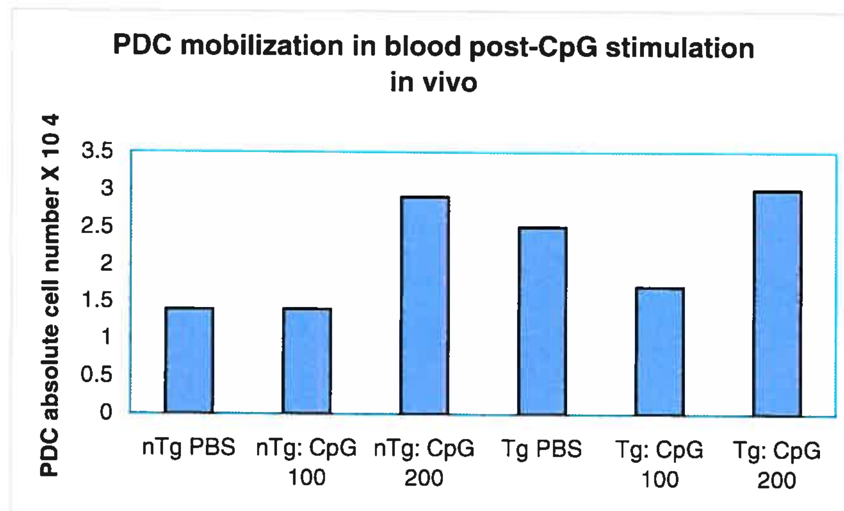
Preliminary results show that pDCs response to CpG in terms of their mobilization into the blood and maturation in pLNs is equivalent between Tg and nTg mice (figure 10.A). Figure 10.B shows that NK cell frequency in Tg mice pLN is increased compared to nTg mice, decreases upon *in-vivo* CpG stimulation in a dose-dependent manner. However, this finding may also be due to downregulation of Dx5 on Tg NK cell population post-CpG stimulation. In addition, NK cells expression of CD69 augments equally in Tg and nTg mice in response to CpG stimulation (figure 10.B). Similarly, as shown in figure 10.C, mDC maturation in response to CpG is conserved in Tg mice. As shown in figure 10.D, peripheral $CD4^{hi}$ T cells are increased and CD4 is up-regulated on $CD4^{+}$ T cells of Tg mice in response to CpG injected at 200 μg and not at 100 μg. Intrestingly, this partial rescue effect on $CD4^{+}$ T cells downregulation and depletion is observed at the same condition (CpG injected at 200 μg), which caused a decrease in abnormally elevated frequency of $CD4^{+}CD25^{+}$ T cells in Tg

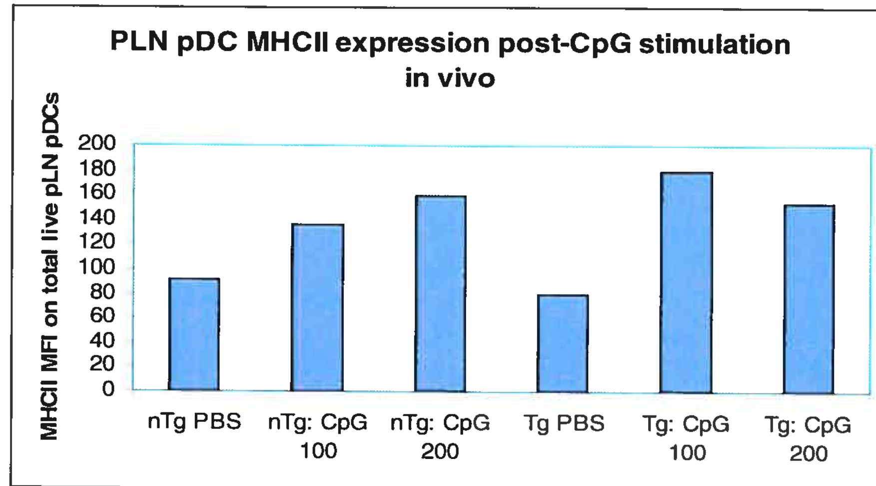
mice. Although, these $CD4^{+}CD25^{+}$ cells are known to correspond to regulatory T cells in normal mice, they have not been characterized as regulatory cells in Tg mice. In contrast to Tg mice in which the immune system is dysfunctional, in nTg mice $CD4^{+}$ T cells are slightly decreased may be because of the slight increase in $CD4^{+}CD25^{+}$ Tr cells population as expected in physiological conditions [60].

FIGURE 10. The effect of *in-vivo* CpG stimulation on immune cells

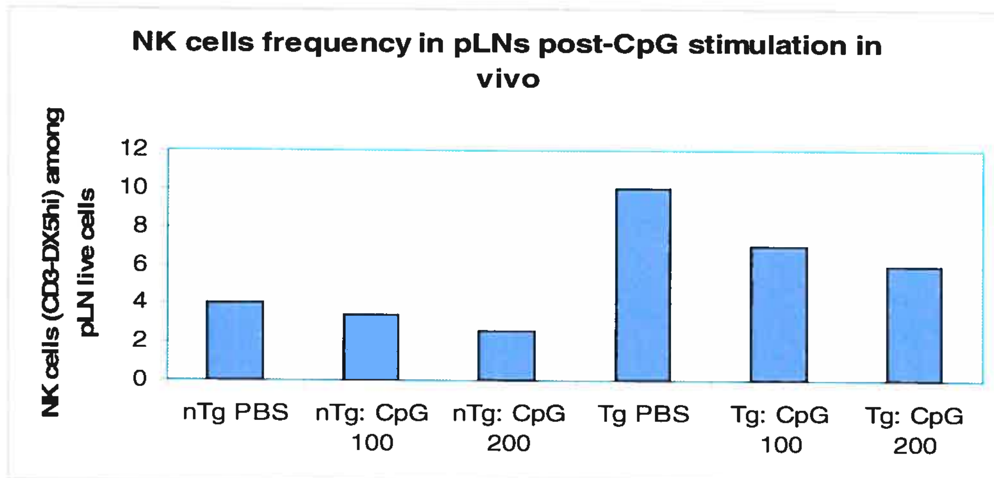
10.A: PDCs mobilization into the blood and maturation in response to CpG



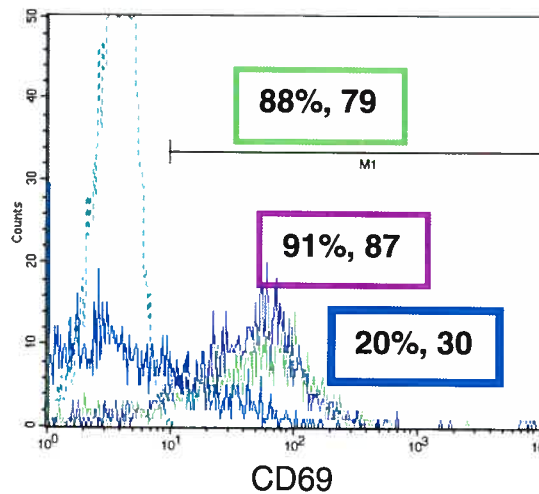


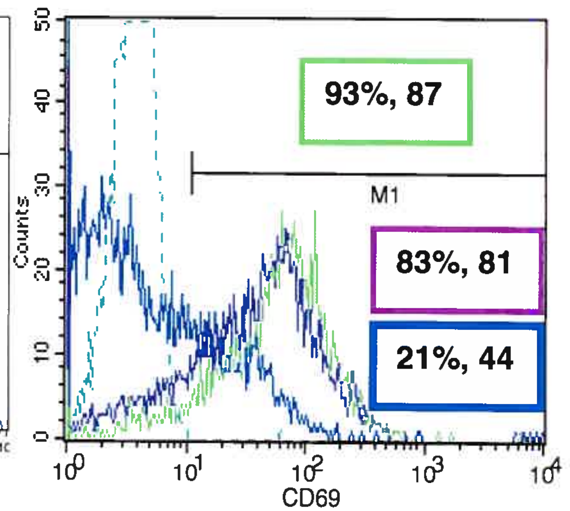


10.B: NK cells frequency and expression of CD69



PLN: nTg NK cells Tg NK cells

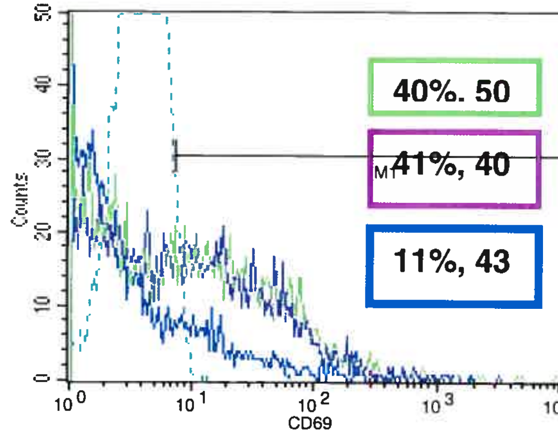


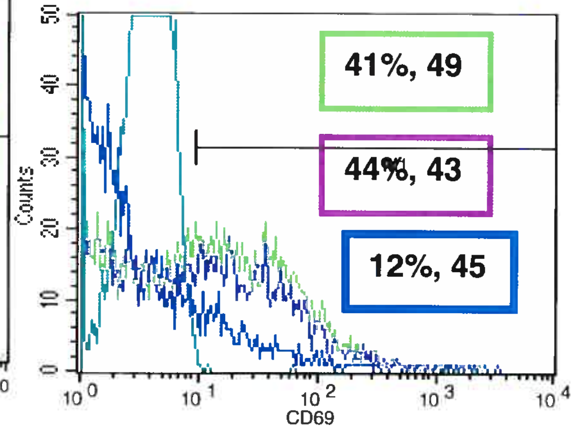


PBS CpG: 100µg CpG: 200µg

Spleen: nTg NK cells

Tg NK cells

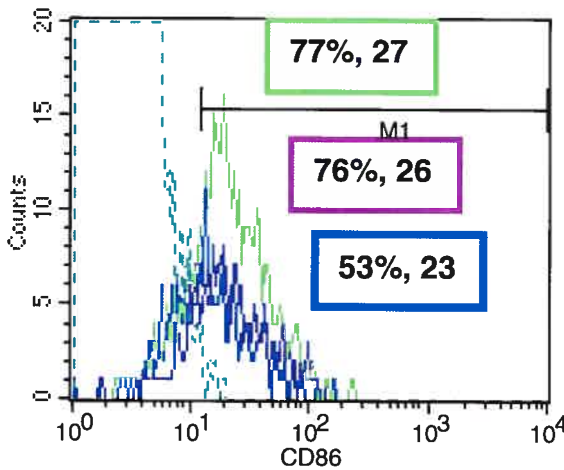


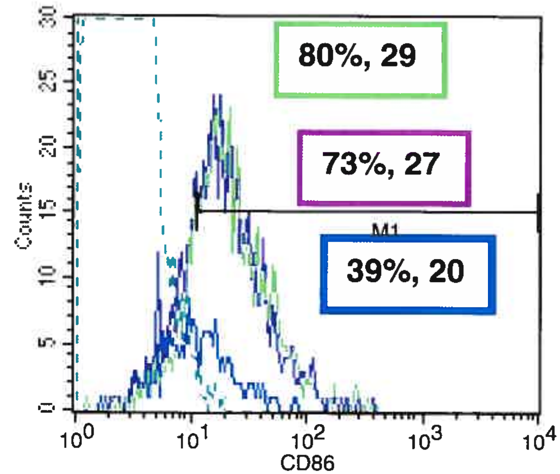


10.C. PLNs MDC maturation

nTg

Tg



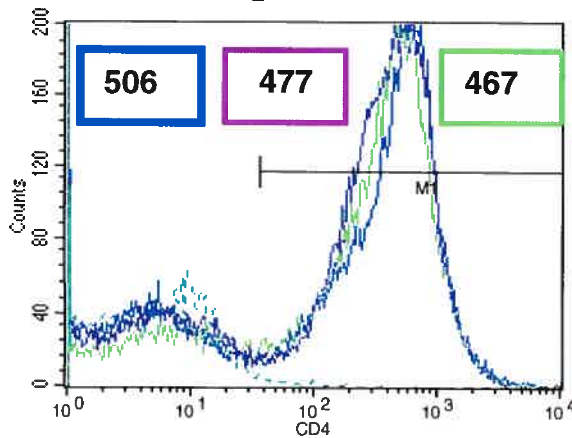


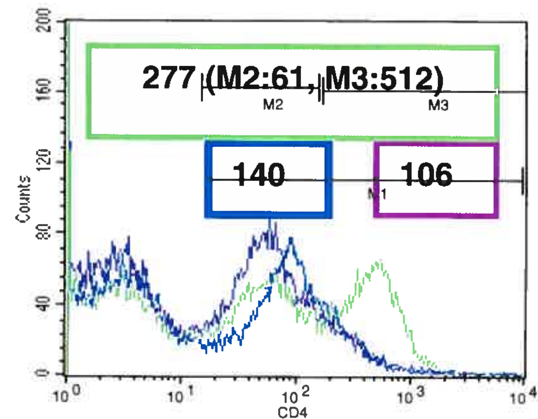
10.D.CD4$^+$ T cells:

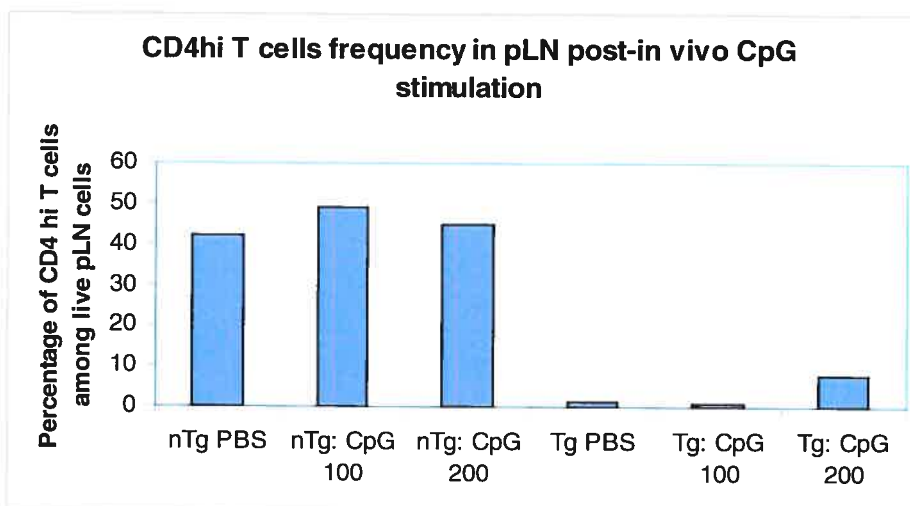
10.D.1. PLN CD4$^+$ T cells: gated on TCRαβ$^+$ cells

nTg

Tg







10.D.2. Splenic $CD4^+$ T cells

nTg mice:PBS

CpG 100 µg

CpG 200 µg

SA050912.044 R3 22% R4 1%

SA050912.043 R3 13% R4 3%

SA050912.042 R3 15% R4 2.3%

CD4

TCRab

Tg mice:PBS

CpG 100 µg

CpG 200 µg

CD4

SA050912.041 R3 0% R4 5%

SA050912.039 R3 0.2% 5%

SA050912.038 R3 2% R4 3.5%

TCRab

10.F. CD4^{+}CD25^{hi} T cells

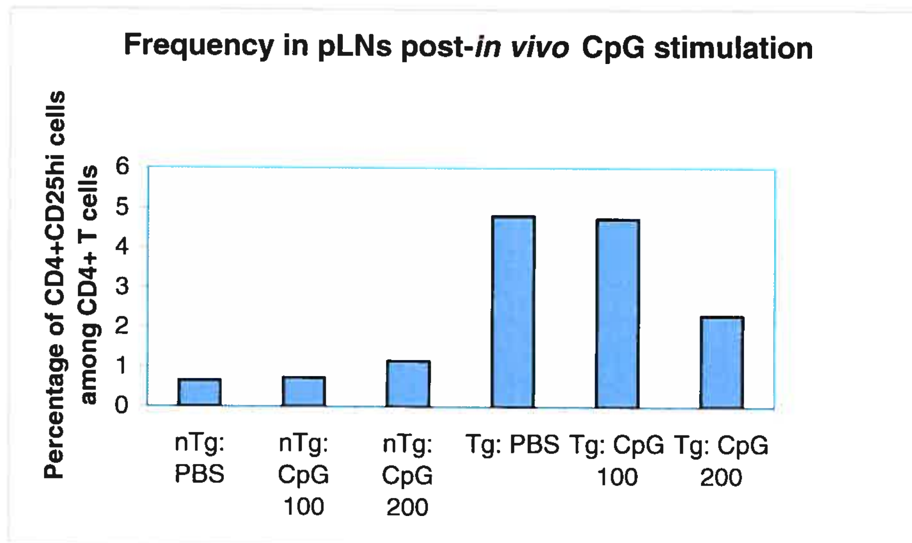


FIGURE 10. The effect of *in-vivo* CpG stimulation on immune cells

A class-C CpG-ODN was injected at a dose of either 100 µg into 2 nTg and 2 Tg mice as described in materials and methods. One nTg and one Tg mice have been injected with PBS as a negative control. Immune-cell population frequency, activation and maturational markers were measured by FACS 24 hours post-injection of CpG. Similar results were obtained in spleen and pLNs.

A) FACS results are reported on gated peripheral pDC population identified as CD11c^{lo}B220^{+}LY49Q^{+} cells. The two first graphs show pDC frequency (% and absolute cell number) in blood. The third graph shows pLN pDC state of maturation reported as MHCII expression (% and MFI).

B) This graph shows NK cell population frequency as percentage of total pLN live cells. The FACS histograms show CD69 expression on gated NK cells in nTg and Tg mice. The percentage and MFI of CD69 are reported for each mouse including, PBS and CpG injected mice. Results obtained from spleen and pLNs are reported for NK cell CD69 expression.

C) FACS histograms show the expression of the costimulatory molecule (CD86) on gated pLN mDCs defined as CD11b^{+}CD11c^{+}B220^{-} cells.

D) **D.1** and **D.2** show FACS analysis on $CD4^+$ T cells in pLNs and spleen, respectively. FACS histograms show CD4 expression on pLN $TCR\alpha/\beta^+$ cells. The graph shows pLNs $CD4^{hi}$ T cell frequency in Tg and nTg mice. In nTg mice, all $CD4^+$ T cells express high level of CD4, while in Tg mice CD4 is down-regulated [138].

E) This graph shows the frequency of $CD4^+CD25^{hi}$ T cells among pLN $TCR\alpha/\beta^+CD4^+$ T cells.

CHAPTER 4: DISCUSSION and FUTURE WORK

In this study, pDCs were examined in the context of the CD4C/HIV^{mutG} Tg mice in which Nef expression in natural target cells for HIV causes a multiorgan inflammatory disease and immune dysfunctions sharing common features with AIDS. PDCs characterization is part of investigations on the immune system dysfunctions in CD4C/HIV Tg mice [104, 115, 117]. Recently, multiple defects in cDC population have been reported in this mouse model [119]. The study of pDCs in CD4C/HIV Tg mice revealed the following characteristics when compared to nTg mice.

In Tg mice, 40 to 60% of splenic and 25% of pLNs pDCs express Nef. PDCs in Tg mice pLNs express higher level of the cell surface marker CD11c and show altered size and granularity as indicated by their light scatter characteristics in FACS analysis. However, Tg mice-derived splenic pDCs show a normal morphology based on Giemsa staining as well as FACS light scatter parameters. Since pDCs residing in these two lymphoid organs may differ in their characteristics, the morphology of pLN pDCs should also be examined by a direct technique as Giemsa staining in order to investigate their altered FACS light scatter features. The frequency of pDCs is increased in blood and pDCs distribution as well as their homing is unusual in the peripheral lymphoid organs of Tg mice. Peripheral pDCs derived from Tg mice display an immature phenotype compared to their nTg equivalents. In addition, in the peripheral lymphoid organs of Tg mice, pDCs surface expression of CD4 and CD8 is reduced and $CD4^+$ as well as $CD8^+$ pDCs are depleted. Moreover, based on preliminary results, the level of intracellular IFN-α is higher in pDCs from Tg mice than from nTg mice.

In vitro assays show that splenic pDCs from Tg mice contrary to nTg mice survive less in stimulated culture conditions compared to MA. *In vitro*, the maturational response of pLNs pDCs to stimulants is lower when they are derived from pLNs of Tg as compared to nTg mice. In contrast to peripheral pDCs, Tg mice derived BM pDCs show a normal maturational profile *ex-vivo*. However, *in vitro,* Tg BM pDCs maturational response to stimulation with a combination of GM-CSF, CD40L and rIL3 is reduced. In addition, pDCs yield from Tg BM post-Flt3L enrichment is slightly but not significantly decreased and their maturational state is significantly lower compared to nTg mice-derived pDCs. However, the maturational response of Flt3L-induced pDCs to CpG is comparable between Tg and nTg

mice. Results obtained from pDCs study in transplantation chimera mouse model suggest that in Tg mice pDCs precursors among BM cells display no defect in their potency to generate pDCs in response to Flt3L enrichment.

Preliminary results obtained from *in vivo* CpG-stimulation show an equivalent response in Tg and nTg mice concerning pDC and mDC maturation and NK cells activation. Since TNFα and IFNα are required for NK cell CD69 up-regulation [23], the secretion of these cytokines by DC subsets in response to CpG might be conserved in Tg mice. In addition, in contrary to humans, in mouse not only pDC but also other DC subsets express TLR-9 and can be directly activated by CpG [68]. This could explain why mDCs maturational response is not altered in Tg mice in contrast to the impairment of monocyte maturation in response to CpG observed in humans [47].

Intrestingly, CpG injected at the dose of 200 μg decreased the abnormally elevated frequency of $CD4^{+}CD25^{hi}$ cells in Tg mice. At this dose of CpG stimulation, a partial rescue effect was observed in CD4 downregulation on $CD4^{+}$ T cell and their depletion. This effect observed in spleen as well in pLNs of Tg mice may be hypothetically due to the augmentation of total CTLs, since NK cells as well as $CD8^{+}$ T cells are activated upon *in vivo* CpG stimulation [46, 136]. These activated cells may lyse $CD4^{lo}$ T cells, which correspond to cells expressing high levels of Nef [138]. This latter effect increases the ratio of survival factors in Tg lymphoid organs, allowing the regeneration of $CD4^{hi}$ T cells known to express low levels of Nef [138]. Moreover, since only at the dose of 200 μg CpG induces a decrease in abnormally elevated levels of $CD4^{+}CD25^{hi}$ T cells, the block in the suppressive effect of Tr cells may allow CTLs to kill viral gene expressing $CD4^{lo}$ T cells and activated DCs to stimulate and expand $CD4^{+}$ T cells [21, 40]. In fact, DCs maturation upon TLR stimulation causes cytokine production such as IL-6, which renders naïve T cells refractory to the suppressive effect of regulatory T cells and induces their proliferation [72]. Also, the depletion of Tr cells may enhance T cell regenerative pathways. However, the mechanisms of TLR-mediated control of adaptive immunity are not yet completely described [72]. In order to test the above hypothesis, the *in-vivo* CpG stimulation should be repeated. Moreover, $CD4^{+}CD25^{hi}$ regulatory T cells must be identified by the use of an Ab against FoxP3, which is a transcriptional factor specifically expressed by these cells [72].

All these defects observed in Tg pDCs could be due either to a direct effect and/or to an indirect effect of Nef expression since only one part of pDCs express Nef. PDCs lower maturational state in the periphery correlates with other DCs immature phenotype in CD4C/HIV Tg mice [119]. In order to further investigate the defects in Tg pDCs MHCII

expression, the level of expression of CIITA, which is a transcriptional coactivator acting as the master regulatory factor for all MHC class II genes could be evaluated [19]. In contrast to peripheral pDCs, BM pDCs from Tg mice do not show a defect in their maturational state. However, since in BM pDCs are precursors that give rise to blood pDCs and are immature compared to peripheral pDCs, a defect in their maturational profile may be undetectable at this stage. Peripheral pDCs immature phenotype as well as the depletion of $CD4^+$ and $CD8^+$ pDCs suggests that pDCs at a less differentiated stage, corresponding to $CD4^-CD8^-$ subset, are accumulating in Tg mice [50]. The accumulation of immature pDCs in Tg mice is also indicated by data showing an increase in blood pDCs, which are mostly composed of $CD4^-$ $CD8^-$ subset and are precursors of pDCs in peripheral lymphoid organs [50].

On the other hand, the phenotypic alterations of pDCs derived from Tg mice can reflect a defect in their function, which correlates with pDCs stage of maturation. In fact, pDCs are known to produce IFN-α at a less differentiated stage and act as APCs upon maturation [42, 50]. Thus preliminary data showing an increase in Tg mice pDCs intracellular IFN-α is in accord with their immature phenotype, if we assume that the level of intracellular IFN-α reflects its production by pDCs. However, since IFN-α is produced rapidly after viral infection [139], the finding of elevated level of this cytokine must be interpreted considering the particular context of Tg mice. In fact, in this mouse model contrary to HIV infection in humans, there is no viral infection and no reinfection cycle occurs in Tg mice. The level of transgene expression in Tg mice is likely to mimic a steady state viral load of virus expression in humans [116]. In addition, to precisely evaluate the production of IFN-α by Tg pDCs, ELISA technique must be used to detect this cytokine in the serum of Tg and nTg mice and in culture supernatant under different pDC stimulation conditions. On the other hand, pDCs Ag-presenting function, which also depends on their maturational state [42], should be evaluated in Tg mice. This function can be assessed by an allogeneic mixte leucocyte reaction (MLR) experiment in which sorted pDCs from pLNs, spleen or Flt3L-enriched BM cells are stimulated O.N. with anti-CD40 Ab. Serial dilutions of *in vitro*-stimulated pDCs are cocultured with allogeneic $CD4^+$ T cells and T cell proliferation is measured by thymidine incorporation assay [35].

The defects in pDCs phenotype and function can contribute to or caused by dysfunctions of the immune system described in Tg mice. For example, immature pDCs have been shown to induce the differentiation of Tr cells [31,58] suppressing Ag-specific T cell proliferation as observed in Tg mice [119]. In addition, since CD40L is required for pDCs maturation in vivo

[50], Tg mice-derived pDCs phenotype may be caused by the decrease in CD40L, as reported in Tg mice [118].

The immature phenotype of peripheral pDCs derived from Tg mice may also contribute to their defective homing in peripheral lymphoid organs. In fact, pDCs migration to T zones of pLNs and splenic white pulp depends on the level of expression of CCR7 on pDCs surface, which is upregulated following their maturation [11, 15]. In addition, only after maturation by stimulants such as CD40L that CCRs on pDCs surface become functional and induce their migration to peripheral lymphoid organs [13]. Therefore, Tg mice-derived pDCs immature phenotype may indicate a defect in pDCs migrational response to CCs causing their unusual homing. In Tg mice, pDCs are reduced in T zones of pLNs while they are absent in T zones of spleen. This difference between spleen and pLNs in pDCs homing may be due to the direct access of blood pDCs to pLNs through HEVs, which are not present in the spleen [13, 64, 67]. This supplementary route of migration to pLNs apart from afferent lymphatics, may explain why a defect in the level of CCR7 expression on pDCs surface do not prevent completely their migration to T zones of pLNs as is the case for spleen. Moreover, specific and different sets of chemokines are required for pDCs trans-HEV homing to pLN directly from blood compared to their splenic homing through afferent lymphatic vessels. Thus, apart from pDCs maturational defect, alteration in chemokines such as CCL19 in the inflammatory context of Tg mice may participate partly in their abnormal trafficking to peripheral lymphoid organs by favoring their homing to inflamed tissues [11, 13, 56]. Measuring pDCs expression level of CCR7, CXCR3 and CD62L, which is necessary for trans-HEV pLN homing on pDCs surface along with the quantification of CCs such as CCL19, CCL21 and CXCL9 in peripheral lymphoid organs, may be helpful for better understanding of the unusual pDCs localization in Tg mice [48, 64]. PDCs lower frequency in T zones of pLNs and their absence in T zones of spleen may contribute to the anergic state observed in Tg mice. In fact, the release of cytokine by pDCs in the microenvironment of T zones is important for shaping a T cell response by linking the innate and adaptive immunity [42].

This study shows an abnormal viability of Tg pDCs *in vitro*. Tg pDCs are more viable than nTg pDCs in MA and less than nTg pDCs in stimulated conditions. The higher viability of Tg mice-derived splenic pDCs in MA is in accordance with the finding that *in vitro* HIV infected pDCs survive more than non-infected pDCs, most probably due to the production of IFN-α, which is a known survival factor [113]. Accordingly, an increase in Tg mice-derived splenic pDCs content of IFN-α was observed as mentioned previously. In the other hand, the lower

viability of Tg mice-derived pDCs in stimulated culture conditions may be due to their higher death following their maturation. In fact, it has been shown that HIV-1 infected pDCs form multinuclear syncytia cells and die upon maturation and both DC subsets depletion in HIV-1 infected patients' blood is partly due to their maturation [45, 140]. Further investigation for pDCs survival *in vivo* could include the measurement of the level of cytokines required for pDCs survival such as TNF-α and IFN-α as well as the expression of TRANCE-R on pDCs [64, 113]. The defect in Tg pDCs survival *in vitro* may reflect pDCs abnormal turnover *in vivo*, which can be examined directly by kinetic assays using 5-bromodeoxyuridine (BrdU) labeling [1].

It should be noted that pDCs are augmented in blood of Tg mice in contrary to their reduction in HIV-1 infected subjects. However, only one of HIV genes is expressed in Tg mice and pDCs disappearance from patients' blood could be a consequence of other HIV proteins functions. The observed augmentation in pDCs frequency in blood mimics a long-term non-progressor status in humans [107, 108]. This can be due to the similarities between AIDS-like disease in Nef Tg mice and a chronic suboptimal infectious condition inducing an anergic immune state.

CD11c is expressed at higher levels in Tg than nTg mice derived pDCs based on values of CD11c MFI in pDC population from pLN and from Flt3L-enriched BM precursor cells stimulated with CpG. Thus, the same shift in CD11c expression is observed in pLN-derived than in CpG stimulated BM-derived pDCs. In addition, since CpG stimulation of pDCs specifically activates the TLR-9 signaling pathway [71] and induces CD11c upregulation in pDCs from nTg and Tg mice (figure 6.F), the similarity observed in the phenotype of Tg pLN and CpG stimulated pDCs, suggest that TLR-9 signaling pathway may be activated on pLN pDCs due to Nef expression in Tg mice. If this hypothesis is exact, Nef-expressing pDCs in pLNs of Tg mice are chronically activated. This suggests that Nef-mediated chronic activation of TLR-9 signaling pathway in pDCs may contribute to the impaired induction of the maturational response of monocytes to CpG observed in HIV-1 infected patients [47].

It is known that pDCs are the homeostatic counterpart of mDCs and they are believed to modulate DC, NK and macrophages functions [50]. Since pDCs play multiple regulatory roles in the innate immune system and because of their impairments observed in Tg mice, the innate immune system may be dysfunctional in these mice. Accordingly, as inflammation is an indicator of the activation of innate immune responses, defects in pDCs characteristics including higher IFN-α production may contribute to the chronic inflammatory organ disease observed in CD4C/HIV Tg mice [116].

In conclusion, since pDCs show many abnormal features in CD4C/HIV Tg mice, they may be implicated in immune dysfunctions of the AIDS-like disease. Since pDCs count correlates with $CD4^+$ T cell count in HIV-1 infected patients, we can examine the effect of pDCs depletion, expansion and/or maturation on the immune system, particularly on $CD4^+$ T cell frequency, in Tg mice. PDC population can be depleted, expanded and or stimulated by injection into Tg and nTg mice of a depleting Ab such as mPDCA-1 [48], Flt3L and CpG respectively, into Tg and nTg mice. Data obtained on Flt3L-induced pDCs suggest that pDCs precursors in BM of Tg mice expand in response to Flt3L, at the same levels than their equivalents in nTg mice. In addition, based on preliminary results obtained from *in-vivo* CpG stimulation, pDCs and IFN-α-mediated response to CpG is equivalent in Tg and nTg mice.

Moreover, the injection of 200µg of CpG induced CD4 upregulation on $CD4^+$ T cells and a slight $CD4^{hi}$ T cell repletion. The partial rescue effect of CpG on CD4 down-regulation of $CD4^+$ T cells and their depletion in $CD4C/HIV^{mutG}$ Tg could be promoted by prior pDC expansion with Flt3L followed by CpG injection at 200µg [141]. In addition, since $CD4^+CD25^+$ regulatory T cells as well as IL-10 are known to inhibit pDC response to CpG, the concomitant injection of an anti-IL-10-neutralizing Ab may optimize the cytokine milieu to allow $CD4^+$ T cell proliferation [16, 136, 142]. In these experiments, pDCs frequency, phenotype, localization, IFN-α production and Ag-presenting function should be assessed. Moreover, other immune cell populations, which are known to be directly or indirectly regulated by pDCs, could be examined. These cell populations include NK cells [43, 63], B cells [37, 58], mDCs [47, 85], $CD4^+$ helper T cells [47, 80] and $CD4^+CD25^+$ Tr cells [60]. This experiment may allow a reversal of $CD4^+$ T cell depletion observed in Tg mice and may help to elucidate the effect of pDC maturation on the immune system homeostasis in CD4C/HIV Tg mice.

CHAPTER 5: CONCLUSION

In Tg mice compared to nTg littermates we observed:

- PLN pDCs show a shift into higher cell surface expression of CD11c and different light scatter characteristics.
- In Tg mouse 40 to 60% of splenic and 20 to 25% of pLN pDCs express Nef.
- In the BM of Tg mice, pDCs maturational state *ex-vivo* is normal while their state of maturation post-Flt3L enrichment is significantly decreased.
- Contrary to BM, peripheral (spleen and pLN) pDCs maturational state is lower *ex-vivo*. PLNs pDCs stimulation with a combination of immunostimulants does not rescue their lower *ex-vivo* maturational state.
- PDCs frequency is augmented in spleen and blood, decreased in pLN and unchanged in BM.
- In addition, in the peripheral lymphoid organs of Tg mice, pDCs surface expression of CD4 and CD8 is reduced and $CD4^{+}$ as well as $CD8^{+}$ pDCs are depleted.
- In vitro culture assays of splenic DCs show that tg-mice pDCs have an increased survival in medium alone and a reduced viability in stimulated conditions.
- PDCs in the lymphoid organs of Tg mice show a homing defect. PDCs are absent in the splenic T cell zone and lowered in the transitional B-T cell zone of Tg versus nTg mice. They are few in nTg MZ and are equally present in Tg and nTg MZ. In Tg pLN, pDCs number is similar to nTg in T cell zone, lower in B-T transitional and enhanced in sub-capsular zone compared to nTg pLN.
- Preliminary results show that Tg mice-derived pDCs contain more IFN-α than nTg pDCs.
- Preliminary results obtained from in-vivo CpG stimulation show that pDCs and mDCs maturational response is conserved in Tg mice.

This research is useful in the context of investigating the effects of each of HIV-1 viral products on cellular functions and their contribution to the development of AIDS. It should be mentioned that part of these results has been obtained *in-vitro* and their physiological relevance remains to be determined by *in-vivo* experiments.

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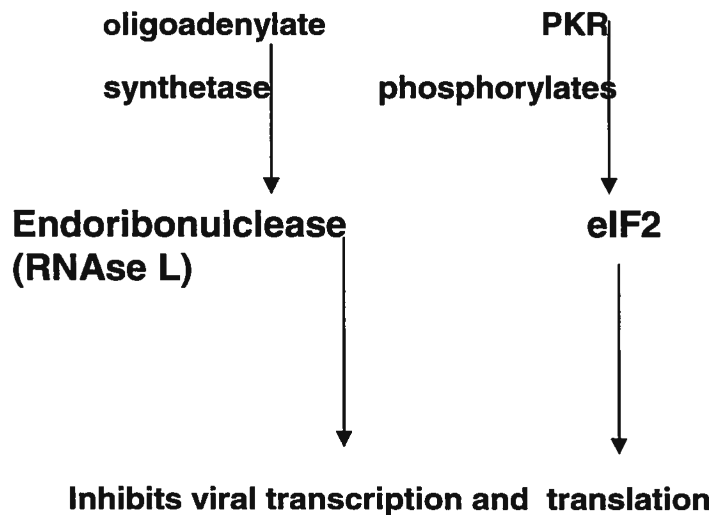
CHAPTER 7: Supplementary Figures

Figure 11: IFN-alpha functions [84]

I. Antiviral effects

1.Antiviral state on cells:



Inhibits viral transcription and translation

2.NK cells and CTL activity stimulation:

Kill viral infected cells

II.Effect on cell growth and division

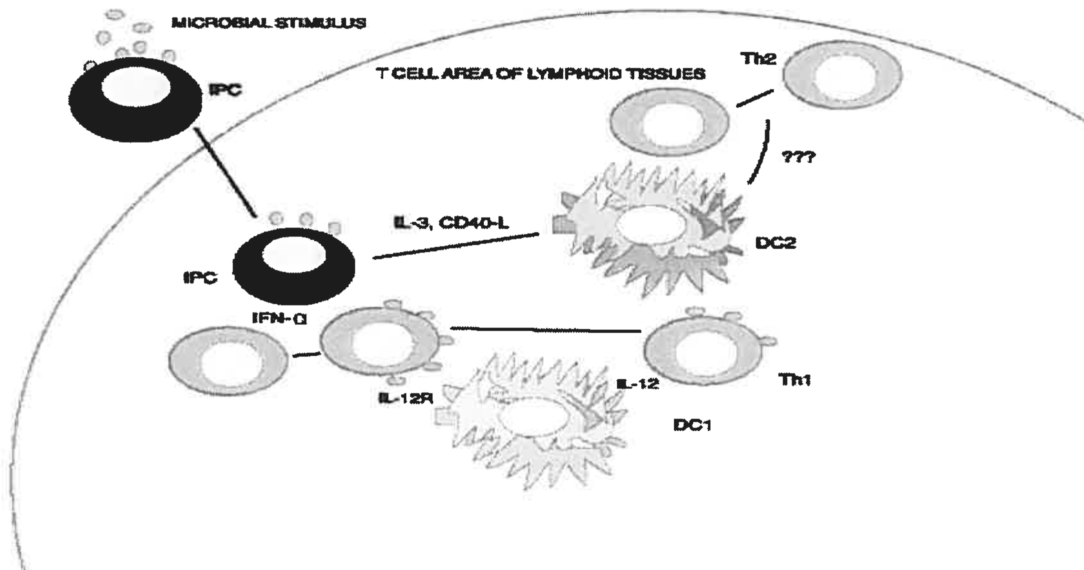
III.MHCI and MHCII expression

IV.Anti-tumoral activity

V.Delayed type hypersensitivity (DTH) up or down-regulation

Figure 12: Interferon-producing plasmacytoid dendritic cells and the pathogenesis of AIDS [139]

A. A model of how interferon-producing cells (pDCs) may play a role in the interface of innate and adaptive immunity



B. A model of the hypothetical role of pDCs in the loss of Th-1 immunity in HIV infection and how these events leads to HIV pathogenesis

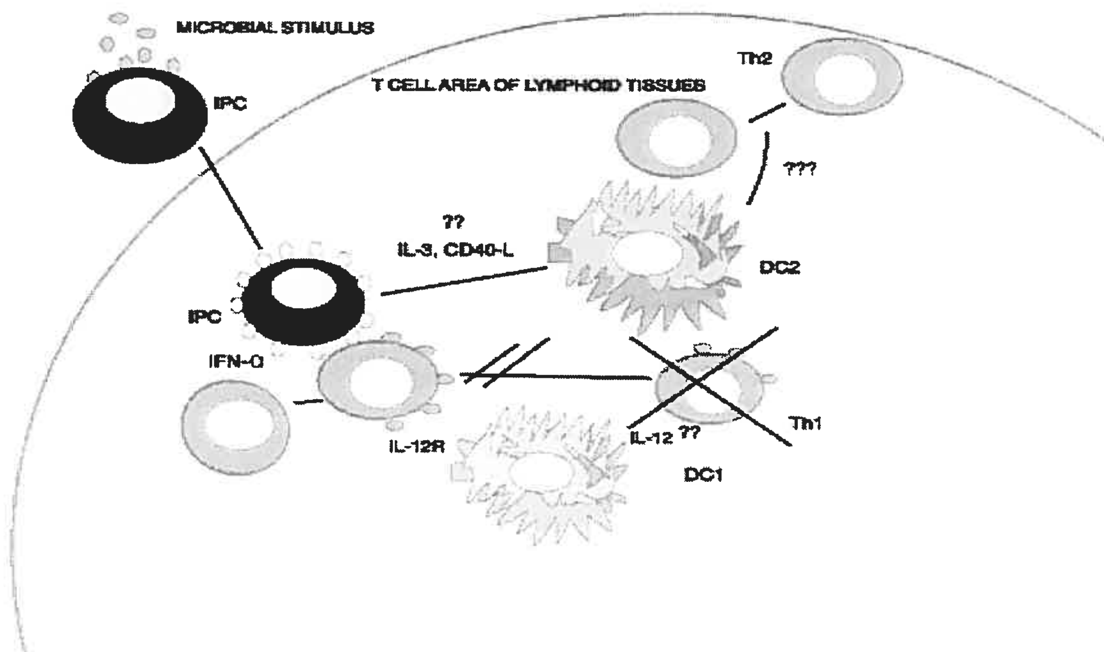
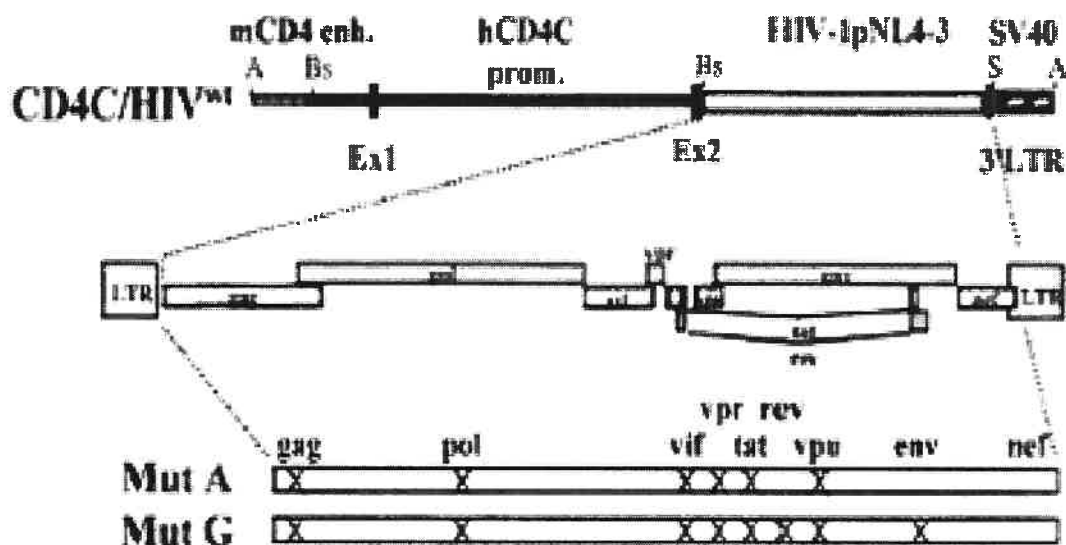


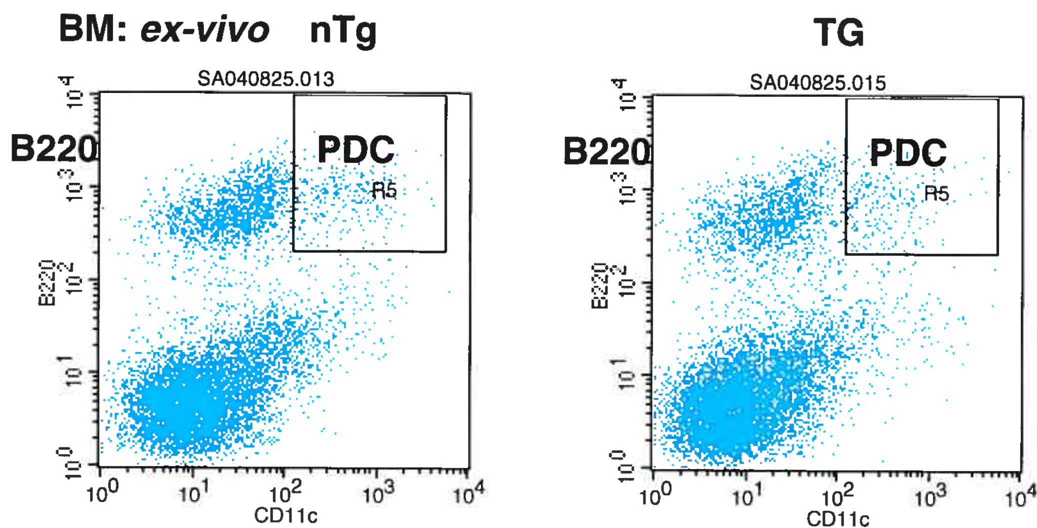
Figure 13: CD4C/HIVmutG transgene

CD4C/HIV^{mutG} transgene was constructed by fusing 14.4 kbp CD4C promoter (Hanna et al., 1994) to 8.8 kbp BssHII-SacI fragment of HIV-1 NL4-3,I, in which all genes except for *nef* gene were mutated, and the simian virus 40 polyadenylation sequences. The CD4C promoter consists of 12.5 kbp upstream sequences of human CD4 gene containing 2.6 kbp of 5' upstream sequences, exon 1, intron 1 and few bases at the beginning of exon 2, as well as the 1.9 kbp mouse CD4 enhancer at the 5' end [116]. CD4C/HIV^{mutA} transgene is essentially similar to CD4C/HIV^{mutG} with the exception that besides HIV-1 Nef gene it also retains the Rev and Env genes of HIV-1 [116].

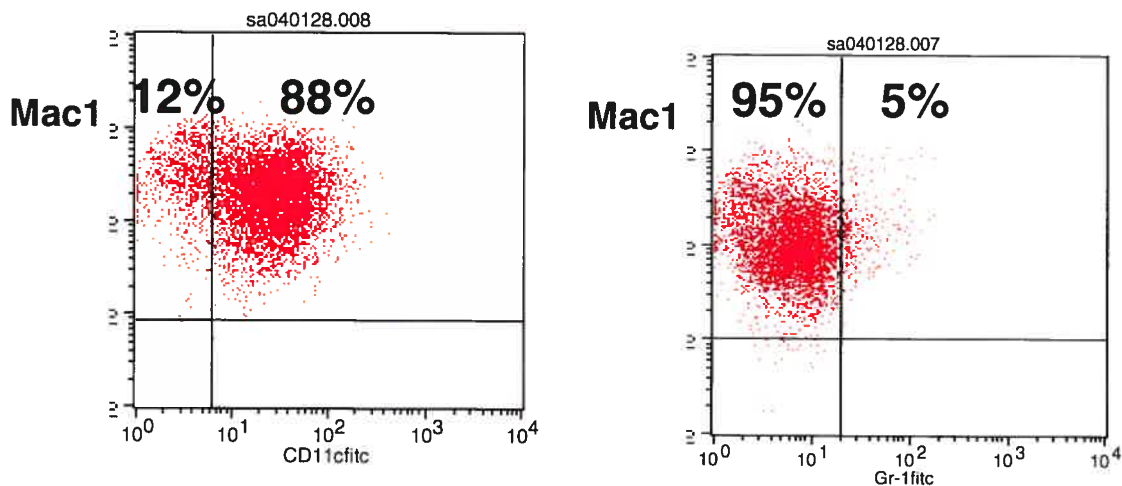


Sturcture of CD4C/HIV^{muts}: The transgene consists of mouse CD4 enhancer (mCD4 enh.) human CD4 promoter (hCD4C prom.), HIV-1 NL4-3 mutant genomes and SV40 polyadenylation sequences. The symbol (x) indicates the interruption of the corresponding HIV genes. Restriction sites: A, AatII; Bs, BssHII; S, SstI. Figure was modified with the permission of the authors [116].

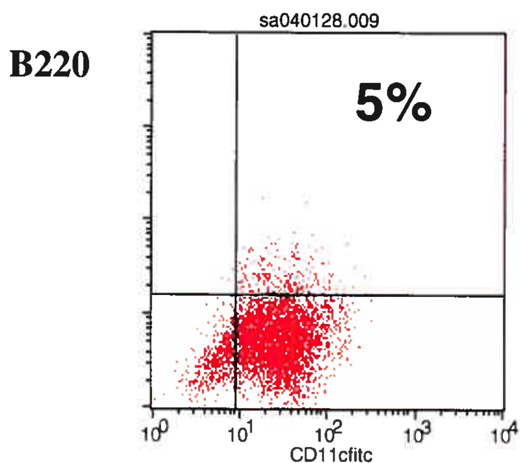
Figure 14: BM-derived DCs and pDCs



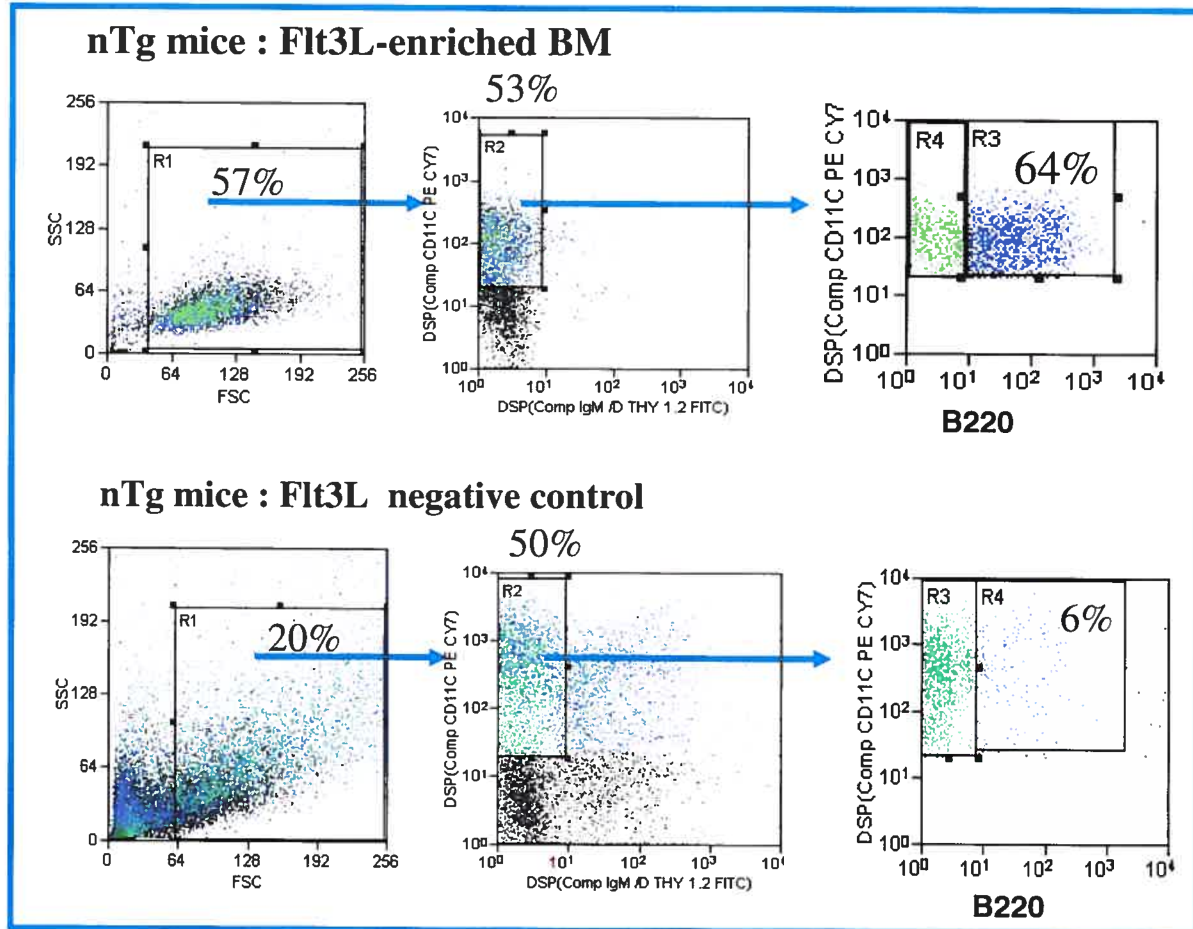
14.A. Advanced method of BMDDC enrichment. : [131]



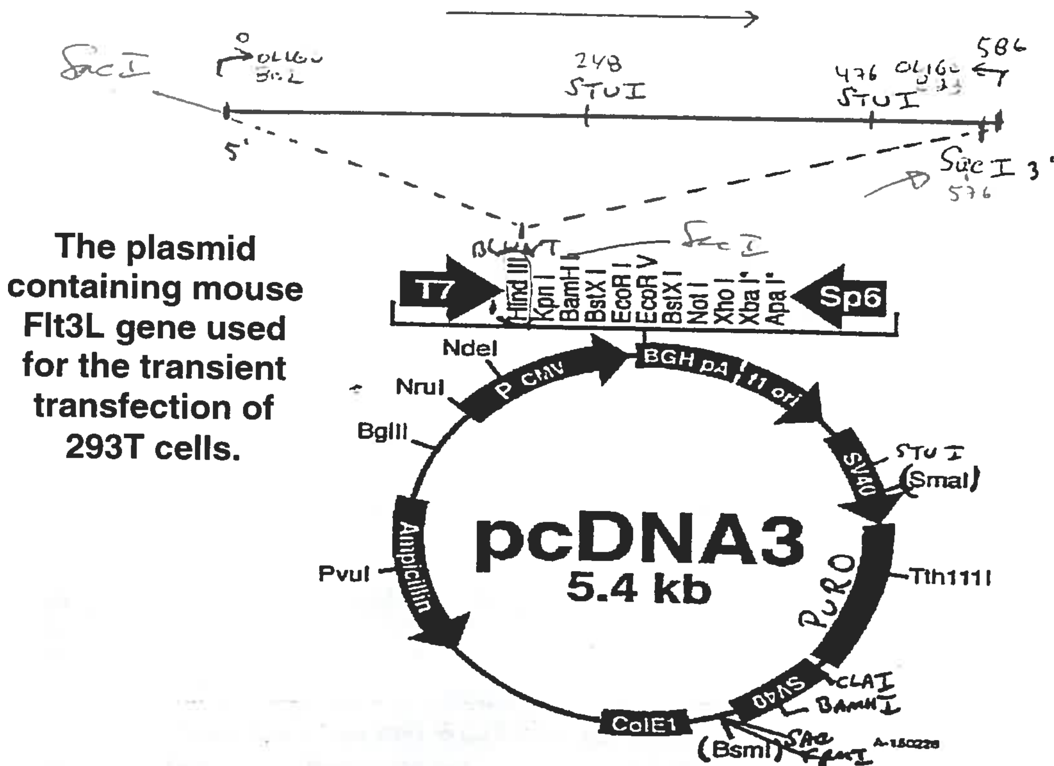
The yield of DC population is 88% and the frequency of granulocytes in this culture is only 5%.



14.B. FLT3L enriched BM [46]

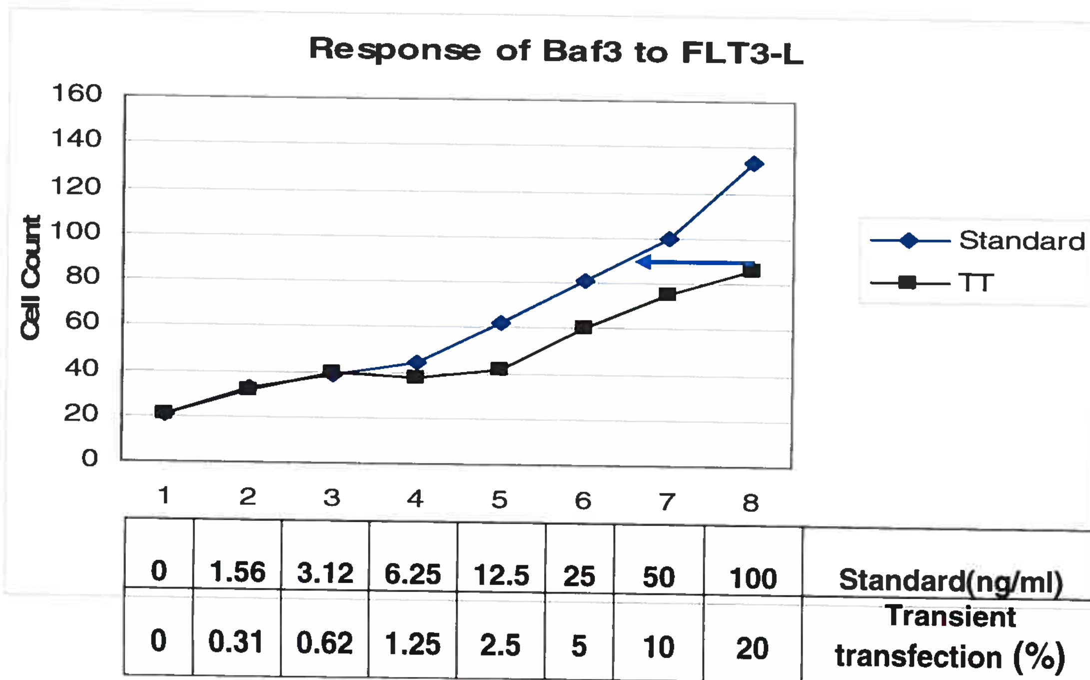


Flt3L is a growth factor for hemopoietic progenitors. It promotes DCs and pDCs expansion and in Flt3L$^{-/-}$ mouse, pDCs are severly disturbed. The upper panels in this figure shows pDC population generated with Flt3L enrichment of BM cells. In this culture Flt3L obtained from transient transfection was used. The lower panels show pDC generation in BM cells culture supplemented with the supernatant obtained from transient transfection using an empty plasmid.



The plasmid containing mouse Flt3L gene used for the transient transfection of 293T cells.

Supernatant containing Flt3L was obtained by transient transfection (TT) of 293 T cells. The graph shows the titration of this supernatant compared to commercial Flt3L. For titration of Flt3L, Baf3 cells were used which are known to proliferate in response to Flt3L. Since the percentage of the supernatant used in the culture media for pDC induction from BM progenitor cells was 20%, the quantity of Flt3L in the culture corresponds to 35 ng (as indicated by the arrow).



0	1.56	3.12	6.25	12.5	25	50	100	Standard(ng/ml)
0	0.31	0.62	1.25	2.5	5	10	20	Transient transfection (%)

Figure 15: BM post Flt3L enrichment in chimeric mice model

pep3B: **CD45.1^{+} CD45.2^{-} control**