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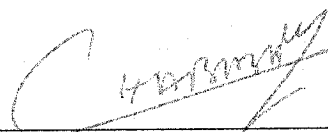
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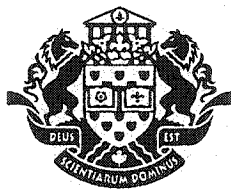
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ABSTRACT

Interleukin 12 plays a central role in NK cell activation and CD4⁺T cell differentiation. IL-12 acts via a receptor composed of IL-12R β 1 and IL-12R β 2 subunits. IL-12R β 1 plays a primary role in ligand binding whereas IL-12R β 2 is responsible for signaling. IL-18 shares some functional activities of IL-12. However, there is a considerable amount of contradictory data in the literature regarding the requirements for IL-12 and IL-18 responsiveness.

This work examined the expression of IL-12R β 2 on murine NK and T cells and the requirements for acquisition of IL-12/IL-18 responsiveness. NK cells stimulated with IL-2+IL-12 or IL-2+IL-18 exhibited rapid upregulation of IFN- γ expression followed by upregulation of IL-10 or IL-13 mRNA, respectively. NK cells from IL-12R β 2-deficient mice responded to IL-2+IL-18 independently of IL-12. Furthermore, flow cytometric analyses revealed that NK cells activated *in vitro* with IL-2 differentiate into two distinct cell subsets expressing different levels of IL-12R β 2.

Like NK cells, NK-T cells also responded to IL-2+IL-12 or IL-2+IL-18 without stimulation of the antigen-specific T cell receptor (TCR). Previously, it was thought that all T cells require activation via the TCR in order to respond to IL-12 or IL-18. Thus, responsiveness of conventional T cells to IL-2+IL-12 or IL-2+IL-18 in the absence of TCR ligation was assessed. Naïve CD4⁺T cells from wild type or DO11.10/Rag2^{-/-} OVA-specific TCR transgenic mice responded to IL-2+IL-12 or IL-2+IL-18 by expressing IFN- γ and cells grown in IL-2+IL-12 exhibited signs of polarization towards a T_H1 phenotype.

Transgenic BALB/c mice constitutively expressing the IL-12R β 2 chain were generated and the role of the IL-12R β 2 in T_H2 phenotype development was analyzed using the T_H1-dependent murine leishmaniasis model. Despite constitutive expression of the IL-12R β 2 chain, transgenic T_H2 cells did not revert to T_H1 when restimulated with IL-12 and mice remained susceptible to *Leishmania*. The role of IL-12R β 2 in T_H1 cell development was also analyzed using the same model. Resistant C57BL/6 mice defective in IL-12R β 2 gene exhibited susceptibility to *L. major* infection and expressed a T_H2 phenotype, consistent with a critical role for IL-12 in this model.

Thus understanding the expression of IL-12R β 2 in NK and CD4⁺T cells and its regulation by cytokines may constitute an important element in studies in cancer and autoimmune disease therapy.

RESUMÉ

Les activités biologiques de la cytokine IL-12 sont médiatisées par un récepteur de haute affinité qui est composé des chaînes IL-12R β 1 et IL-12R β 2. Cette dernière est responsable de transduire les signaux de IL-12. La chaîne IL-12R β 2 est exprimée sur les cellules NK (Natural Killer) et les lymphocytes T et est considérée comme le commutateur moléculaire qui détermine la différenciation des cellules T_H1. Cependant, la régulation de la chaîne IL-12R β 2 sur les cellules NK n'a pas été profondément étudiée. En utilisant l'antisérum IL-12R β 2, les résultats démontrent qu'après activation *in vitro* avec IL-2, les cellules NK de la rate de souris se différencient en deux sous-populations cellulaires exprimant différents niveaux de IL-12R β 2 : IL-12R β 2^{bas} et IL-12R β 2^{élevé}. Le développement de ces deux sous-populations est généralement accompli vers le 10^{ème} jour de culture cellulaire. La caractérisation phénotypique indique que plusieurs marqueurs associés aux cellules NK tels que NK1.1, DX5, Ly-49A et Ly-49C se répartissent de façon égale entre les deux sous-populations cellulaires excepté pour le récepteur inhibiteur Ly-49G2 qui est restreint à la sous-population IL-12R β 2^{élevé}. Les deux sous-populations répondent à IL-12 par prolifération, production de l'interféron gamma (IFN- γ) et par une activité cytolytique accrue.

IL-12 agit en synergie avec IL-18 pour induire une expression intense d'IFN- γ dans les cellules T et NK du système immunitaire. Dans le présent travail, une étude cinétique de l'expression des gènes des cytokines des cellules NK stimulées avec IL-2+IL-12 et/ou IL-2+IL-18 a été réalisée. Les résultats démontrent que la réponse à IL-12 ou à IL-18 dépend de la présence de IL-2 ou IL-15. En réponse à IL-2+IL-12 ou IL-2+IL-18, les cellules NK ont montré une expression rapide et maintenue d'IFN- γ (6 heures) suivie d'une accumulation tardive des ARNm de IL-10 ou IL-13 respectivement. Pendant la stimulation avec IL-2+IL-12+IL-18 l'expression des ARNm de l'IFN- γ et de IL-13 a été maintenue; cependant l'expression de l'ARNm de IL-10 a été réprimée. De plus, l'analyse de l'IFN- γ intracellulaire a démontré que les cellules NK stimulées avec IL-2+IL-12+IL-18 se différencient en deux populations de cellules produisant différentes quantités d'IFN- γ . En outre, les résultats ci-joints constituent une évidence solide affirmant que les cellules NK de souris répondent à IL-18 indépendamment de IL-12. En

effet, les cellules NK de souris normale et de souris présentant une déficience dans le gène IL-12R β 2 (souris IL-12R β 2KO) répondent de façon similaire à la stimulation avec IL-2+IL-18 en produisant IFN- γ et IL-13.

Les cellules NK de rate de souris sont composées d'une majorité de cellules exprimant les marqueurs cellulaires CD3⁺DX5⁺ (cellules NK) et d'une minorité de cellules exprimant les marqueurs CD3⁺DX5⁺ (cellules NK-T). Les résultats rapportés ici montrent que les cellules NK-T répondent à la stimulation aux cytokines indépendamment des signaux transmis par l'activation du récepteur des cellules T (TCR). De plus, en réponse à IL-2+IL-12 ou IL-2+IL-18, les cellules NK-T produisent une quantité plus élevée d'IFN- γ que les cellules NK. Ces résultats nous ont incités à examiner si les cellules conventionnelles CD4 naïves (CD4⁺T) répondent à l'activation avec IL-2 en présence ou en absence des cytokines IL-12 et/ou IL-18 et en l'absence de ligature du TCR. Lorsque les cellules CD4⁺T naïves de souris DO11.10/Rag2^{-/-}, qui expriment un TCR transgénique, sont activées avec 1000 U/ml de IL-2, elles répondent à IL-12 ou à IL-18. En effet, les cellules CD4⁺T activées avec IL-2 prolifèrent et secrètent de l'IFN- γ en réponse à IL-12 ou IL-18. Plus intéressant encore, les cellules CD4⁺T naïves stimulées avec IL-2+IL-12 montrent des signes de polarisation envers le phénotype T_H1 après ré-activation avec les anticorps CD3 et CD28. En outre, les cellules CD4⁺T naïves adoptent un phénotype activé (CD25⁺CD44^{hi}CD45^{lo}CD62L^{lo}) en réponse à ces cytokines. Le terme "bystander polarization" a été ainsi adopté pour décrire ce processus et nous spéculons que ce phénomène peut se produire *in vivo* pendant des réactions immunitaires intenses de manière indépendante de l'antigène.

Il a été rapporté que la différenciation des cellules CD4⁺T naïves envers le phénotype T_H2 a comme conséquence la répression de la chaîne IL-12R β 2 qui provoque la perte de capacité de ces cellules à répondre à IL-12. Afin de comprendre le rôle de la chaîne IL-12R β 2 dans le développement et la stabilité du phénotype T_H2 *in vitro* et *in vivo*, des souris transgéniques de race BALB/c qui expriment constitutivement le gène IL-12R β 2 ont été produites. Les résultats démontrent qu'en dépit de l'expression constitutive du gène IL-12R β 2, les cellules CD4⁺T naïves de ces souris transgéniques se différencient normalement en cellules T_H2 en présence de IL-4 *in vitro*. Cependant, les cellules T_H2 polarisées de ces souris transgéniques ne se convertissent pas en T_H1 une

fois stimulées avec IL-12. De même, l'expression constitutive de la chaîne IL-12R β 2 ne protège pas les souris BALB/c transgéniques de l'infection au parasite intracellulaire *L. major* et par conséquent ces souris continuent à développer une réponse immunitaire fatale de type T_H2 en réponse au parasite. Donc les résultats indiquent que l'expression de la chaîne IL-12R β 2 n'est pas essentielle au développement ni à la stabilité des cellules T_H2 *in vitro* et *in vivo*.

Le rôle de la chaîne IL-12R β 2 dans le développement des cellules T_H1 *in vivo* a aussi été analysé en utilisant le même modèle de l'infection au parasite *L. major*. Des souris génétiquement résistantes qui présentent une déficience dans le gène IL-12R β 2 (IL-12R β 2KO) ont été utilisées. Les résultats démontrent que les souris IL-12R β 2KO sont devenues susceptibles à l'infection et que les nodes lymphatiques expriment un taux élevé de cytokines T_H2 au niveau des ARNm et des protéins. De plus, les souris IL-12R β 2KO démontrent un taux sérique élevé des anticorps de type T_H2 (IgG1 et IgE) alors que les anticorps de type T_H1 (IgG2a) n'ont pas pu être détectés. Ces résultats suggèrent que contrairement au phénotype T_H2, la chaîne IL-12R β 2 est essentielle au développement du phénotype T_H1 et elle est nécessaire pour résister à l'infection au parasite *L. major*.

En conclusion, comprendre l'expression de la chaîne IL-12R β 2 par les cellules NK et les lymphocytes T et sa régulation par les cytokines peut constituer un élément important dans les études de thérapie du cancer et des maladies auto-immunitaires.

In memory of Abdessalam Chakir (Casablanca 1938-2001)

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LIST OF ABBREVIATIONS

α	Anti
aa	Amino-acid(s)
Ab	Antibody
Ag	Antigen
Amp	Ampicillin
AP	Alkaline phosphatase
ATCC	American Type Culture Collection
Bp	Base pairs
BSA	Bovine serum albumin
β -2ME	Beta-2-mercaptoethanol
$^{\circ}\text{C}$	Degrees Celsius
CaCl_2	Calcium chloride
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's Adjuvant
Chlph	Chloramphenicol
CIAP	Calf intestinal alkaline phosphatase
CMV	Cytomegalovirus promoter
ConA	Concanavalin A
cpm	Counts per minute
d	Day(s)
DC	Dendritic cells
ddH ₂ O	Double distilled water
Depc-treated H ₂ O	Diethyl pyrocarbonate-treated water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECD	Extracellular domain

EDTA	Ethylenediaminetetra-acetic acid disodium salt
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Fix/Perm	Fixation and permeabilization
gDNA	Genomic deoxyribonucleic acid
x g	Force of gravity
GFP	Green fluorescent protein
HCl	Hydrochloric acid
H&E	Hematoxylin-eosin
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid
hi	High
His	Histidine
hr	Hour(s)
HRP	Horseradish peroxidase
huIL-2	Human interleukin-2
IFA	Incomplete Freund's adjuvant
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IL-	Interleukin
IL-12R	Interleukin-12 receptor
IP	Immunoprecipitation
IPTG	Isopropylthio- β -D-galactoside
Kb	Kilobase
kDa	KiloDalton
KO	Knock out
LAK	Lymphokine activated killer cells
LB medium	Lauria-Bertani medium
<i>L. major</i>	<i>Leishmania major</i>
LmAg	<i>Leishmania major</i> antigen

LN	Lymph node
lo	Low
LFA-2	Lymphocyte function-associated antigen-2
LPS	Lipopolysaccharide
M	Molar
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
μ Ci	Microcurie(s)
mCi	Millicurie(s)
mg	Milligram(s)
MgCl ₂	Magnesium chloride
mIL-12	Murine IL-12
min	Minute(s)
ml	Milliliter(s)
mM	Millimolar
MNC	Mononuclear cells
mRNA	Messenger ribonucleic acid
μ g	Microgram(s)
μ l	Microliter(s)
μ m	Micrometer(s)
MW	Molecular weight
Na ₂ CO ₃	Sodium carbonate
NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
NaN ₃	Sodium azide
NaOH	Sodium hydroxide
Neo	Neomycin called also geneticin (G418)
NH ₄ Cl	Ammonium chloride
NBT/BCIP	Nitroblue tetrazolium chloride/5-Bromo-4-chloro-3-indolylphosphate p-Toluidine salt
Ni-NTA	Nickel-nitrilotriacetic acid

NK cells	Natural killer cells
nm	Nanometer(s)
NRS	Normal rat serum
O.D	Optical density
ORF	Open reading frame
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohemagglutinin
PIPES	Piperazine-N, N'-bis-[2-ethansulfonic acid]
PMA	Phorbol 12-myristate 13-acetic acid
PMSF	Phenylmethanesulphonyl fluoride
poly (I:C)	Poly i(nosinic acid) + c(ytidylic acid)
Rag 2	Recombinase activating gene
RBC	Red blood cells
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute Medium-1640
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SAV	Streptavidin
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec	Seconds
SEM	Standard Error of the Mean
STAT	Signal Transducers and Activators of Transcription
TAE	Tris acetate-EDTA buffer
TCR	T cell receptor
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine

TGF- β	Transforming growth factor
TNF- α	Tumor necrosis factor alpha
T _H	T helper
Tween-20	Polyoxyethylene Sorbitan Monolaurate
U	Unit(s)
UTR	Untranslated region
UV	Ultraviolet
Zeo	Zeocin

CHAPTER I

GENERAL INTRODUCTION

The general aims of this study are: 1) to analyze the regulation of the beta-2-transducing subunit of the interleukin (IL) 12 receptor (IL-12R β 2 chain) on murine NK and T cells and 2) to study the IL-12 and IL-18 responsiveness of these cell populations *in vitro* and *in vivo*. The results of these studies will help understand the consequences of IL-12R β 2 expression on the modulation of the innate and the adaptive immune systems.

Phagocytes and NK cells are components of the innate immune system, which provide a first line of defense against infection. Innate immunity is characterized by a lack of memory and specificity to antigen. Complete eradication of invasive pathogens is accomplished by the adaptive immune system made up of antigen-specific B and T lymphocytes. A strong cross-talk exists between the innate and the adaptive immune systems. This cross-talk is mediated by cellular interactions and by cytokines released from phagocytic cells (for example IL-1, IL-6, IL-12, IL-18 and tumor necrosis factor (TNF) α) and from lymphocytes (such as IFN- γ , granulocyte/macrophage colony stimulating factor (GM-CSF), IL-4, IL-10 and IL-13) (1,2).

1.1. T helper cells and their differentiation

Upon antigen encounter, a naïve T cell becomes an effector T cell that participates in the adaptive immune response to eradicate the antigen that triggered its activation. Two classes of effector T cells have been described on the basis of recognizing peptides in the context of MHC class I and II molecules. CD8⁺T cells, which differentiate into cytotoxic T cells, recognize peptides from intracellular pathogens that multiply in the cytoplasm and are presented by MHC class I molecules. CD4⁺T cells recognize peptides derived from organisms multiplying inside intracellular vesicles or derived from extracellular pathogens and presented by MHC class II molecules.

CD4⁺ T helper cells can be further divided into at least three major classes (T_H1, T_H2 and T_H3) based on the pattern of cytokines they produce (3). The T_H1 and T_H2 paradigm has been the subject of many studies (4-11). T_H1 cells produce IL-2, TNF- α and IFN- γ . These cytokines are effective in activating macrophages, mediating delayed-type hypersensitivity and organ-specific autoimmune diseases (12). IFN- γ also has the capacity to trigger switching to T_H1-associated antibody isotypes (IgG2a in mouse and IgG2 in humans) (9). In contrast, T_H2 cells specifically produce IL-4, IL-5, IL-6, IL-9,

IL-10 and IL-13, which down-regulate macrophage functions and promote antibody switching to T_H2 -associated antibody isotypes such as IgG1 and IgE. The T_H2 -associated cytokines mainly promote the development of humoral immune responses (3,6,13). T_H3 cells form a particular $CD4^+$ regulatory subset generated from gut-associated lymphoid tissues (GALT) and are characterized by increased levels of TGF- β production accompanied by IL-4 and IL-10 cytokine secretion [reviewed in (14)]. TGF- β has been reported to play a role in oral tolerance and is considered as costimulatory factor for IgA production. T_H3 cells show suppressive characteristics on both T_H1 and T_H2 effector cells. The factors inducing differentiation of TGF- β -secreting cells are not completely defined (15).

The selective differentiation of $CD4^+$ T cell precursors into T_H1 and T_H2 cells is established during the initial priming of these cells and is influenced by multiple extracellular factors. Factors such as strength of stimulation with antigen, type of APCs, nature of costimulatory molecules, the presence of key cytokines in the milieu, expression of specific cytokine receptors and differential expression of transcription factors have all been reported to regulate T_H1/T_H2 differentiation (4,5,16-22). The cytokine environment has been considered to play the most decisive role in such differentiation (19,23). Thus, cytokines present in the early stages of antigen driven $CD4^+$ T cell activation help to determine the specific pattern of T_H phenotype that develops (23). T_H1 development is enhanced when naïve T cells are activated in the presence of IL-12 (23). IL-12-induced IFN- γ assists T_H1 development initially through a mechanism consistent with promoting the ability of naïve T cells to respond to IL-12 (23,24). Conversely, T_H2 cells develop when IL-4, but not IL-12, is present during activation of naïve T cells (25,26). Analyzing the development and stability of T_H1 and T_H2 phenotypes, it has been demonstrated that the commitment of the two developing T_H cells differs substantially (27). IL-4 can reverse early T_H1 differentiation, but IL-12 cannot reverse early T_H2 differentiation. T_H1 reversibility results from maintenance of IL-4 signal transduction, whereas T_H2 commitment results from loss of IL-12 signaling (12,27,28). Indeed, the presence of IL-4 during *in vitro* (23,27,29) or *in vivo* (30,31) stimulation of naïve T cells, has been reported to result in the downregulation of IL-12R β 2 resulting in a loss of responsiveness to IL-12 (32). Moreover, a cross-regulation

between T_H1 and T_H2 cells takes place, so that development of one class is inhibited by cytokines produced by the other (6,17,19). IL-4 dominates over IL-12 *in vitro* for effects on T_H cell development when both cytokines are present during naïve T cell activation (12,23,27,33). However, positive cross-regulation during T_H1/T_H2 cell development has also been reported. For example, IL-4 (considered to be a T_H1 antagonist) has been reported to stimulate the proliferation of both T_H1 and T_H2 cells (19). In contrast with evidence suggesting that IL-4 downregulates IL-12R, the combination of IL-4 plus IL-12 has been reported to act synergistically to induce proliferation of T_H2 cells and APCs (34). Furthermore, IL-4 has been reported to synergize with IL-12 to induce murine erythropoiesis *in vitro* (35). Other unexpected data demonstrated that IL-4 increases the production of bioactive IL-12 by murine and human dendritic cells (DC) (36). A recent study also indicated that IL-4 promotes T_H1 development when present during the initial phase of APC maturation (37). On the other hand, although IL-4 exerts the inhibitory effects on IL-12 signaling via the downregulation of IL-12Rβ2 expression, IL-12 was reported to act positively on IL-4-induced effects. It was also shown that in the absence of endogenous IFN-γ, IL-12 stimulates IL-4 and IgE synthesis promoting T_H2 development (38). A synergistic effect of IL-12 and IL-4 has also been reported in human NK cells where IL-12 upregulates IL-12 and IL-4 receptors (39).

1.2. T_H1/T_H2 transcription factors

T_H1 and T_H2 development is also regulated by specific transcription factors. Recent studies have demonstrated that T-bet (T-box protein expressed in T lymphocytes) and GATA-3 (zinc finger factor) act as master switches in T_H1 and T_H2 cell differentiation respectively (18,22,40,41). Indeed, T-bet, a T_H1-specific transcription factor, is expressed in T_H1 and not T_H2 cells and is induced in response to IL-12 and TCR stimulation. Ectopic expression of T-bet resulted in a strong expression of IFN-γ and several T_H1-specific cell surface receptors [reviewed in (18,22,42)]. Over-expression of T-bet also resulted in repression of IL-4 and IL-5 cytokines in T_H2 cells suggesting that T-bet controls the development of T_H1 phenotype by promoting T_H1 stability and by repressing T_H2 development [reviewed elsewhere (18,22,43)]. In T_H2 cells, IL-4-induced STAT6 activation was linked to the expression of the T_H2-specific transcription factor

GATA-3 (44). GATA-3 expression is restricted to T_H2 cells and it regulates the transcription of IL-4 and IL-5 target genes (44-46). Through its capacity to induce T_H2 cytokine expression and IgE production, GATA-3 plays a critical role in eosinophilic airway inflammation and asthma (47). In T_H1 cells, IL-12 signaling via STAT4 fully represses GATA-3 expression, whereas in T_H2 cells, IL-4 signaling via STAT6 inhibits IFN- γ production and T_H1 cell development [reviewed in (48)].

I.3. Murine leishmaniasis as a model for T_H1/T_H2 cell development studies

Experimental infection of mice with *L. major*, an obligate intracellular protozoan parasite that resides inside macrophages (49,50), provides an excellent model for defining the conditions required for the generation of T_H1 or T_H2 cells and for the study of T_H1-T_H2 cell development and regulation *in vivo* (50,51). The outcome of murine leishmaniasis depends on the genetic background of infected mice. Moreover, the curative immune response to *L. major* infection is mediated mainly by CD4⁺T helper cells since protective immunity against this parasite in BALB/c, known as genetically susceptible strain mice, was achieved by adoptive transfer of a CD4⁺T cell clone that secretes IFN- γ when encountering a specific *Leishmania* antigen (52). The role of CD4⁺T cells in susceptibility was also demonstrated by CD4⁺T cell depletion from susceptible mice, which resulted in resistance to the disease (53). Infection of C3H mice (resistant strain) with *L. major* leads to a dominant T_H1 type response and healing, while infection of BALB/c mice results in the development of a T_H2 response and an ultimately fatal infection (16,54). It has been demonstrated that during the initial period of infection, IL-12 and IL-4 play critical roles in mediating the differentiation of T_H1 and T_H2 subsets as well as the ability to contain or the failure to contain lesion development (50,51,55). The T_H2 response that develops in BALB/c mice after infection with *L. major* depends on the production of IL-4 early after infection (25,31,32,56). This is supported by previous studies showing that BALB/c mice, in contrast to mice of resistant strains, exhibit a burst of IL-4 expression in draining lymph nodes within 16 hours of infection (16,30-32). This burst of IL-4 occurs within a subpopulation of CD4⁺T cells expressing V α 8 V β 4 TCR (57). Also, it has been found that IL-12 is produced soon after infection of C3H mice (resistant strain) and that the IL-12 activates NK cells as well as other cell types to

produce the IFN- γ required for the development of T_H1 cells and resistance to this parasite (1,58,59). IFN- γ production by NK cells seems to not be a requisite since mice lacking NK cells developed a normal T_H1 response to *L. major* infection (60). Macrophages and DC might constitute a source of IFN- γ needed for T_H1 development during leishmaniasis since these cell populations have been recently reported to respond to IL-12 *in vitro* by secreting high levels of IFN- γ (61-64).

A role for IL-12 in T_H1-mediated protective immunity to leishmaniasis has been demonstrated by treatment of *L. major*-susceptible BALB/c mice with IL-12. Administration of IL-12 to susceptible BALB/c early during the infection resulted in a protective T_H1-type response and resistance to re-infection (27,51,70,71). The protective effect of IL-12 required the presence of this cytokine within the first week after infection, prior to the establishment of a T_H2-type response (29) and was dependent on IFN- γ production (29,50). The critical role of IL-12-induced IFN- γ was further demonstrated in resistant strains of mice deficient in IFN- γ (72) or IFN- γ receptor (73) which displayed susceptibility to *L. major* infection. In addition, the use of anti-IL-12 antibodies has illustrated the importance of endogenous IL-12 in the response to intracellular pathogens. Anti-IL-12 antibody treatment exacerbated *L. major*-infection in normally resistant C57BL/6 or C3H mice (74-76). Other studies have demonstrated the role of IL-12 and IL-12 signaling in T_H1/ T_H2 cell development during intracellular *Leishmania* infection by gene targeting. Mice of normally resistant backgrounds that are defective in IL-12 or the IL-12 signaling molecule STAT4 are also unable to control *L. major* infection (77, 78).

IL-4 is a T_H2-specific cytokine that antagonizes T_H1-cell development. The role of IL-4 in *L. major* susceptibility has been widely investigated. Although contradictory data were reported regarding the immune responses developed by IL-4-deficient mice in response to *L. major* infection (26,65), different investigators agree on the importance of IL-4 and its signaling in T_H2 development and consequently in susceptibility to *L. major* infection (25,56,66-69).

In addition to the murine leishmaniasis model, infection with extracellular pathogens such as intestinal nematodes (*Nippostrongylus brasiliensis*) was used to study T_H2 cell polarization. Indeed, infection of mice with *N. brasiliensis* induces antibody

responses by B cells that require help from T_H2 cells to secrete specific antibodies. T_H2 cells secrete the T_H2 -associated cytokines IL-4, IL-5, IL-6 and IL-13 which, in conjunction with the co-stimulatory molecules (such as CD40L/CD40), activate mature B cells to promote antibody switching to T_H2 -associated IgG1 and IgE isotypes and consequently clear the infection [reviewed elsewhere (79)].

The *L. major* and *N. brasiliensis* models of infection describe the contrasting role of $CD4^+$ T helper cells as important regulators in the adaptive immune system. $CD4^+$ T cells have the ability to differentiate towards T_H1 or T_H2 effector cells, which facilitate cellular and humoral immune responses respectively (3,6,80). Thus, differentiation of specific $CD4^+$ T helper cells can determine specific pathogen resistance or susceptibility and underlies development of effective immune responses (27).

1.4. IL-12 and its receptor

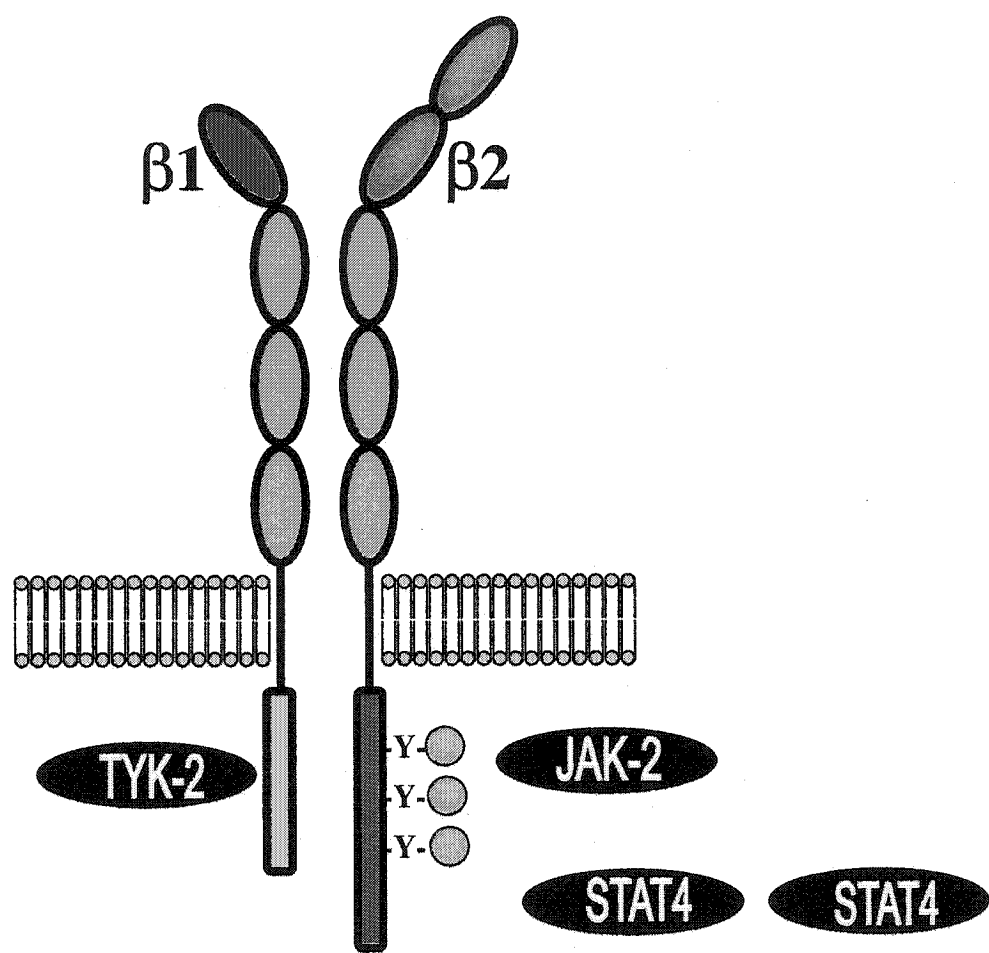
IL-12, previously termed NK cell stimulatory factor (NKSF), has been described as a key initiator of cell-mediated immune responses (12,81). IL-12 is a 70-kDa heterodimeric cytokine composed of two disulfide-bonded subunits, p35 and p40 based on their apparent molecular weight (12,82,83). IL-12 is primarily produced by macrophages, B cells and dendritic cells (12,28). IL-12 has pleiotropic effects on T and NK cells including stimulation of cell proliferation, induction of IFN- γ secretion and enhancement of CD8, NK and lymphokine-activated killer (LAK) cell-mediated cytotoxicity (84-86). IL-12 also plays a central role in promoting T_H1 -type immune responses and thus cell-mediated immunity (12,87,88). Indeed, knockout mice lacking the IL-12 p35 subunit (77) or the IL-12 p40 subunit (89,90) are severely compromised in IFN- γ production, T_H1 immune cell responses and delayed type hypersensitivity (DTH). T_H1 cells, via their production of IFN- γ and TNF- β (known as a powerful lymphotoxin), control cell-mediated immune responses and play a key role in the defense against bacterial and intracellular parasitic infections (12,71,85,91,92). Furthermore, the ability of IL-12 to enhance the cytotoxicity of CD8 and NK cells might also explain the potential therapeutic role attributed to IL-12 as a powerful anti-tumor agent. Indeed, early studies performed on mice demonstrated that administration of IL-12 resulted in regression of established tumors and upregulation of anti-tumor activity (93,94). However, IL-12 has

been reported to induce lethal toxicity in mice and cancer patients following high dose administrations (12,95). IL-12 has also been implicated in the development of autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA) and insulin-dependent diabetes mellitus (IDDM, type I diabetes) [reviewed in refs (12,28,96,97)]. The role of IL-12 has been extended to red blood cell genesis as IL-12 has been shown to enhance erythropoiesis in anemic mice *in vitro* and *in vivo* (35,98,99).

IL-12 manifests its biological functions through interaction with a heterodimeric cell surface receptor (IL-12R) [(28) and Fig. 1.1]. The cDNAs for two IL-12R subunits have been cloned from human and mouse and designated as IL-12R β 1 (100,101) and IL-12R β 2 (102). They are type-I transmembrane glycoproteins, with molecular sizes of about 100 kDa (IL-12R β 1) and 130 kDa (IL-12R β 2). Cells transfected with each of the two IL-12R subunits express dimers / oligomers on the cell surface. The formation of these structures is ligand independent (28). When expressed individually on COS cells, each human IL-12R subunit binds labeled IL-12 with only low affinity. Co-expression of IL-12R β 1 and IL-12R β 2 is required for the generation of high affinity IL-12 binding sites *in vitro* (102). It has been shown that the IL-12R β 2 subunit is partially capable of transducing proliferation signals in IL-12R β 2-transfected-Ba/F3 cells and that IL-12R β 1 chain contributes primarily to an increased affinity of IL-12 binding (102).

Critical to understanding the role of IL-12 in the development of the T_H1 cell phenotype is understanding how the IL-12 receptor is regulated. Early studies have shown that T_H1 and T_H2 cells differ in their expression of a functional IL-12 receptor (23,27,54), and that the inability to efficiently activate IL-12 signaling pathways is responsible for the development of a T_H2 phenotype *in vivo* (103,104). Resting naïve T cells do not express IL-12R (100,105), however, activation of T cells with mitogens or via the T cell receptor (TCR) results in rapid, but transient, expression of high affinity IL-12R on the cell surface (12,28,105). Furthermore, a number of reports have demonstrated that whereas the IL-12R β 1 chain is constitutively expressed after TCR stimulation, IL-12R β 2 chain is regulated by the presence of key cytokines at the time of stimulation (13,23,31). Surprisingly, IL-12 itself has regulatory effects on the surface expression of its own IL-12R β 2 chain on T cells and monocytes (106,107). Indeed, recent data

Fig. 1.1. Structure of the IL-12 receptor. IL-12 receptor belongs to the gp 130 subgroup of the cytokine receptor family. It is a type I transmembrane glycoprotein of a molecular weight of about 100 kDa for IL-12R β 1 chain and 130 kDa for IL-12R β 2 chain. The IL-12R β 1 subunit is required for the formation of high affinity IL-12 binding sites, whereas IL-12R β 2 chain containing tyrosine residues in the cytoplasmic tail is known as the signal transducing subunit. IL-12-responsiveness is mediated via the JAK/STAT pathway involving specifically, JAK2 and Tyk2 tyrosine kinases as well as STAT4.



indicated that IL-12 induces IFN- γ production by murine macrophages (61,62) and DC (63,64) suggesting that the expression of IL-12R β 2 subunit is not restricted to T and NK cells only and that IL-12 has an autocrine effect on its own producers. Together these mechanisms of IL-12R β 2 modulation have profound implications for the development of an ensuing immune response considering the strong ability of IL-12 to drive T_H1 development.

IL-12R β 2 is the signal transducing component of IL-12R (100-102) which signals through a number of different pathways. Treatment of human T cells with IL-12 induces rapid tyrosine phosphorylation of both receptor-associated JAK2 and Tyk2 tyrosine kinases. Physical association between JAK2 and IL-12R β 2 chain and between Tyk2 and IL-12R β 1 chain has been demonstrated. However, although IL-12R β 1 binds to Tyk2 kinase (90,108-110), the role of IL-12R β 1 chain in transducing IL-12 signals has not been reported. JAK2 and Tyk2 kinases activate the signal transducer and activator of transcription (STAT)1, STAT3 and STAT4 proteins (111-113). IL-12, IFN- α and IL-23 are the only cytokines capable of inducing STAT4 activation (112,114,115), moreover, STAT4 is the main transcription factor implicated in T_H1-cell development since only STAT4 deficiency, and not the other signaling pathways, completely abrogates T_H1 cell development (12,119,120). In response to IL-12, phosphorylated STAT4 dimerizes and translocates to the nucleus where it binds to specific DNA response elements in the promoter regions of IL-12-inducible genes and mediates transcription of IL-12-responsive genes responsible for proliferation, cytokine production and cell cytotoxicity (90). Requirement for IL-12R β 1 in T_H1 cell development has been recently reported in humans. Indeed, deficiency in IL-12R β 1 gene in humans leads to severe infections by poorly pathogenic bacteria due to low production of IFN- γ (90,116). Mice deficient in IL-12R β 1 (117), IL-12R β 2 (118) and STAT4 (119,120) exhibited abrogated IL-12-induced IFN- γ production, cellular proliferation and NK cell cytotoxicity with impairment of T_H1 development in response to either IL-12 or *Listeria monocytogenes*. Mice deficient in Tyk2 tyrosine kinase have been recently generated and they displayed a defect in IL-12-induced T and NK cell functions, STAT3 activation and viral clearance (121-123) suggesting special requirements for the JAK/STAT pathway in IL-12-induced functions.

However, mice deficient in both STAT4 and STAT6 (IL-4-specific signaling protein) were still capable of mounting T_H1 immune response due to the presence of an alternate STAT4-independent signaling pathway that induces IFN- γ . This pathway is apparently suppressed by IL-4-induced STAT6 (124). An early study on human NK cells reported that IL-12 also induces tyrosine phosphorylation of a 56-kDa protein, identified in Western blot experiments as p56^{lck} (125), however, the binding of p56^{lck} to IL-12R as well as the role of its activation have to be determined. IL-12 has also been demonstrated to involve additional signaling pathways. Recently, IL-12 has been shown to activate members of the NF- κ B family in murine DC cells to induce IL-12 production without any activation of STAT proteins (63). In addition, IL-12 was reported to induce cell proliferation and IFN- γ production in human CD8⁺T cells via activation of p38 mitogen-activated protein (MAP) kinase pathway (126). Moreover, two different studies reported recently that in activated murine CD4⁺ T and T_H1 cells, IL-12 treatment promoted IFN- γ mRNA upregulation via the activation of two different p38 MAP kinase pathways; STAT4-independent via mitogen-activated protein kinase kinase (MKK) 3/6 (127) and STAT4-dependent pathway via MKK6 (128). Despite the involvement of different signaling pathways in IL-12-induced functions, the JAK/STAT pathway seems to play the most important role since STAT4-deficient mice display obvious impaired responsiveness to IL-12 and disrupted T_H1 differentiation (119,120).

1.5. IL-23 cytokine

A recent study reported the discovery of a new protein subunit (p19) closely related to IL-12p35 and IL-6. p19 combines with the p40 subunit of IL-12 to form a bioactive cytokine named IL-23 (129). p19 is produced by activated macrophages, DCs (IL-12p40 cell producers), T cells and endothelial cells (115). Expression of p19 is upregulated in response to bacterial infections (130). IL-23 (p40/p19 heterodimer) binds to the IL-12R β 1 chain but not to IL-12R β 2 and induces STAT4 phosphorylation in PHA blasts (129). In contrast to the bioactive IL-12p70, IL-23 induces proliferation and IFN- γ secretion from memory but not from naïve T cells (115).

I.6. NK cells

NK cells are bone marrow derived large granular lymphocytes that play an important role in the first line of defense against microbial infections [reviewed elsewhere (131)]. NK cells do not express surface TCR and exert their effector functions independently of antigens. However, NK cells also mediate antibody-dependent cytotoxicity (ADCC) as well as spontaneous cytotoxicity (131). This later function is increased by cytokines such as IL-2 and IL-15, two NK survival factors (132-134). IL-2 and IL-15 receptors share the beta (β) and gamma (γ) subunits but have specific alpha (α) chain subunits (135,136). Studies in the murine and human systems, have demonstrated the potential role played by NK cells in tumor therapy (137-140). NK cells express IL-12R and are very sensitive to monocyte and T cell-derived cytokines (133,135,141,142). NK cells thus constitute a model to study the regulation of IL-12R β 2 and its role in the innate immune system. Indeed, both IL-12 and IL-18 have a significant biological impact on NK cells (12,76,131,143,144). Although the regulation of IL-12 receptor expression on NK cells is poorly understood, some investigators have reported that IL-12R expression is up-regulated after activation of NK cells with IL-2 or IL-15 as indicated by responsiveness to exogenous IL-12 (133,145,146). In contrast, other studies have reported that resting NK cells constitutively express the IL-12R at low levels (39,147).

I.7. IL-18 and its receptor

IL-18 is another powerful cytokine that promotes secretion of IFN- γ from T and NK cells (143,148,149). IL-18 is an IL-1-related protein originally identified as IFN- γ -inducing factor (IGIF) in the livers of mice challenged with *Propionibacterium acnes* and LPS (150,151). IL-18 is produced by a wide range of cells including macrophages, DC, Kupffer cells, keratinocytes and microglia (148,151-153). The IL-18 gene encodes an inactive precursor (pro-IL-18) of 192 aa containing a leader sequence of 35 aa. The generation of the active IL-18 of 18 kDa requires cleavage by caspase-1, named also IL-1 β converting (ICE) enzyme (149,154).

IL-18 exerts its effects through binding to a heterodimeric IL-18 receptor. IL-18R, a member of the IL-1R family, is comprised of a ligand binding chain known as IL-1 receptor related protein (IL-1Rrp) or IL-18R- α and a signaling chain designated as accessory protein like (AcPL) or IL-18R- β (155-158). *In vitro* transfection assays showed that IL-18R- β chain alone was unable to bind IL-18 ligand (154,159). Although it has significant homology with IL-18, IL-1 is unable to bind the IL-18R (156). Naïve T cells are reported to lack receptors for IL-18 until activation via TCR signaling (160). However, in contrast to T cells, NK cells are reported to constitutively express IL-18R (147,161,162). In T_H1 cells, but not in T_H2 cells, IL-18R transduces a signal that activates the myeloid differentiation (MyD) 88 adaptor protein, IL-1R-associated kinase (IRAK), and TNF receptor associated factor (TRAF) 6 resulting in activation and nuclear translocation of NF- κ B and c-Jun N-terminal kinase (JNK) (144,159,163,164). However, recent reports also demonstrated that, in NK cells, STAT3, p38 and p44^{erk} MAP kinases are activated in response to IL-18 (141). Mice deficient in MyD88 (165), IL-18R α (166), IRAK kinase (144) or caspase-1 (167) showed abrogated IL-18-induced functions in T and NK cells.

IL-18 is a cytokine with a broad range of targets and actions. IL-18 acts on NK, NK-T, macrophages, CD4, CD8 and B cells (149,150,168-170). This cytokine induces IFN- γ production and increases cytolytic activity in NK and T cells. It also augments the T_H1 -promoting activity of IL-12 but IL-18 is reported to be incapable of inducing T_H1 polarization by itself (149,163). IL-18 also plays a role in autoimmune diseases such as insulin-dependent diabetes mellitus (IDDM) and multiple sclerosis (MS) [reviewed in (149,158,171)]. In clinical studies, IL-18 was also found to be more efficient than IL-2 and/or IL-12 in inducing tumor-specific cytolytic T cell activity (172). Such function may attribute to IL-18 the privilege to be considered as a useful candidate in cancer therapy. Evidence showed that IL-18 also promotes T_H2 cytokine secretion by CD4⁺T cells (149,160,173,174) and by NK cells (146,175). Indeed, aberrant expression of IL-18 in IL-18-transgenic mice results in the increased production of T_H1 and T_H2 cytokines. Overexpression of IL-18 *in vivo* induced IgE, IgG1, IL-4 and IFN- γ production in mouse (176). More interestingly, recent data demonstrated that, in the presence of IL-2, IL-18 induces CD40L upregulation and modest IL-4 secretion by naïve CD4⁺T cells in the

absence of TCR engagement (177). TCR signaling further increased T_H2 cytokine secretion leading to T_H2 cell polarization (177,178). Furthermore, IL-18 induces B cells to secrete high levels of IgE antibodies (177,179) and stimulates basophiles to secrete IL-4 and histamine (180). Therefore, IL-18 is considered as a unique cytokine capable of simultaneously regulating both T_H1 and T_H2 immune responses (149,150,158,170).

I.8. Synergy between IL-12 and IL-18

Although different studies demonstrated that IL-18 synergizes with IL-2 and IL-10 to induce NK cell activity (181-183), the synergy between IL-18 and IL-12 generates more potent effects in both NK and T cells. IL-12 and IL-18 have a strong synergistic effect on T_H1 cell polarization and IFN- γ production. Indeed, administration of IL-12+IL-18 to mice resulted in high toxicity and a fatal systemic inflammation (141,184). Together IL-12 and IL-18 mediate resistance to T_H1 -associated infectious diseases such as leishmaniasis and leprosy (54). Nevertheless, a different group of authors demonstrated that mice deficient in IL-18 were able to develop a T_H1 -specific immune response and cure *L. major* infection (185). In the presence of IL-18, IL-12-mediated innate immunity to *Toxoplasma gondii* is upregulated in SCID mice (186).

Studies examining the synergy between IL-12 and IL-18 have generated controversial results adding more complexity to the phenomenon of IL-12/IL-18 interaction. For example, several recent reports have indicated that the mechanism underlying the synergy between IL-12 and IL-18, at least in T cells, is the induction of IL-18R by IL-12 (160,187-190) suggesting that an intact IL-12 signaling pathway is required for IL-18-induced activity (123,191). However, other studies demonstrated that IL-18 induces IFN- γ production by established T_H1 cells independently of IL-12 (192). A third group reported that this synergistic effect resides in the capacity of IL-18 to promote IL-12R β 2 expression (193,194). A fourth group of authors demonstrated that the synergy between IL-12 and IL-18 results from reciprocal induction of IL-12 and IL-18 receptors (195). However, other data showed that these receptors are constitutively expressed on T cells (190,196,197). IL-12 and IL-18 also act together on NK cells. However, some aspects of the IL-12 and IL-18 signaling pathways appear to be distinct between these two cell types. For example, the mechanisms for initiating IL-12R expression must be

distinct between T and NK cells because NK cells do not signal through a TCR. NK cells stimulated with IL-12+IL-18 do not induce any reciprocal upregulation of IL-12R/IL-18R suggesting that these receptors are constitutively expressed on resting NK cells (39,145,147,198,199).

In summary, IL-12R β 2 signaling is considered as a key receptor for activation of NK cells and the switch igniting T_H1 effector cell development. Therefore, an understanding of how IL-12R β 2 expression is regulated will not only provide insights into the mechanisms governing IL-12 responsiveness in NK, monocytes and T cells, but may also result in the identification of molecular factors or mechanisms that are involved in the generation of the appropriate immune responses required to clear intracellular infections and to develop therapeutic strategies to control autoimmune diseases and cancer.

The present dissertation is composed of five related chapters that have been submitted for publication as described in the preface section. The overall objective of these chapters is to understand how the expression of IL-12R β 2 chain is modulated on NK and T cells and to define whether signaling through this IL-12R subunit is required for IL-18 responsiveness in these cells. Furthermore, the importance of IL-12R β 2 expression for the development of T_H1 phenotype *in vivo* as well as the possible role of the newly discovered IL-23 in T_H1 cell differentiation was also investigated. Similarly, the role of IL-12R β 2 subunit in the differentiation and the stability of T_H2 cells was studied *in vitro* and *in vivo*.

CHAPTER II

Murine DX5⁺NK cells differentiate into two distinct cell populations based on the expression of the signal transducing subunit of the IL-12R

II.1. Summary

IL-12 is required for the development of T_H1 cells which play an important role in protective cell-mediated immunity against a variety of intracellular pathogens (28). IL-12 also induces IFN- γ production and increases cytolytic activity of natural killer (NK) cells. This property qualifies IL-12 as a powerful anti-tumor agent in cancer therapy (93,137). The biological activities of IL-12 are mediated through the heterodimeric IL-12R comprised of the IL-12R $\beta1$ chain and the signal transducing IL-12R $\beta2$ subunit. This receptor is expressed on T cells and NK cells and was recently reported to be expressed by dendritic cells (DC) and macrophages (61,62). IL-12R $\beta2$ has been suggested to function as a molecular switch in determining the differentiation of T_H cells into T_H1 cells and its expression in $CD4^+$ T cells as well as its modulation by cytokines have been well documented (51,62,105,106,118,200-210). In contrast to $CD4^+$ T cells, little is known about the regulation of IL-12R $\beta2$ in NK cells. In an attempt to understand IL-12 responsiveness of cells from the innate immune system, the expression of IL-12R $\beta2$ by murine NK cells was investigated. For this purpose, a specific rat anti-mouse IL-12R $\beta2$ antiserum was generated and used to monitor the expression of IL-12R $\beta2$ by flow cytometry. Flow cytometric analyses of IL-2-activated NK cells, using anti-IL-12R $\beta2$ antiserum, revealed the development of two distinct NK cell populations characterized by the expression of different levels of IL-12R $\beta2$. When compared to IL-2-activated NK cells stained with non-immune serum, these two NK populations corresponded to IL-12R $\beta2^{lo}$ and IL-12R $\beta2^{hi}$. Further analysis of surface marker expression confirmed that the two cell populations were of NK origin and that they differentiated from each other by the expression of the inhibitory receptor Ly49G2 that was restricted to IL-12R $\beta2^{hi}$ subset. Both NK populations showed similar cell proliferation, IFN- γ production and cytolytic activity in response to exogenous IL-12. Furthermore, similar to T cells, murine NK cells were able to skew towards NK1 and NK2 phenotypes when activated in the presence of T_H1 and T_H2 polarizing cytokines respectively.

Thus, the emergence of two NK cell populations expressing variable levels of IL-12R $\beta2$ chain is a novel finding proving that the regulation of IL-12R $\beta2$ is a complex process at least in murine NK cells.

II.2. Hypothesis

In murine NK cells, the expression of IL-12R β 2 signaling subunit is regulated differently from T cells. This differential regulation has an impact on the innate and the adaptive immune systems.

II.3. Objective

The main objective is to analyze the expression of the IL-12R β 2 on murine NK cells using the specific rat anti-IL-12R β 2 antiserum and to compare some phenotypic and functional characteristics between the two emerging populations of NK cells that express different levels of IL-12R β 2 transducing subunit.

II.4. Materials and Methods

II.4.1. Animal care approval for animal use

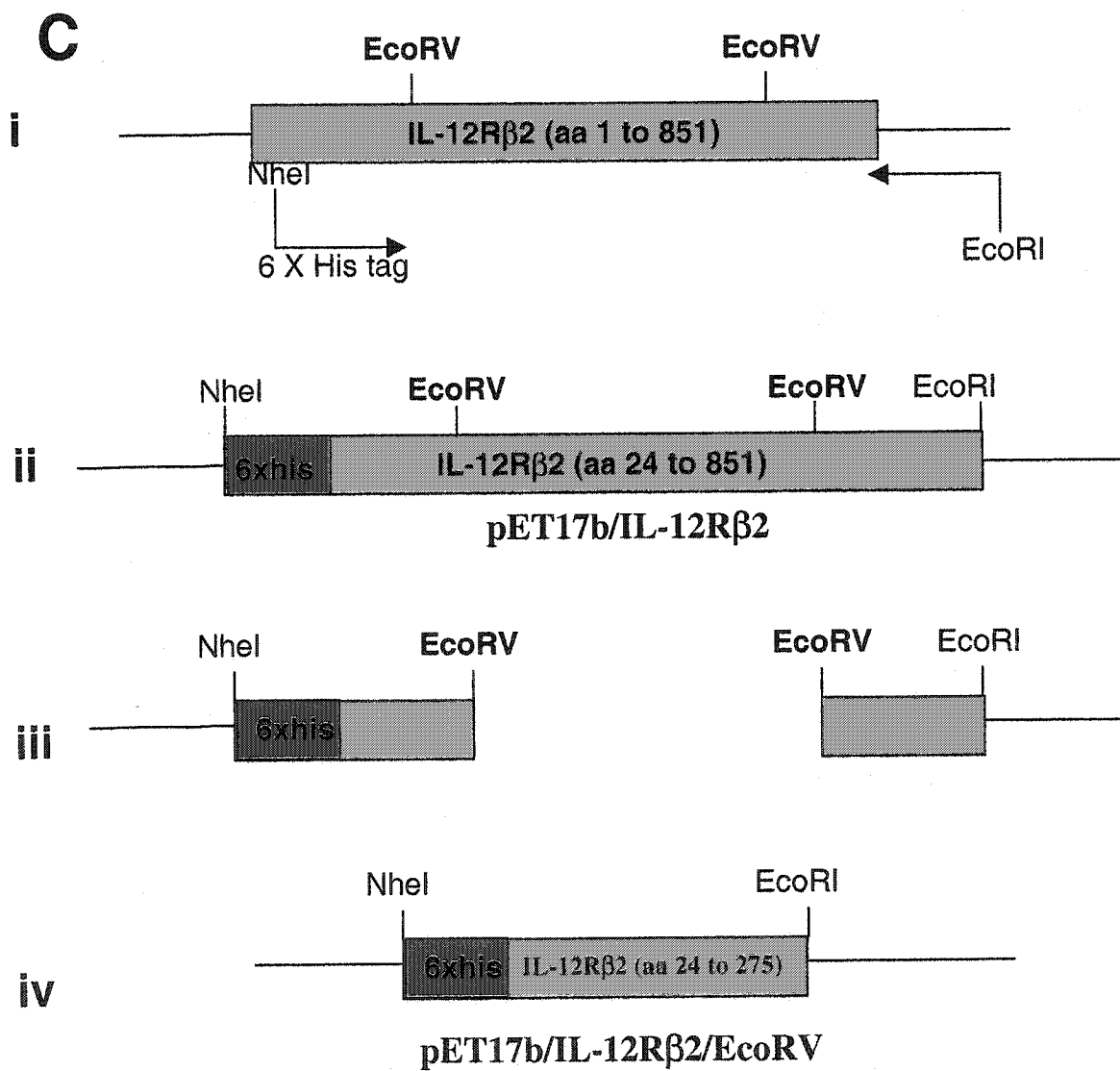
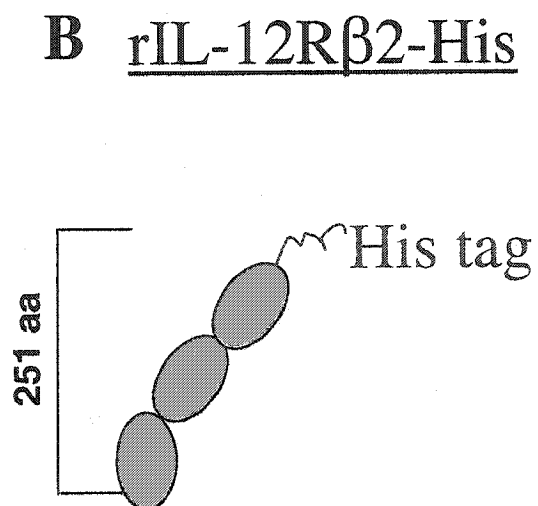
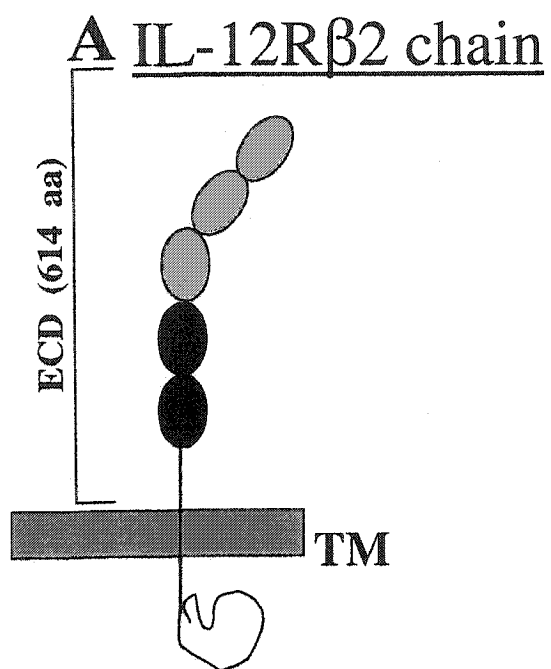
Ethics approval for experiments using mice, rats and rabbits was obtained from the Animal Care Committee of the University of Ottawa (Ottawa, Ontario).

II.4.2. Generation of specific anti-mouse IL-12R β 2 antisera

The extracellular domain of the murine IL-12R β 2 gene was amplified by PCR from the murine IL-12R β 2 cDNA plasmid (kindly provided by Dr. Kenneth M. Murphy, Washington University) with addition of an NheI site at the 5' end and an EcoRI site at the 3' end (Fig. 2.1). The sequence of the specific primers is reported in Appendix A.5.3. Also, a histidine tag (6xHis) was added to the 5' extremity to facilitate subsequent protein purification by affinity chromatography (Fig. 2.1 B and C). 6xHis tag has been reported to be poorly immunogenic and would not interfere with the induced-protein (The QIAexpress system: QIAGEN Handbook for high-level expression and purification of 6xHis-tagged proteins). The PCR product was electrophoresed and purified from agarose gel using PrepA gene DNA purification system kit following the manufacturer's protocol (BioRad). Purified PCR product was then cloned into the pET17b high level bacterial

Fig 2.1. Generation of specific antibodies to mouse IL-12R β 2 chain. (A) Structure of the mouse IL-12R β 2 chain. Total IL-12R β 2 protein contains about 851 aa. The murine IL-12R β 2 extracellular domain (ECD) is composed of 614 aa, the trans-membrane (TM) domain contains 24 aa and the intracellular domain (ICD) is comprised of 213 aa (212). (B) To generate specific anti-IL-12R β 2 antibodies, a recombinant IL-12R β 2 protein corresponding to aa 24-275 was expressed in BL21 (DE3) pLysS *E. coli* and a 6XHis-tag was integrated to the N-terminal extremity to facilitate rIL-12R β 2 protein purification. (C) Complete mouse IL-12R β 2 cDNA clone (kindly provided by Dr. Kenneth Murphy, Washington University) was amplified by PCR with addition of an NheI and an EcoRI sites at the 5' and 3' ends respectively (C.i). Also, a 6 x His tag was added to the 5' terminus (C.ii). PCR product was cloned into pET17b high level bacterial expression system to form pET17b/IL-12R β 2 construct (C.ii). pET17b/IL-12R β 2 construct was then digested with EcoRV (C-iii). The digested fragment encoding the ECD of the mouse IL-12R β 2 (aa 24 to aa 275) was re-ligated to form pET17b/IL-12R β 2/EcoRV (C.iv). The expressed rIL-12R β 2 protein was purified and used to immunize Lewis rats and New Zealand white rabbits.

aa: amino-acid; His: histidine



expression system (Novagen) digested with NheI and EcoRI restriction enzymes. The DNA construct obtained (pET17b/IL-12R β 2) was digested with EcoRV restriction enzyme (Life Technologies) giving a linear fragment encoding a segment of the amino-terminal domain of IL-12R β 2 (aa 24-275) (Fig. 2.1 B and C). After electrophoresis of the EcoRV-digestion products, the DNA band was purified from the agarose gel then re-ligated using Rapid DNA ligation kit (Boehringer Mannheim) to form the plasmid pET17b/IL-12R β 2/EcoRV. This plasmid was used to transform competent *E. coli* strain (BL21 (DE3) pLysS) (Novagen) to direct the expression of a 6xHis-recombinant IL-12R β 2 protein of 32 kDa (see Appendix A.6.7 for competent cell production and cell transformation protocols).

BL21 (DE3) pLysS *E. coli*, transformed with pET17/IL-12R β 2/EcoRV construct were induced to express the IL-12R β 2 protein using IPTG (BioShop Canada). The expressed protein, termed recombinant IL-12R β 2 (rIL-12R β 2), was then purified to homogeneity by nickel-based affinity chromatography using Ni-NTA chromatography affinity (QIAGEN) according to the manufacturer's instructions. Briefly, BL21 (DE3) pLysS *E. coli* transformed with the pET17b/IL-12R β 2/EcoRV plasmid were induced with IPTG for 4h to express the rIL-12R β 2 protein. Bacterial cells were then harvested, lysed and rIL-12R β 2 protein was extracted from the bacterial insoluble fraction using 6M guanidine buffer pH8 (6M guanidine hydrochloride (BioShop) plus 10 mM 2 β -ME (Bio-Rad) in 50 mM NaH₂PO₄ (pH8) containing 300 mM NaCl). A Ni-NTA column loaded with the rIL-12R β 2 protein was washed with 6M guanidine buffer at pH 8 then with the same buffer at pH 6.3. The purified rIL-12R β 2 protein was eluted from the column using 6M guanidine buffer, pH 4.5. The eluted fractions containing highly purified rIL-12R β 2 protein were pooled together and dialyzed 24h against PBS using Snake Skin Dialysis Tubing (Pierce). Precipitated rIL-12R β 2 protein was solubilized in 10 mM Tris (pH 8.0) containing 2% SDS and 5% 2 β -ME.

The purified rIL-12R β 2 was quantified using Coomassie Plus Protein Assay Reagent (Pierce) following the manufacturer's instructions. 500 μ g or 100 μ g were mixed with CFA and used to immunize New Zealand white rabbits and Lewis female rats

(Charles River Laboratories) respectively for the generation of the anti-murine IL-12R β 2 polyclonal antibodies (see immunization protocol in Appendix A.6.1).

II.4.3. Cell isolation and cultures

II.4.3.1. Separation of mouse spleen mononuclear cells

After homogenization of spleen tissues in RPMI-1640 (Life Technologies) using the frosted end of two sterile glass micro-slides, cells were pelleted at 260 x g, resuspended in 5ml ice cold 0.17 M NH₄Cl lysis buffer per spleen and incubated 10 min on ice to lyse the RBCs. Cells were washed three times with 10 ml RPMI-1640 to remove all trace of NH₄Cl. MNC were then resuspended in RP-10 medium (RPMI 1640 supplemented with 10% FCS, 25 mM HEPES, 50 μ M 2 β -ME, penicillin/streptomycin and 10 μ l/ml of 200 mM L-glutamine. The medium was filtered in 500 ml vacuum driven filtration system (Stericup)). Cell viability was determined by trypan blue exclusion. Cells designated for immediate culture were kept in RP-10 medium; whereas cells requiring further cell purification were resuspended in MACS buffer (PBS containing 2 mM EDTA and 0.5 % BSA) for subsequent enrichment by positive magnetic selection.

II.4.3.2. Generation of murine LAK cells and ConA blasts from bulk MNC

Isolated murine MNC were plated at a specific density of 1x10⁶ cells/ml in RP-10 medium. LAK cells were generated from bulk MNC by supplementing the cultures with 1000 U/ml huIL-2 (provided by Dr. Craig Reynolds, NCI, Bethesda, MD) for at least 10 days. ConA blasts were generated by supplementing the MNC culture with 2.5 μ g/ml ConA (Sigma) plus 10 U/ml huIL-2 for 3 days. LAK cells and ConA blasts were used to analyze the expression of IL-12R β 2 chain on NK and T cells respectively using the rat anti-mouse IL-12R β 2 antisera.

II.4.3.3. Enrichment of murine DX5⁺NK cells by positive magnetic selection

Murine DX5⁺NK cells were purified from splenic MNC by positive magnetic selection using 10 μ l/10⁷ cells of anti-DX5 microbeads (Miltenyi Biotec) at a final volume of 10⁷ cells/100 μ l in MACS buffer following the manufacturer's protocol. Cells were incubated with the microbeads for 20 min at 4°C. The excess microbeads were removed by washing the cells twice with 10 to 20 x the labeling volume of MACS buffer. The anti-DX5 labeled cells were then resuspended in 0.5 ml degassed MACS buffer and

passed through MS⁺ mini-MACS magnetic columns. The columns were washed three times with 0.5 ml degassed MACS buffer and the flow-through containing non-labeled cells was recovered in new 15 ml centrifuge tubes. The positively selected cells were recovered in new 15 ml centrifuge tubes by flushing the magnetic columns with 1 ml MACS buffer. Aliquots (50 µl) from various purification steps were resuspended in 100 µl FACS buffer (1% BSA and 0.1 % NaN₃ in PBS) and double stained with 1:50 (v/v) anti-NK1.1-PE (100 µg/ml) or anti-DX5-PE (200 µg/ml) plus 1:50 (v/v) anti-CD3-FITC (100 µg/ml) mAbs. DX5⁺NK cell separation was analyzed by flow cytometry.

Flow cytometric analysis of CD3 expression on enriched DX5⁺ cells revealed the presence of two populations: DX5⁺CD3⁻ cell population corresponding to NK cells and DX5⁺CD3⁺ cells corresponding to NK-T cells. The term “DX5⁺NK cells” used in this dissertation indicates total murine DX5⁺ cells composed of NK and NK-T cells unless specified. Enriched DX5⁺NK cells were then used to generate LAK cells as described above for bulk splenic MNC.

II.4.4. Anti-mouse IL-12Rβ2 antisera testing

II.4.4.1. Western blot

Serum samples from rIL-12Rβ2-immunized animals were taken after each boost and were analyzed for the production of specific anti-mouse IL-12Rβ2 antibodies by Western blot using rIL-12Rβ2 protein as a source. Therefore, 50 ng per well of purified rIL-12Rβ2 protein were separated by electrophoresis using 12% SDS-polyacrylamide gel as per Laemmli (213). Pre-stained high molecular weight proteins (Life Technologies) were used as standard markers. The electrophoresed proteins were blotted onto nitrocellulose membranes (Protran, Schleicher & Schuell) using Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) and incubated overnight at 4°C in the Western blocking buffer (1% BSA dissolved in PBS plus 0.1% Tween 20). Blocked membranes were incubated 1h at RT with 1:500 (v/v) rat and rabbit pre-immune sera (used as negative controls) or anti-mouse IL-12Rβ2 antisera in Western blocking buffer. Nitrocellulose membranes were then washed 2 x 10 min with 10 ml Western washing buffer (PBS, 0.1% Tween-20) then incubated 1h at RT with 1:10,000 (v/v) AP-

conjugated anti-rat or anti-rabbit IgG (Sigma) depending on the primary antibody. The nitrocellulose blots were washed 2 x 10 min with Western wash buffer then 1 x 10 min with AP buffer (12.1 g Tris base; 5.8 g NaCl, 1 g MgCl₂ in 1L ddH₂O). The detection was carried out using NBT/BCIP reagents (Life Technologies) in AP buffer. The reactions were stopped using AP stop buffer (50 ml PBS, 0.2 ml of 0.5 M EDTA at pH 8.0 in 1L ddH₂O).

II.4.4.2. Flow Cytometry

For flow cytometry analyses, only rat anti-mouse IL-12R β 2 antiserum was used. Cell surface IL-12R β 2 expression was monitored by flow cytometry using an Epics XL-MCL (Beckman Coulter) flow cytometer. Murine Con A blasts (d 3) or LAK cells (d 10 to 15) were resuspended in 100 μ l of FACS buffer and incubated with 1:50 (v/v) of anti-IL-12R β 2 antiserum for 25 min at 4°C. As a control, cells were incubated with 1:50 (v/v) of rat pre-immune serum. Cells were washed twice with FACS buffer and were resuspended in 100 μ l FACS buffer containing 1:100 (v/v) of 0.5 mg/ml FITC-conjugated goat anti-rat Ig Ab (Pharmingen) for 25 min at 4°C. Cells were then washed twice with FACS buffer and resuspended in 0.4 ml of the same buffer. A minimum of 2 x 10⁴ events was analyzed by flow cytometry. Flow data were analyzed using WinMDI 2.8 software.

II.4.4.3. Immunoprecipitation

2 x 10⁷ murine DX5⁺NK cells were harvested, resuspended in 0.5 ml FACS buffer in 1.5 ml Eppendorf tubes and briefly pelleted at 350 x g. Cells were stained with 1:50 (v/v) of rat pre-immune serum or rat anti-mouse IL-12R β 2 antiserum and incubated 25 min at 4°C. Cells were washed twice with FACS buffer. They were then stained with 1:100 (v/v) of 0.5 mg/ml biotinylated polyclonal anti-rat Ig (Pharmingen) for 20 min at 4°C. After 2 washes in 0.5 ml FACS buffer, 5 μ l aliquots were resuspended in 100 μ l FACS buffer and stained with 1:100 (v/v) of 0.5 mg/ml SAV-FITC (Pharmingen) for flow cytometry confirmation of the expression of IL-12R β 2. Remaining cells were pelleted and lysed in 100 μ l IP lysis buffer (300 mM NaCl, 50 mM Tris pH 8, 0.5 % Triton X-100 and one protease inhibitor cocktail tablet (Roche) in ddH₂O) for 30 min on ice. Cell lysates were centrifuged 15 min at 10,000 x g to pellet the nuclei and the supernatants were mixed with 15 μ l SAV-agarose beads (Oncogene) by rotating the

samples 30 min at 4°C using Tube Rotator (Sepco). The materials were centrifuged 1 min at 10,000 x g, the immunoprecipitates (pellet fractions) were washed three times with IP lysis buffer and resuspended in 2 x SDS sample buffer (0.5 M Tris-HCl pH 6.8; 1/10 (v/v) Glycerol; 10% (w/v) SDS; 1/20 (v/v) 2-βME and 1% (w/v) bromophenol blue in ddH₂O). The immune complexes were boiled for 5 min, electrophoresed through 10% SDS polyacrylamide gels and subjected to Western blot as explained above.

II.4.5. Analysis of NK cell surface markers by flow cytometry and cell sorting

All flow cytometric stains were performed as follows: 1: 50 (v/v) rat anti-murine IL-12Rβ2 antiserum as primary Ab or rat pre-immune serum as a negative control. After washing the cells twice with FACS buffer, 1: 100 (v/v) FITC-conjugated goat anti-rat Ig (at 0.5 mg/ml) was used as secondary Ab. Cell surface markers were detected using biotin-conjugated or PE-conjugated Abs against second surface marker. Biotin-conjugated Abs were detected with 1: 100 (v/v) of 0.5 mg/ml STV-PE (Sigma). Biotin or PE-conjugated Abs were used at 2 to 10 µg per ml in FACS buffer. The different antibodies used for cell surface characterization and their sources are reported in Appendix A, table 2. The different controls used are reported in Appendix A, table 4.

For cell sorting of IL-12Rβ2^{lo} and IL-12Rβ2^{hi}, DX5⁺NK cells (stimulated with 1000 U/ml huIL-2 for 15 days) were stained for IL-12Rβ2-FITC or Ly49G2-FITC (as a surrogate marker for IL-12Rβ2) and sorted on an ASTRA cell sorting system (Coulter) or separated by magnetic cell separation using anti-FITC microbeads (Miltenyi Biotec) following the manufacturer's instructions. Sorted cells to more than 98% purity were returned to culture in RP-10 medium in the presence of IL-2.

II.4.6. Analysis of IL-12Rβ2 and IFN-γ transcript levels by real-time PCR

All real-time PCR experiments reported in the present dissertation were performed using the protocol reported below. DX5⁺NK cells isolated from C57BL/6 mice by positive magnetic selection were harvested immediately after purification (resting or naïve DX5⁺NK cells) or cultured in RP-10 medium and stimulated with 1000 U/ml huIL-2 for 6 days. At d6, cells were left in IL-2 or incubated overnight with IL-2 plus one ng/ml murine IL-12 (from Genetics Institute, Cambridge, MA). Total RNA was prepared

using Trizol reagent (Life Technologies) and was sent to our collaborators at GlaxoSmithKline (Collegeville, PA, USA). Real-time PCR analyses were performed under the supervision of Dr. Ruth Tal-Singer, as described elsewhere (214), using ABI-PRISM 7700 Sequence Detection System (PE Biosystems) loaded with the sequence detector software v 1.7 (PE Biosystems). This real-time system (referred to as fluorescence-based simultaneous amplification and product detection) uses the TaqMan assay, which uses a fluorogenic probe, designed to hybridize to an internal region of the PCR product, to enable the detection of the specific PCR products as they accumulate during PCR and so enable accurate and reproducible quantitation of cDNA. One to 4 μ g RNA were subjected to a DNase treatment to eliminate any contaminating gDNA following the manufacture's instructions (Standard DNase kit, Qiagen). The RNA samples were then subjected directly to the RT-PCR reactions to generate cDNA using Reverse Transcription Superscript Pre-amplification kit (Life Technologies). The expression of the transcripts of the cytokines and cytokine receptors was analyzed using specific probes and primers (see Appendix A.5.5 for probe and primer sequences). Relative copy numbers of cytokine and cytokine receptor transcripts were determined from a standard curve generated for each assay using PCR standards. Transcript levels of the ribosomal protein L19 (rl19) were used as internal controls.

II.4.7. Detection of IFN- γ by intracellular flow cytometry

Detection of the intracellular IFN- γ reported in the present dissertation was performed with or without cell surface marker staining. The intracellular IFN- γ was detected by flow cytometry as follows:

II.4.7.1. Cell stimulation

LAK cells (grown in RP-10 containing 1000 U/ml huIL-2) were seeded at 1×10^6 cells/ml in 24-well plates and stimulated overnight with or without 1ng/ml mL-12 at 37°C. In the next morning, 3 μ M of the intracellular protein transport inhibitor Monensin A sodium salt (Sigma) (from 4 mM stock in 100% ethanol) was added to the cells and the cultures were re-incubated for 4h at 37°C. Cells were then recovered in FACS buffer and pelleted at 350 x g.

II.4.7.2. Cell surface marker staining

Pelleted cells were resuspended in 100 µl FACS buffer and stained with 2 to 10 µg/ml FITC-conjugated anti-mouse Abs or biotinylated Abs against external cell markers for 25 min at 4°C. After two washes in FACS buffer, cells primarily stained with biotin-conjugated Abs were re-stained with 1: 100 (v/v) of 0.5 mg/ml STV-FITC for 25 min at 4°C. After two washes in FACS buffer, the cells were ready for intracellular cytokine staining.

II.4.7.3. Cell permeabilization and fixation

Saponin is a reversible permeabilization agent and its activity is abolished upon withdrawal. Therefore, all buffers for fixation/permeabilization, washes and intracellular staining contain 0.1% saponin (Sigma). After extracellular staining, cells were resuspended in 250µl fixation / permeabilization buffer (4% paraformaldehyde (w/v) and 0.1 % saponin (w/v) in PBS) for 25 min at RT then were washed twice in intracellular wash buffer (1% BSA (w/v), 0.1% saponin (w/v) and 0.1 % NaN₃ (v/v) in PBS). At this step, fixed cells could be stored at 4°C for 2 to 3d.

II.4.7.4. IFN-γ intracellular staining

After fixation/permeabilization, cells were resuspended in 100 µl intracellular blocking buffer (1% BSA (w/v), 1% NRS (v/v), 5% skim milk powder (w/v), 0.1 % (w/v) saponin and 0.1 % NaN₃ (v/v) in PBS) for 10 min before addition of 1: 100 (v/v) of 0.2 mg/ml PE-conjugated anti-IFNγ or 1: 100 (v/v) of 0.2 mg/ml matching PE-conjugated isotype control. The cells were stained for 30 min at RT in the dark and washed twice with intracellular wash buffer. The cells were then resuspended in FACS buffer and analyzed by flow cytometry.

II.4.8. Analysis of cytokine secretion by ELISA

The following protocol was used for all the ELISA experiments performed in the present thesis. 96 well-plates flat bottom (NUNC) were coated with 50 µl/well of capture antibody at 1 µg/ml in ELISA binding buffer (0.1 M Na₂CO₃, 0.1 M NaHCO₃ in ddH₂O, pH 9.6.) for 1h at 37°C or overnight at 4°C. The plates were washed 6 times using ELISA wash buffer (0.05% tween-20. (Sigma) in PBS) then blocked for 1h at RT or overnight at 4°C with 200 µl/well of ELISA blocking buffer (1% BSA in 1 x PBS). After 6 washes

with ELISA wash buffer, 100 μ l/well of cytokine standards and samples diluted in ELISA dilution buffer (1% BSA in PBS containing 0.05% tween-20) were plated in duplicate and the plates were kept overnight at 4°C. ELISA dilution buffer was used as blank. Next day, the plates were washed 6 times, soaked 10 min then washed 6 times with ELISA wash buffer. 100 μ l/well of biotinylated detection Abs (0.5 to 1 μ g/ml) in ELISA dilution buffer were added for 1h at RT. The plates were washed as mentioned above and were incubated with 100 μ l/well of 1:5000 (v/v) of avidin-HRP (Pharmingen) in ELISA dilution buffer for 30 min at RT. The plates were washed then the cytokines were detected with 100 μ l/well of TMB peroxidase substrate (MANDEL). The reactions were stopped using 100 μ l/well of 1N HCl in ddH₂O. The plates were read at 450 nm using a WinSelect microplate reader (Tecan). Capture Abs, biotinylated detection Abs and standards specific for each cytokine analyzed are reported in Appendix A.2.

II.4.9. Proliferation of murine NK cells in response to IL-12

2 x 10⁴ cells / well of sorted IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK in a total volume of 200 μ l were plated in 96-well plates and were stimulated with varying concentrations of murine IL-12 in RP-10 medium containing 1000 U/ml huIL-2. After 16 h incubation, the cells were pulsed with 1 μ Ci per well of [³H]-thymidine (Amersham Pharmacia Biotech) for an additional 6 h. Cells were then harvested onto glass fiber filters (Packard) using Harvester 96 (TomTec). Incorporated radioactivity was measured as cpm in a Matrix beta counter (Packard).

II.4.10. Analysis of cytokine gene expression by RNase Protection Assay (RPA)

2 x 10⁶ of sorted IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK cells, expanding in RP-10 medium containing 1000 U/ml huIL-2, were left untreated or stimulated overnight with 1 ng/ml murine IL-12 or with 50 ng/ml PMA and 1 μ M ionomycin (Sigma) for the last 3 h. Cells were harvested and total RNA was prepared using Trizol reagent. 1 μ g RNA per condition was examined for cytokine mRNA expression using the mouse common cytokine set multiprobe template set mCK1 (RiboQuant, Pharmingen) according to the manufacturer's protocols (refer to Appendix A.3 for mCK1 composition). In general, RiboQuant multi-Probe RNase Protection Assay System is a complete kit available for detecting cytokine transcripts. The kit contains multiprobe cDNA templates encoding for

common cytokines. These templates were used for the synthesis of specific [^{32}P]-radiolabeled anti-sense RNA probes. The transcribed radiolabeled probe set was hybridized to target total RNA. Free probes and non-hybridized RNA (single stranded RNA) were digested with RNase. Hybridized RNA or "protected RNA" was purified, was separated on denaturing polyacrylamide gels and was detected by autoradiography. As control, probes for housekeeping genes incorporated into the sets were used to quantify the levels of individual mRNA between samples. RNase-protected probes were resolved on 6 % polyacrylamide gels using 0.5 X TBE running buffer (for 10 X TBE buffer, 108 g Tris base, 55 g boric acid (BDH) and 0.5 M EDTA pH8.0 in 1L ddH₂O). As size markers, 1000 to 2000 cpm/well of undigested [^{32}P]-radiolabeled probe set were run on the gel.

II.4.11. Cytotoxicity Assay

To further examine the biological significance of murine DX5⁺NK cell subsets, sorted IL-12R β 2^{lo} and IL-12R β 2^{hi} cell subsets were tested as effector cells in a ^{51}Cr release assay using NK-sensitive YAC-1 target cells kindly provided by Dr. Sylvia Vidal (BMI, faculty of medicine, University of Ottawa, ON). The cytolytic capacity of DX5⁺NK cell populations was analyzed in the absence and the presence of exogenous IL-12 as follows:

II.4.11.1. Effector NK cells

3 x 10⁵ cells per well of sorted IL-12R β 2^{lo} and IL-12R β 2^{hi} effector LAK cells (d 15), generated from purified DX5⁺ cells, were plated overnight at 37°C in 2 ml RP-10 medium containing 1000 U/ml huIL-2 in 2 wells of 24 well-plates. One well (1 ml) was left in IL-2 only. The other well was treated overnight with 1 ng/ml murine IL-12. The next day, cells were washed twice with RP-10 medium and resuspended in 800 μl RP-10 medium per tube. Effector cells were then diluted 5 x 2 fold dilutions in Eppendorf tubes to be plated with the YAC-1 target cells.

II.4.11.2. YAC-1 target cells

YAC-1 target cells, in log phase of growth, were cultured overnight at 1 x 10⁶ cells/ml in RP-10 medium. Next day, 1 x 10⁶ cells of YAC-1 target cells were pelleted and resuspended in 100 μl of 1mCi / ml Na⁵¹CrO₄ (Amersham Pharmacia biotech) in a 15 ml centrifuge tube. YAC-1 target cells were then incubated for 1h at 37°C. The cells were

gently shaken every 15 min. In the next step, YAC-1 target cells were washed twice with 10 ml RP-10 medium and radioactive supernatants were decanted in the appropriate liquid waste container. Finally, target cells were diluted in RP-10 medium at 5×10^4 cells/ml. 100 μ l containing 5×10^3 ^{51}Cr -labeled target cells were plated in triplicate in 96 well-plates (round bottom) and incubated with serial dilutions of IL-12R β ^{lo} or IL-12R β ^{hi} effector cells (stimulated or not with IL-12) for 4 hours at 37°C. 100 μ l supernatants were harvested in glass tubes and the ^{51}Cr released from YAC-1 target cells was counted in a gamma-counter (Gamma 5500, Beckman). As controls, ^{51}Cr -labeled YAC-1 target cells were incubated with 100 μ l of Triton X-100 to determine 100% lysis and with 100 μ l RP-10 medium to define spontaneous ^{51}Cr release. Percent specific lysis was calculated as follows:

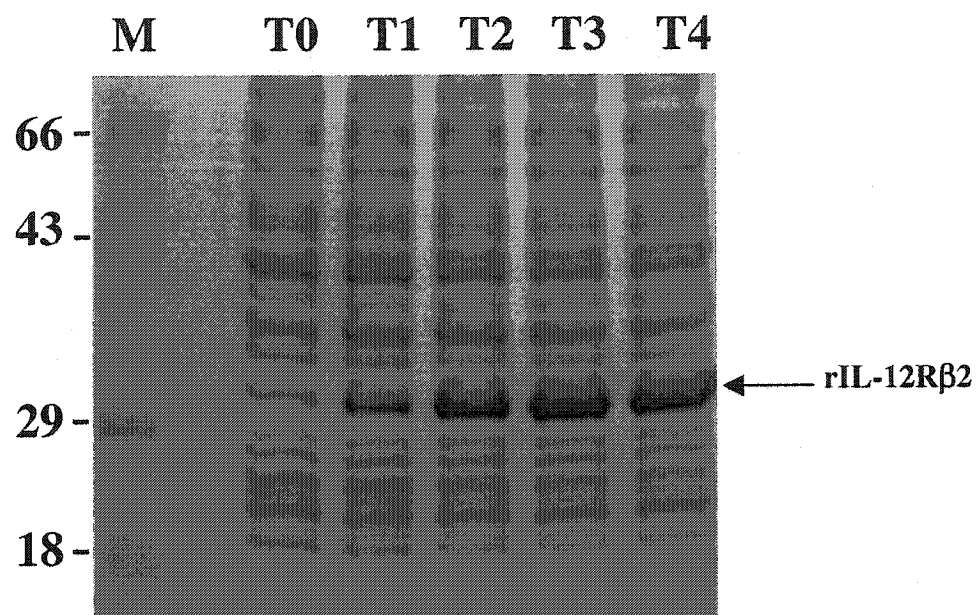
$$\% \text{ lysis} = 100 \times \frac{\text{Mean experimental (cpm)} - \text{mean spontaneous release (cpm)}}{\text{Mean 100\% release (cpm)} - \text{mean spontaneous release (cpm)}}$$

II.4.12. Murine NK1 and NK2 cell polarization

II.4.12.1. Generation of NK1 and NK2 cells

Murine DX5⁺NK cells, purified from spleen cells by positive magnetic selection, were plated at 5×10^5 cells/ml in a 12 well-plate and stimulated with 1000U/ml huIL-2 in the presence or absence of the T_H1/T_H2 polarizing cytokines. DX5⁺NK grown in huIL-2 only were termed NK0 cells. NK1 cells were cultured with huIL-2 plus 0.1 ng/ml mIL-12 plus 10 μ g/ml anti-mIL-4 (11B11). NK2 cells were incubated with hIL-2 plus 100 U/ml IL-4 plus 1: 250 (v/v) rat anti-mouse IL-12p40 (C17.8) hybridoma supernatant. Cells were stimulated in the indicated conditions for 6 d then expanded in 5 x the stimulation volume of fresh RP-10 containing 1000 U/ml huIL-2 only. At d6 of cytokine stimulation, cell-free supernatants were harvested and analyzed for IL-5 and IFN- γ secretion by capture ELISA. At d10, cells were analyzed for IL-12R β 2 expression by flow cytometry using the rat-anti IL-12R β 2 antiserum.

Fig. 2.2. SDS-PAGE analysis of recombinant IL-12R β 2 expression in BL21 competent *E. coli*. IL-12R β 2 gene was cloned into pET17b high level bacterial expression system and the construct was used to transform competent BL21 (DE3) pLysS *E. coli*. rIL-12R β 2 protein expression was induced with addition of IPTG for 4h to the bacterial culture. Aliquots of uninduced (T0) and induced bacterial cell lysates (T1 to T4) were analyzed for protein expression kinetics (1h to 4h) by electrophoresis. The gel was stained with Coomassie blue to detect total proteins. Arrow indicates the position of the rIL-12R β 2 protein with the expected MW of 32 kDa. Pre-stained high molecular weight proteins (Life Technologies) were used as standard markers (M).



II.5. Results

II.5.1. Generation of rabbit and rat anti-mouse IL-12R β 2 antisera

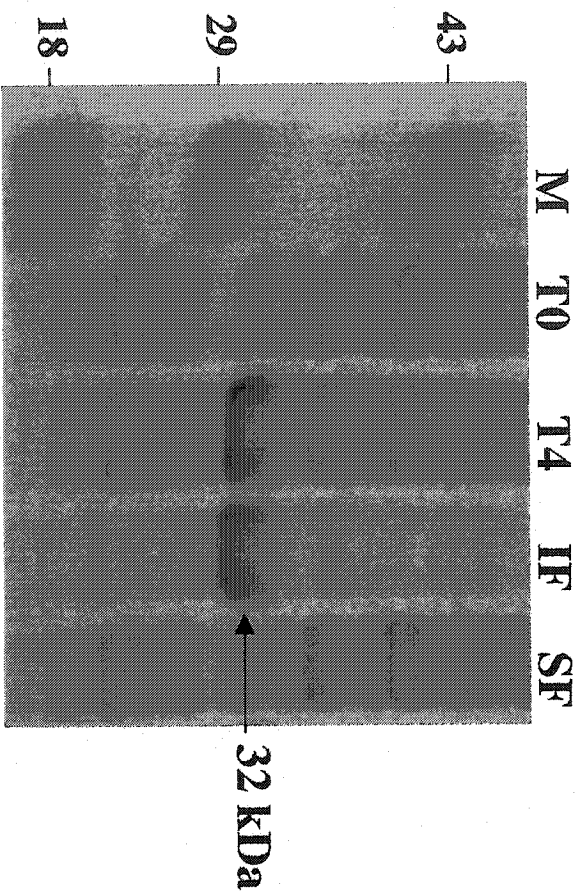
The expression of murine IL-12R β 2 chain on the surface of T or NK cells can be monitored by flow cytometry using IL-12R β 2 chain-specific antibody. However, no such murine antibody was available, thus the mouse IL-12R β 2 gene was amplified by PCR from a mouse IL-12R β 2 cDNA clone, kindly provided by Dr. Kenneth M. Murphy (Washington University), using specific primers. Also, a 6 x His tag was integrated into the 5' end of IL-12R β 2 ORF. The PCR-amplified fragment was cloned into pET17b high level expression protein and a DNA construct encoding the N-terminal IL-12R β 2 extracellular domain (ECD) (aa 24-275) was generated (Fig. 2.1). rIL-12R β 2, with the expected MW of 32 kDa, was expressed in BL21 *E. coli* (Fig.2.2). The level of the expressed fusion protein increased with time to reach a maximum at T = 4 h after IPTG induction. rIL-12R β 2 was found predominantly in the bacterial inclusion bodies fraction (insoluble fraction) after gel staining with Coomassie blue (Fig. 2.3 A). Western blot of the induced rIL-12R β 2 protein revealed a positive immunoblot staining with anti-His mAb at T = 4h after *E. coli* treatment with IPTG confirming the expression of the correct recombinant protein (Fig. 2.3 B). This rIL-12R β 2 protein was purified to homogeneity by Ni-NTA chromatography affinity (Fig. 2.4).

II.5.2. Rat anti-murine IL-12R β 2 antiserum exhibited high specificity for IL-12R β 2 expressed on murine DX5⁺NK cells

The main goal during the preparation of anti-murine IL-12R β 2 antibody was to obtain an antiserum able to recognize the native form of the IL-12R β 2 chain. Therefore, the antisera generated were tested for their specificity using different techniques such as Western blot, flow cytometry on mouse LAK cells and ConA blasts from splenic cells and IP. The results, presented in Fig. 2.5, showed that when IPTG-induced BL21 *E. coli* samples (T4) were immuno-blotted with both rabbit and rat anti-murine IL-12R β 2 test bleed antisera, they revealed the presence of a unique band corresponding to the expected recombinant protein of 32 kDa. Immuno-blot controls

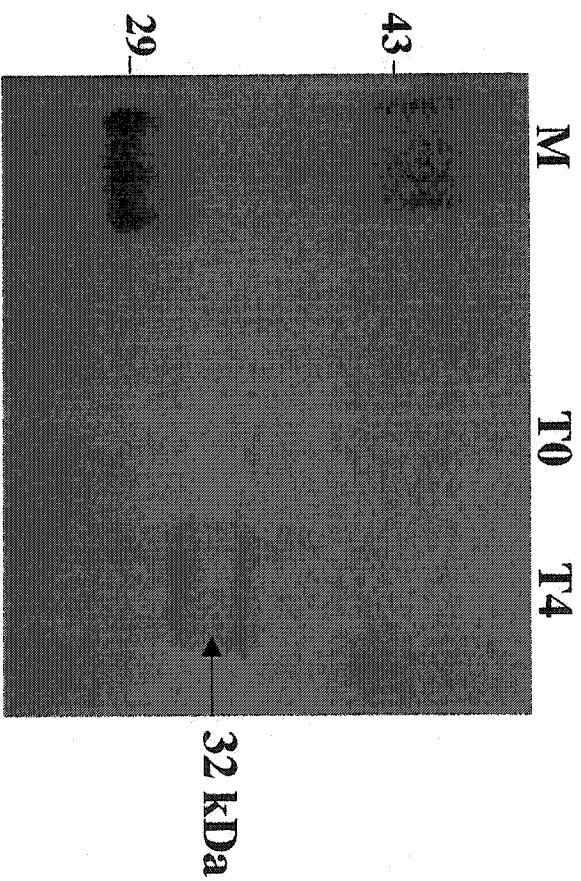
Fig 2.3. Analysis of rIL-12R β 2 protein expression. Competent BL21 (DE3) pLysS *E. coli* transformed with pET17b-IL-12R β 2/EcoRV construct were lysed. Aliquots from insoluble fraction (inclusion bodies) (IF) and soluble fraction (SF) were analyzed by electrophoresis to determine where the protein was concentrated. The first gel was stained with Coomassie Blue to detect total proteins (A). The second gel was blotted onto nitrocellulose and probed with anti-His mAb. His tag-bearing rIL-12R β 2 protein with the expected MW (32 kDa) was detected (B). M: Pre-stained high molecular weight proteins markers; T0: aliquots from uninduced bacterial cell lysates; T4: aliquots from bacterial cell lysates induced with IPTG for 4h. Arrow indicates the position of rIL-12R β 2 protein.

A



Coomassie Blue staining

B



Anti-His tag mAb

Fig. 2.4. Purification of expressed rIL-12R β 2 protein by affinity chromatography. rIL-12R β 2 protein was purified to homogeneity by Ni-NTA nickel chelate affinity chromatography (Qiagen). Samples from different purification steps were analyzed by electrophoresis and the gels were stained with Coomassie blue. BB (insoluble fraction resuspended in 6M guanidine buffer (pH8), before binding to Ni-NTA resin); FT (flow-through); P8: wash with 6M guanidine buffer (pH 8); P6: wash with 6M guanidine buffer (pH 6.3); F: eluted fractions. The fractions F1-F6 were pooled together and used to immunize rats and rabbits. Arrow indicates the position of the purified rIL-12R β 2 protein. Pre-stained high molecular weight proteins were used as markers.

BB FT P8 P6 F1 F2 F3 F4 F5 F6 F7

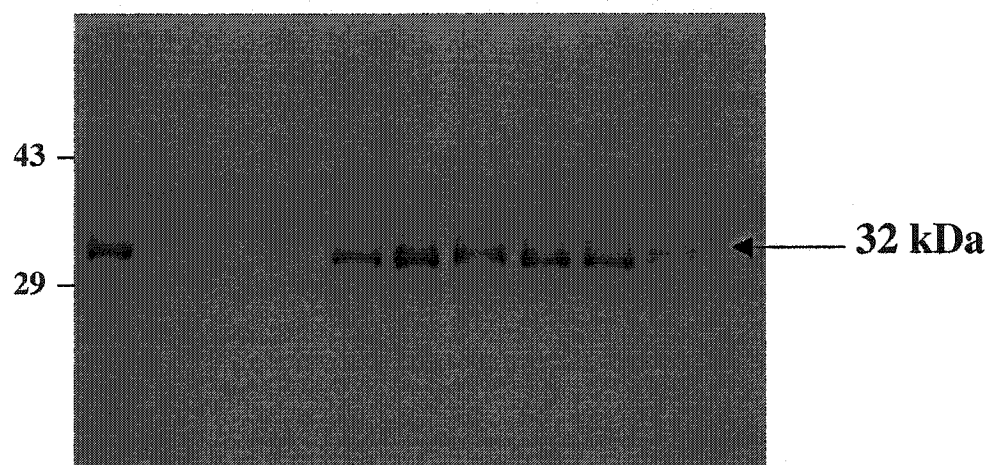
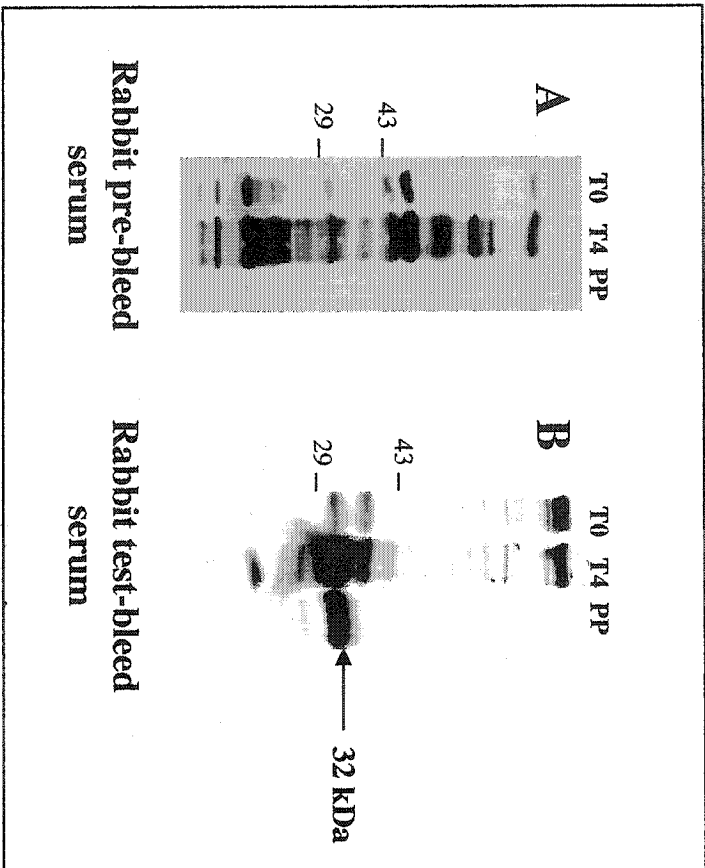
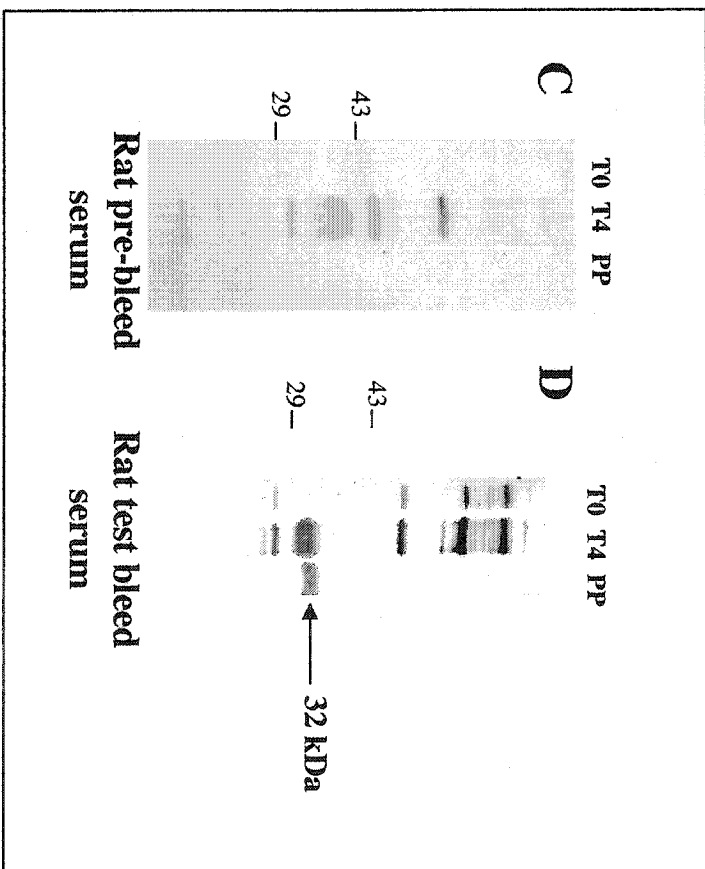


Fig. 2.5. Western blot analysis of rabbit and rat immune sera for specific anti-mouse IL-12R β 2 antibody production. Samples from uninduced (T0), induced bacterial cell lysates (T4) and purified rIL-12R β 2 protein (PP) were separated by electrophoresis and analyzed by western blotting using test-bleed sera from immunized rat and rabbit. Pre-immune sera from the non-immunized animals were used as negative controls. Arrow indicates the position of the reactive rIL-12R β 2 protein. Pre-stained high molecular weight proteins were utilized as markers.

Rabbit



Rat

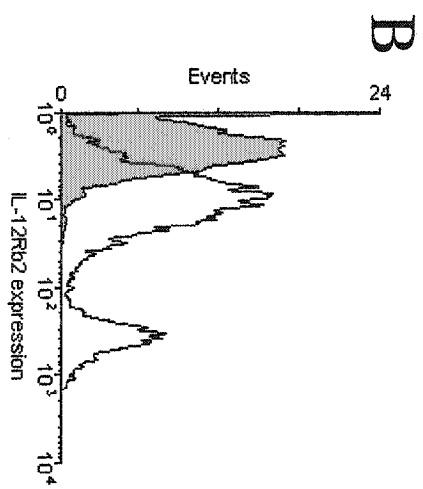
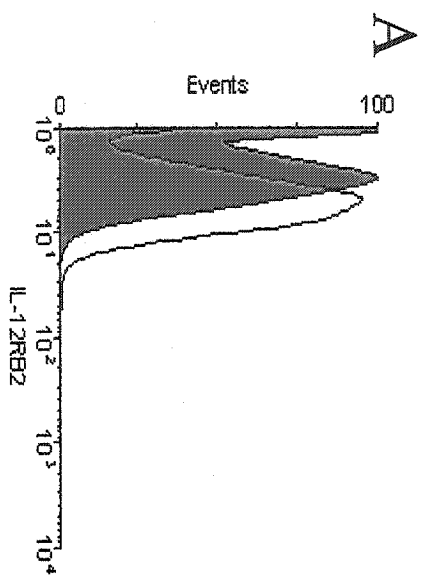


incubated with pre-immune sera revealed no bands. These data indicate that anti-IL-12R β 2 antisera generated in both rabbit and rat recognized the SDS-denatured form of rIL-12R β 2 protein by Western blot. To test for the ability of anti-IL-12R β 2 antiserum to recognize the native form of murine IL-12R β 2 chain, ConA blasts, generated from murine MNC were stained with anti-IL-12R β 2 antisera and analyzed by flow cytometry. The results indicate that stimulation of murine MNC with ConA for 3d induced a moderate upregulation of IL-12R β 2 subunit, which was revealed with rat anti-IL-12R β 2 antiserum only (Fig. 2.6.A). DX5⁺NK cells from C57BL/6 mice, expanding in the presence of 1000 U/ml huIL-2 for 12 d, were also analyzed for IL-12R β 2 expression. Flow cytometric studies repeatedly showed the development of two subsets of IL-2-activated murine LAK cells expressing different levels of IL-12R β 2 chain cells when stained with rat anti-IL-12R β 2 antiserum (Fig. 2.6.B). When compared to control DX5⁺NK cells stained with rat pre-immune serum, these two subsets corresponded to IL-12R β 2^{lo} and IL-12R β 2^{hi} cells. Development of two distinct populations of NK cells based on the expression of IL-12R β 2 chain was a consistent pattern that was routinely observed in MNC or DX5⁺NK cells stimulated with 1000 U/ml huIL-2. This NK pattern was also observed on LAK cells from different mouse genetic backgrounds (C57BL/6, BALB/c and FVB/N mouse strains) indicating that this phenomenon is strain independent.

Specificity of the rat antiserum was also investigated by immunoprecipitation of murine IL-12R β 2 subunit from DX5⁺NK cells stimulated for 12 d with 1000 U/ml huIL-2. Fig. 2.6.C indicates that under reducing conditions, rat anti-IL-12R β 2 antiserum successfully immunoprecipitated a protein of the approximate molecular mass of 98 kDa.

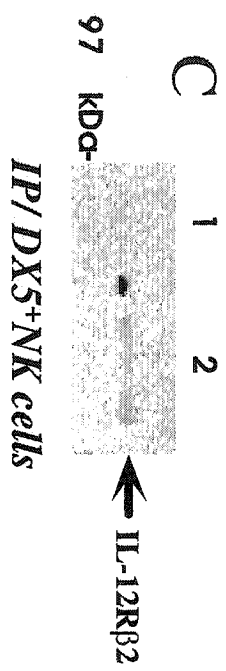
Rat anti-murine IL-12R β 2 antiserum specificity was further analyzed by a competitive binding assay. IL-2-activated DX5⁺NK cells were stained with rat anti-IL-12R β 2 antiserum in the absence (Fig. 2.7.A) or the presence (Fig. 2.7.B) of soluble rIL-12R β 2 protein as competitor. In the presence of high concentrations of rIL-12R β 2 (500 ng/ml), binding of rat anti-IL-12R β 2 antiserum to the surface of DX5⁺ cells was completely abrogated, whereas binding of DX5 mAb was unaffected. Using varying concentrations of purified rIL-12R β 2 protein, competitive binding of rat anti-mouse IL-

Fig. 2.6. Rat anti-mouse IL-12R β 2 antiserum recognizes the native form of IL-12R β 2 subunit. Splenic MNC from C57BL/6 mice were stimulated with ConA (2.5 μ g/ml) and huIL-2 (10 U/ml) for 3 days (A), or with huIL-2 (1000 U/ml) for 8 to 10 days (B) and analyzed for surface expression of IL-12R β 2 by flow cytometry. Cells were stained with rat anti-mouse IL-12R β 2 antiserum (clear curve) or non-immune rat serum (shaded curve) followed by FITC-conjugated goat anti-rat Ig. Cells in (A) were first gated on the CD3⁺ blast population. Cells in (B) were gated on the NK blast population. Specificity of anti-mouse IL-12R β 2 antiserum was also demonstrated by immunoprecipitation (IP) (C). DX5⁺ IL-2 blasts (2.5×10^7) were incubated with non-immune rat serum (lane 1) or with rat anti-mouse IL-12R β 2 antiserum (lane 2) followed by biotin-conjugated goat anti-rat Ig. Cells were lysed and biotin-conjugated complexes were recovered using streptavidin-agarose beads. Immune complexes were analyzed by western blot using rat anti-mouse IL-12R β 2 antiserum. A reactive protein with the expected molecular weight is indicated by the arrow.



Con A blasts

LAK cells



12R β 2 antiserum to the DX5⁺NK cell surface showed a dose-dependent inhibition (Fig. 2.7.C). Taken together, these results confirm the specificity of the rat anti-mouse IL-12R β 2 antiserum and demonstrate the importance of this tool in murine IL-12R β 2 detection by flow cytometry.

II.5.3. Kinetics of IL-12R β 2 expression on IL-2-activated DX5⁺NK cells

To better understand the IL-12R β 2^{lo} and IL-12R β 2^{hi} NK pattern developed by NK cells, LAK cells generated from C57BL/6 bulk splenocytes were analyzed on days 0-11 for the kinetics of subset development (Fig. 2.8, A panel). Cells were double stained for DX5 marker and IL-12R β 2 expression and analyzed by flow cytometry. Resting splenocytes (d0) contain a small population of DX5⁺NK cells (8%) but did not express detectable amounts of IL-12R β 2. After addition of 1000 U/ml huIL-2 to the cell cultures for 5d, a rapid expansion of the DX5⁺NK population reaching 34% of total MNC was observed, and differentiation of the DX5⁺NK cells into IL-12R β 2^{lo} and IL-12R β 2^{hi} was evident. Complete differentiation of DX5⁺MNC into two distinct populations was achieved by d11 of the cell culture.

To determine whether formation of IL-12R β 2^{lo} and IL-12R β 2^{hi} pattern was inherent to the DX5⁺ cells or required the presence of other MNC types in the cell culture, DX5⁺ cells were purified from C57BL/6 mice by positive magnetic selection for the generation of LAK cells (Fig. 2.9). DX5⁺ cells, enriched to 97 % purity, showed the presence of low percent of DX5⁺NK-T cells, usually ranging from 5 to 11% of total spleen MNC, after flow cytometric double staining using anti-DX5 and anti-CD3 mAbs.

As observed with bulk splenocytes, enriched DX5⁺NK cells stimulated with 1000 U/ml huIL-2 showed a gradual differentiation into IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets (Fig. 2.8, B panel). At day 11, the cells were completely differentiated and the percentages of the two DX5⁺NK cell subsets were approximately equal. Together, these data show that the capacity of murine LAK cells to differentiate into IL-12R β 2^{lo} and IL-12R β 2^{hi} is inherent to DX5⁺NK cells upon stimulation with IL-2.

Fig. 2.7. Specificity of the rat anti-mouse IL-12R β 2 antiserum. DX5⁺NK cells purified from C57BL/6 splenocytes by positive magnetic selection were stimulated with huIL-2 (1000 U/ml) for 11 days to generate DX5⁺ IL-2 blasts. Cells were then stained with rat anti-mouse IL-12R β 2 antiserum and FITC-conjugated goat anti-rat Ig plus PE-conjugated anti-DX5 Ab (A-C). Specific anti-IL-12R β 2 Ab binding was performed in the absence (A) or presence (B) of 500 ng/ml competitor rIL-12R β 2. (C), titration of competitive inhibition of specific Ab binding shown in (A) and (B). Data is shown as percentage of inhibition of anti-mouse IL-12R β 2 binding or anti-DX5 binding in the presence of varying concentrations of rIL-12R β 2 as measured by flow cytometry.

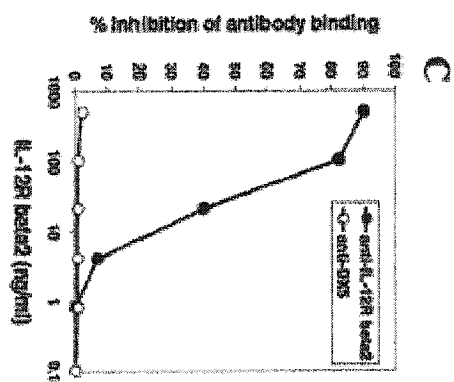
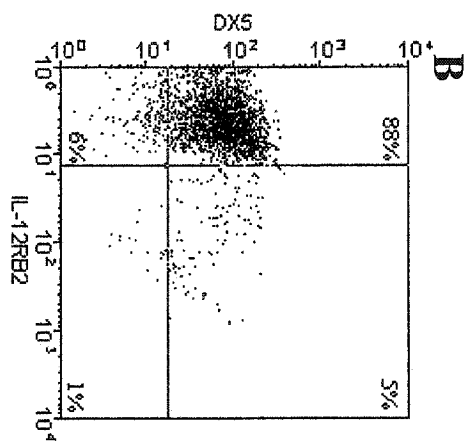
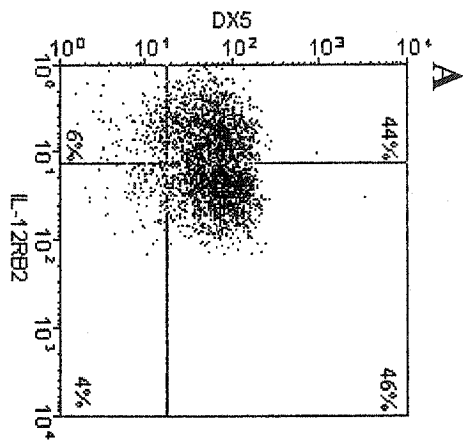
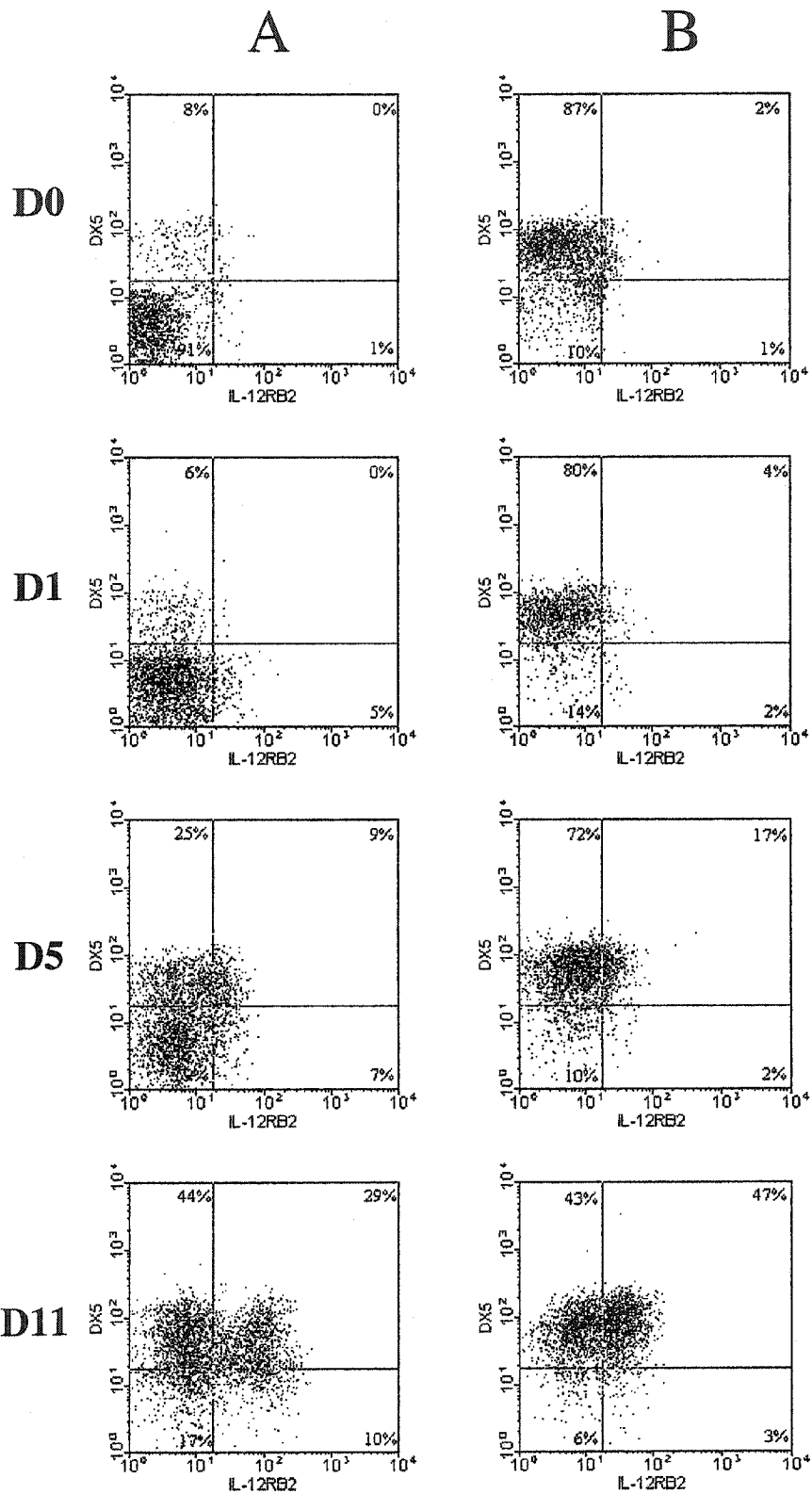


Fig. 2.8. Kinetics of IL-12R β 2 expression on murine LAK cells. C57BL/6 bulk splenic MNC (A panel) or DX5⁺NK cells purified from C57BL/6 splenocytes by positive magnetic selection (B panel) were grown in the presence of huIL-2 (1000 U/ml) for 11 days and analyzed by flow cytometry at the indicated time points using the rat anti-IL-12R β 2 antiserum and FITC-conjugated goat anti-rat Ig plus PE-conjugated anti-DX5.



Bulk IL-2 Blasts

IL-2-activated DX5⁺ NK

II.5.4. Phenotypic characterization of IL-12R β 2^{lo} and IL-12R β 2^{hi} LAK cells

The surface phenotype of the two DX5⁺NK cell populations was further analyzed using two-color flow cytometry. IL-2 activated DX5⁺NK cells (d11) were double stained for IL-12R β 2 using rat anti-IL-12R β 2 antiserum and for the expression of different cell surface markers (DX5, CD3, CD19, B220, Ly49A, Ly49C and Ly49G2) using specific mAbs. As reported in Fig. 2.10, IL-2-activated DX5⁺NK cells were comprised of approximately equal populations of IL-12R β 2^{lo} and IL-12R β 2^{hi} cells when co-stained for DX5. Moreover, the two populations showed a positive staining for NK1.1 surface marker, further confirming that both subsets are from NK cell origin. When co-stained with anti-CD3 mAb, both IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets showed the presence of a small subpopulation of NK-T cells. However, the two subsets were not positive for CD19 staining indicating that they were free from any B cell contamination. B220, known to be a marker for activated NK cells (215), was upregulated in both IL-12R β 2 subsets suggesting that they both were activated. As reported earlier (216), these IL-2-activated DX5⁺NK cells were heterogeneous in term of expression of the Ly-49 receptor family (NK cell surface receptors). Indeed, 23% of DX5⁺NK cells were stained positively for Ly-49A, 23% for Ly49C and 57% for Ly-49G2. The Ly-49A⁺ and Ly-49C⁺ cells were equally distributed between IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK cell subsets. Surprisingly, Ly-49G2 cell surface marker expression was restricted to the IL-12R β 2^{hi} population, resulting in a delineation of DX5⁺NK cells as being either Ly-49G2⁻ IL-12R β 2^{lo} or Ly-49G2⁺ IL-12R β 2^{hi}.

II.5.5. IL-12R β 2 transcripts were upregulated in DX5⁺NK cells in response to IL-2 activation

Transcript levels of IL-12R β 2 and IFN- γ were analyzed by real-time PCR to confirm the upregulation of IL-12R β 2 chain observed by flow cytometry on DX5⁺NK cells in response to IL-2 activation. Splenic DX5⁺NK cells enriched from C57BL/6 mice were used immediately after purification (resting cells) or were stimulated with huIL-2 for 6d. Total cellular RNA was analyzed for IL-12R β 2 and IFN- γ mRNA expression.

Fig. 2.9. Enrichment of splenic DX5⁺NK cells C57BL/6 DX5⁺NK cells were isolated from splenic MNC by positive magnetic selection using anti-DX5 microbeads (Miltenyi) following the manufacturer's protocol. Cells were stained with FITC-conjugated anti-CD3 and PE-conjugated anti-DX5 and analyzed by two-color flow cytometry.

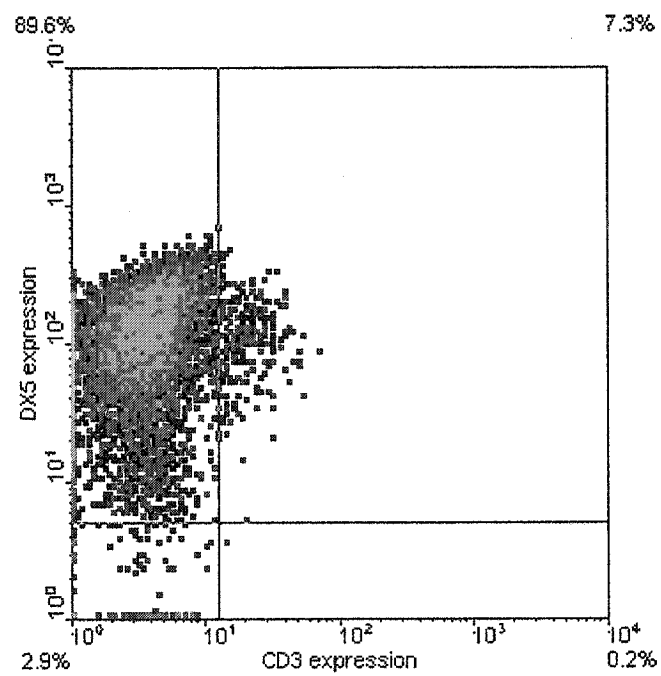


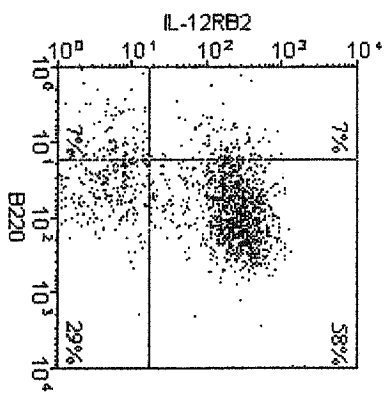
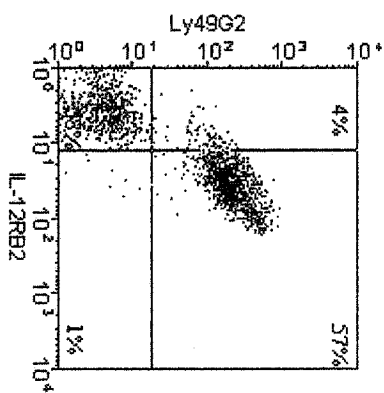
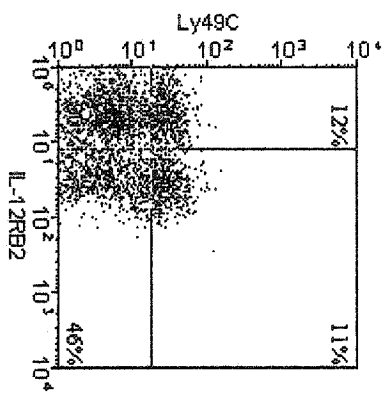
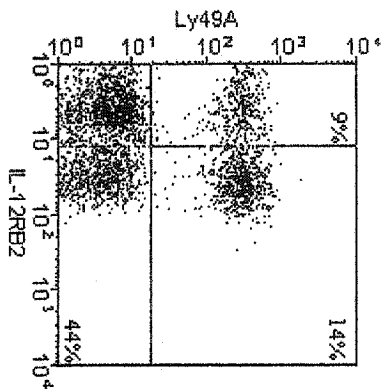
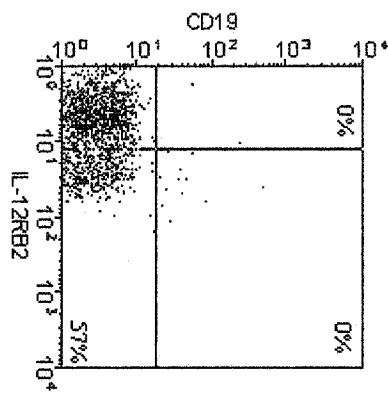
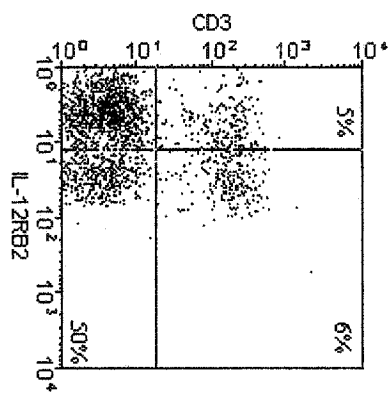
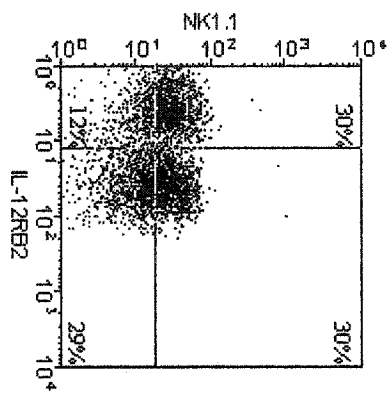
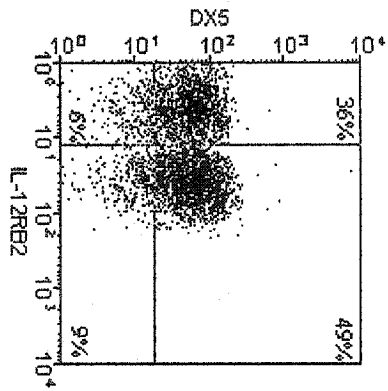
Fig. 2.10. Phenotypic characterization of IL-12R β ^{lo} and IL-12R β ^{hi} LAK cell subsets. DX5⁺NK cells, purified from C57BL/6 splenocytes by positive magnetic selection were stimulated with 1000 U/ml huIL-2 for 11 days. Cells were stained consecutively with rat anti-mouse IL-12R β 2 antiserum and FITC-conjugated goat anti-rat Ig followed by PE-conjugated anti-DX5, NK1.1, CD3 and CD19 or biotin-conjugated Ly-49A, Ly-49C and Ly-49G2 and PE-conjugated streptavidin. For B220 marker detection, cells were stained with rat anti-IL-12R β 2 antiserum and PE-conjugated goat anti-rat Ig followed by FITC-conjugated anti-B220. Cells were then analyzed by two-color flow cytometry.

DX5

NK1.1

CD3

CD19



Ly49A

Ly49C

Ly49G2

B220

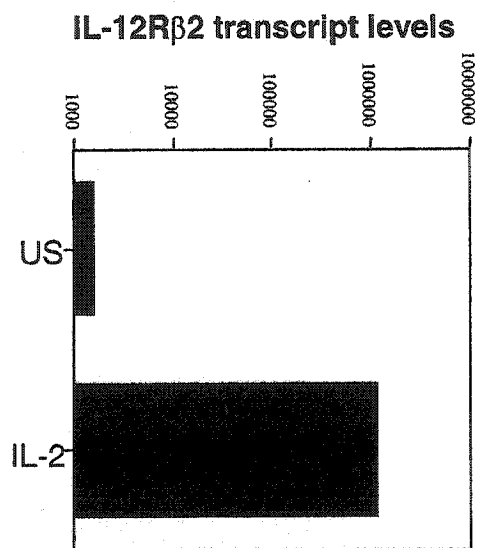
Resting DX5⁺NK cells constitutively produced very low levels of IL-12R β 2 mRNA. However, IL-2 activation of these cells resulted in a dramatic upregulation of IL-12R β 2 transcript levels (Fig. 2.11 A). Furthermore, resting DX5⁺NK expressed residual IFN- γ transcripts (Fig. 2.11 B). Although the IL-2-activation of DX5⁺NK cells resulted in a dramatic accumulation of IL-12R β 2 transcripts, no IFN- γ mRNA upregulation was observed in response to IL-2. The mRNA level of the ribosomal protein L19 (r19) was used as an internal control to ensure that the increase in IL-12R β 2 mRNA is not due to a higher concentration of mRNA tested (Fig. 2.11 C).

II.5.6. IL-12-induced IFN- γ by IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK cells

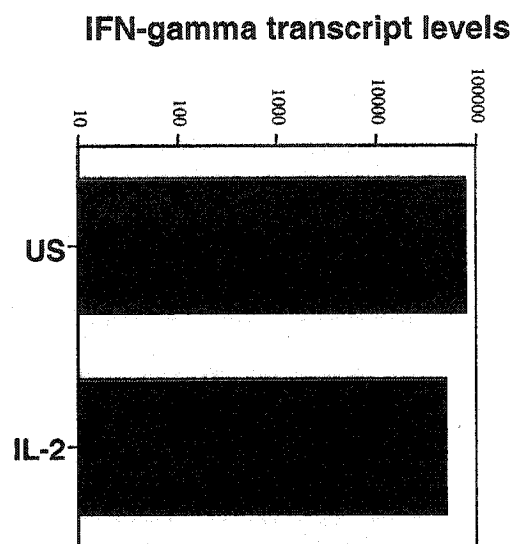
The following step was to determine whether IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK subsets differ in their capacity to produce IFN- γ in response to IL-12 stimulation. IL-2-activated DX5⁺NK cells (d10) were incubated overnight in the presence or the absence of 1 ng/ml mIL-12 and were assessed for intracellular IFN- γ production by flow cytometry. Both IL-12R β 2^{lo} and IL-12R β 2^{hi} populations produced significant levels of IFN- γ after overnight treatment with exogenous IL-12 (Fig. 2.12 A and B).

To address whether IL-12 was acting directly on both subsets or whether IL-12R β 2^{hi} population was influencing the IL-12R β 2^{lo} through an indirect mechanism, the experiment was repeated using sorted IL-12R β 2^{lo} and IL-12R β 2^{hi} populations. In addition, the cells were incubated overnight in the presence of varying concentrations of IL-12 to determine whether the IL-12R β 2^{hi} cells were more sensitive to IL-12 stimulation than IL-12R β 2^{lo} cells. Although the percentage of IFN- γ -producing cells was higher in the IL-12R β 2^{hi} population at high concentrations of IL-12, both the IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets clearly responded to IL-12 by increasing the intracellular IFN- γ (Fig. 2.12.C).

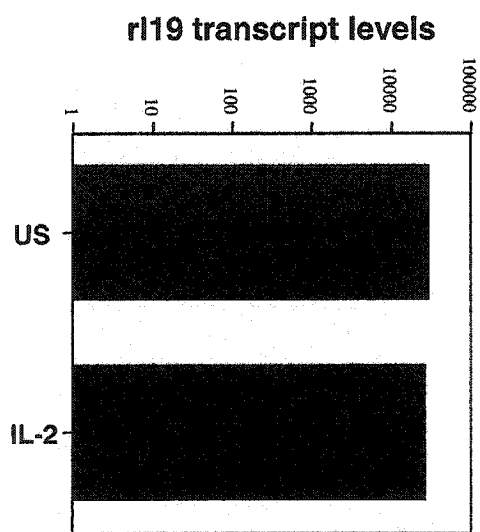
Fig. 2.11. Expression of IL-12R β 2 and IFN- γ transcripts by DX5⁺NK cells in response to IL-2 activation. DX5⁺NK cells, purified from C57BL/6 splenocytes by positive magnetic selection, were used immediately (resting NK cells) or stimulated with 1000 U/ml huIL-2 for 6 days. Total RNA was prepared and analyzed for gene expression of IL-12R β 2 (A) and IFN- γ (B) by real-time PCR. Ribosomal protein L19 (rl19) RNA was used as internal control to normalize the RNA levels (C). Transcripts levels correspond to the relative copy numbers that were calculated from a PCR standard curve specific for each assay (IL-12R β 2 and IFN- γ).



A

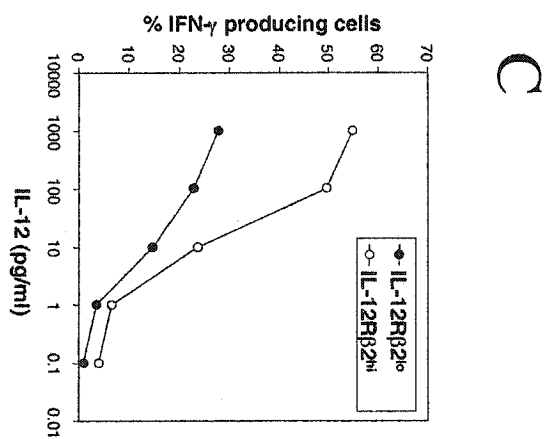
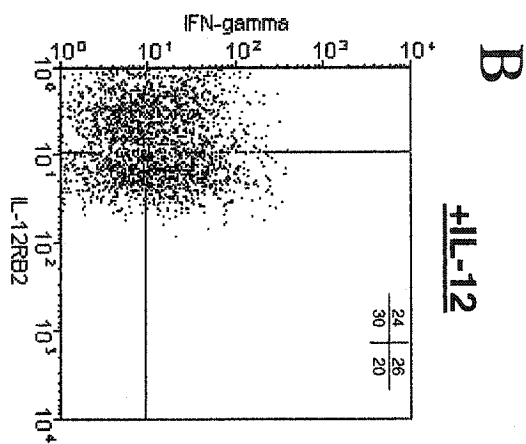
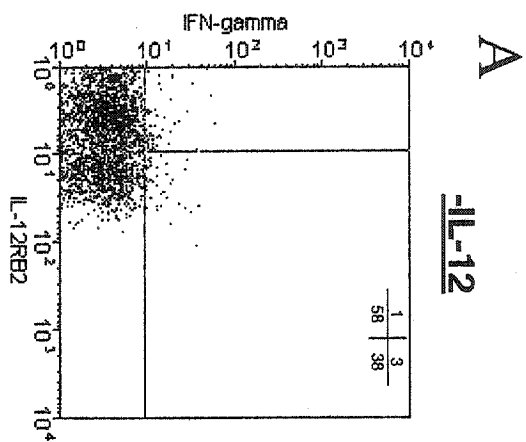


B



C

Fig 2.12. IL-12-induced IFN- γ production by IL-12R β ^{lo} and IL-12R β ^{hi} DX5⁺NK cells. DX5⁺NK cells purified from C57BL/6 splenocytes by positive magnetic selection were stimulated with huIL-2 (1000 U/ml) for 10 days then were incubated overnight in the absence (**A**), or presence (**B**), of IL-12 (1 ng/ml) and analyzed for intracellular IFN- γ by flow cytometry. Cells were surface stained with rat anti-mouse IL-12R β 2 antiserum and FITC-conjugated goat anti-rat Ig then fixed with paraformaldehyde, permeabilized with saponin and stained with PE-conjugated anti-IFN- γ . (**C**), Titration of intracellular IFN- γ production in response to varying amounts of IL-12. Sorted IL-12R β ^{lo} and IL-12R β ^{hi} cells were stimulated overnight in the presence of ten-fold serial dilutions of mouse IL-12 and the data are plotted as the percentage of IFN- γ -producing cells within each subset.



II.5.7. Murine IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK cells exhibit similar proliferative activity in response to IL-12 stimulation

To further investigate IL-12 responsiveness of IL-12R β 2^{lo} and IL-12R β 2^{hi} cell subsets, sorted IL-12R β 2^{lo} and IL-12R β 2^{hi} cells were assayed for the proliferative response to varying concentrations of exogenous IL-12 (Fig. 2.13). Both IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets showed high sensitivity to exogenous IL-12. Indeed, both subsets showed the same incorporation level of [³H]-thymidine in response to varying concentrations of murine IL-12. These results indicated that unlike IL-2, IL-12 stimulation was able to illicit IFN- γ as well as DX5⁺NK cell proliferation. It is worthwhile to report that murine DX5⁺NK cells do not survive in very high concentrations of IL-12 (for example 10 ng/ml) and they die after few days (data not shown). The IL-12-induced LAK cell death mechanism has not been investigated in this project.

II.5.8. Cytokine gene expression demonstrated that IL-12R β 2^{lo} DX5⁺NK cells do not correspond to human NK2 cell population

Recent studies reported that human NK cells were able to differentiate into NK1 or NK2 subsets depending on the cytokines present in the milieu at the time of stimulation (211). To determine whether IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK populations corresponded to these human NK cell subsets, the cytokine gene profile was analyzed by RNase Protection Assay (RPA) (Fig. 2.14). Sorted murine IL-12R β 2^{lo} and IL-12R β 2^{hi} (d18) were stimulated or not with PMA and ionomycin or with IL-12. Neither subset showed detectable amounts of cytokine mRNAs in the absence of stimulation, however, both IL-12R β 2^{lo} and IL-12R β 2^{hi} populations upregulated IFN- γ transcripts in response to PMA and ionomycin. Furthermore, both populations dramatically upregulated IFN- γ mRNA in response to exogenous IL-12. In accordance with previous reports (211), IL-10 mRNA was highly expressed by both IL-12R β 2^{lo} and IL-12R β 2^{hi} cells in response to IL-12 stimulation. Neither subsets produced IL-5 or IL-13 mRNAs either in the presence or in the absence of any stimulation, indicating that despite

Fig. 2.13. Proliferation of IL-12R β ^{lo} and IL-12R β ^{hi} DX5⁺NK cells in response to IL-12. DX5⁺NK cells were purified from C57BL/6 splenocytes by positive magnetic selection and were stimulated with huIL-2 (1000 U/ml) for 10 days. Cells were then stained with rat anti-IL-12R β 2 antiserum/FITC-conjugated goat anti-rat Ig or FITC-conjugated anti-mouse Ly-49G2 as surrogate then cells were sorted using anti-FITC microbeads (Miltenyi). Cells (2x10⁴ cells/well) were cultured in triplicate in a 96-well plate. They were left in huIL-2 only or stimulated 16h in the presence of five-fold serial dilutions of mIL-12. Cells were then pulsed with 1 μ Ci per well of [³H]-thymidine for an additional 6 h. The plotted values are the mean of replicate wells and error bars represent the SEM. IL-12R β ^{lo} and IL-12R β ^{hi} cells showed similar proliferative response to exogenous IL-12.

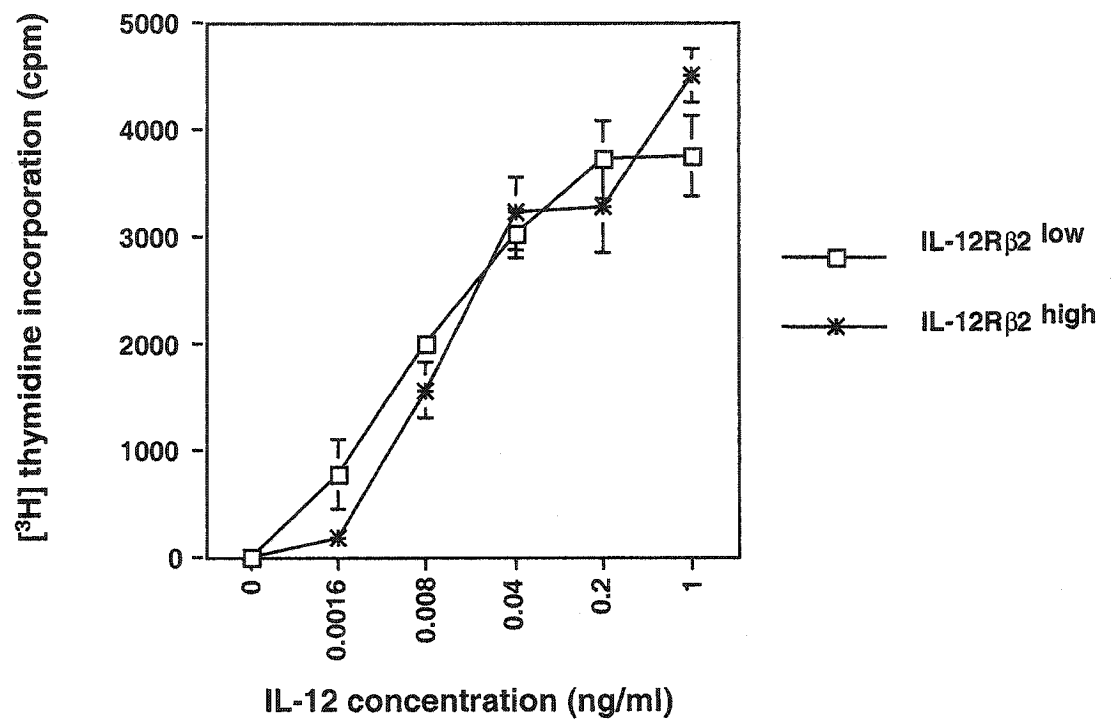
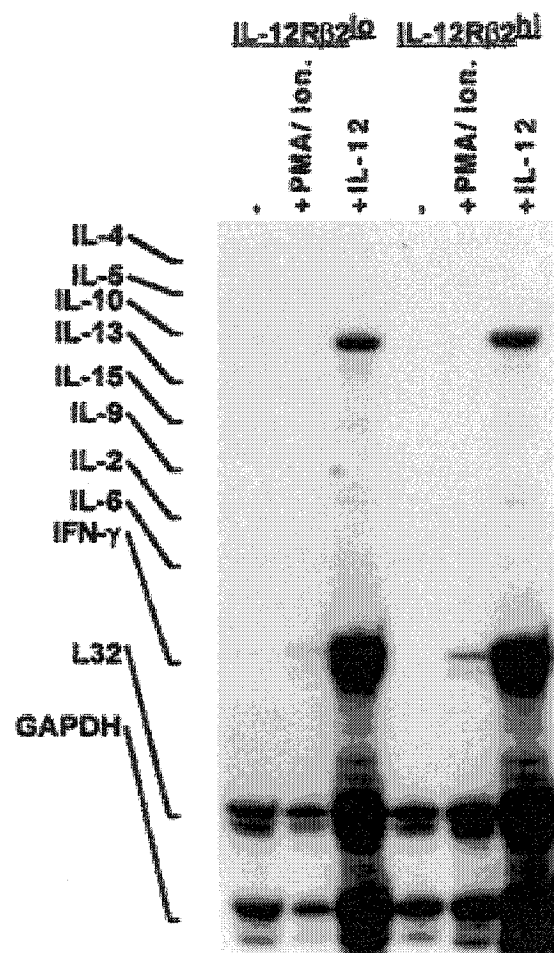


Fig. 2.14. Analysis of cytokine gene expression in IL-12R β ^{lo} and IL-12R β ^{hi} DX5⁺NK populations. DX5⁺ IL-2 blasts purified from C57BL/6 mice (d15) were sorted into IL-12R β ^{lo} and IL-12R β ^{hi} subsets and cells (1x10⁶/well) were cultured overnight in the absence (-) or presence of IL-12 (1 ng/ml). A third aliquot of cells was stimulated with PMA and ionomycin for the final 3h of culture. Total cellular RNA was prepared and cytokine mRNA expression was detected by RPA using the mCK-1 probe set as described in materials and methods. Significant production of IFN- γ and IL-10 was evident in both the IL-12R β ^{lo} and IL-12R β ^{hi} subsets after stimulation with IL-12. Undigested radiolabelled probe was used as markers.



different levels of IL-12R β 2 expression on the surface of IL-12R β 2^{lo} and IL-12R β 2^{hi} cells, these subsets do not correspond to the previously described human NK2 subsets, but have the cytokine profile of human NK1 cells (211). L32 and GAPDH are internal controls.

II.5.9. IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK cells exhibit high cytolytic activity in response to IL-12 stimulation

To determine the biological significance of IL-12R β 2 expression on IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets, sorted IL-12R β 2^{lo} and IL-12R β 2^{hi} cells were used as effector cells in a standard cytotoxicity assay. Both subsets of cells were highly efficient at lysing NK-sensitive YAC-1 targets even at very low effector to target (E:T) ratios (Fig. 2.15). Addition of exogenous murine IL-12 augmented this already high level of cytotoxicity in both populations to reach 80 % cell lysis at the highest E:T ratio. The percentage lysis showed an E:T dose dependent response. We conclude that IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK populations exhibit similar cytolytic activity against YAC-1 target cells in the presence or absence of IL-12 stimulation despite the expression of different levels of IL-12R β 2 chain on the surface of these two cell subsets.

II.5.10. Polarization of murine DX5⁺NK cells towards NK1 and NK2 subsets

Since RPA results showed that IL-12R β 2^{lo} DX5⁺NK cells do not express the NK2 phenotype reported for human NK cells, the capacity of DX5⁺NK cells to respond to T_H1/T_H2 cytokines and to polarize towards NK1 and NK2 subsets was investigated. Enriched DX5⁺NK cells were activated with huIL-2 only (for NK0), IL-2+IL-12+anti-IL-4 mAb (for NK1) and IL-2+IL-4+anti-IL-12 (for NK2) phenotypes. At d6, cell culture supernatants were analyzed for IFN- γ and IL-5 production by ELISA (Fig.2.16). IL-5 was measured as a T_H2-associated cytokine because NK cells were reported to not produce IL-4 (211). In the presence of IL-2 only, DX5⁺NK cells (NK0) showed no IL-5 or IFN- γ production. In accordance with data reported earlier for human NK1 cells (211), IL-2-activated DX5⁺NK cells co-activated with exogenous IL-12 did not show any detectable IL-5 production but secreted high level of IFN- γ . However, similar to human NK2 cells

Fig. 2.15. Effect of IL-12 on the cytolytic activity of IL-12R β 2^{lo} and IL-12R β 2^{hi} NK cells. DX5⁺ IL-2 blasts from C57BL/6 mice (d11) were sorted into IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets and cells were used as effector cells in a standard 4h cytotoxicity assay against YAC-1 target cells. Sorted cells were stimulated overnight in the presence or absence of IL-12 (1 ng/ml) and then washed prior to being incubated with ¹⁵¹Cr-labeled YAC-1 target cells (5x10³) at the indicated effector to target (E:T) ratios. The plotted values are the mean of triplicate wells and error bars represent the SEM. Both DX5⁺NK cell subsets showed high cytolytic activity against YAC-1 cells. The cytolytic activity increased in the presence of IL-12.

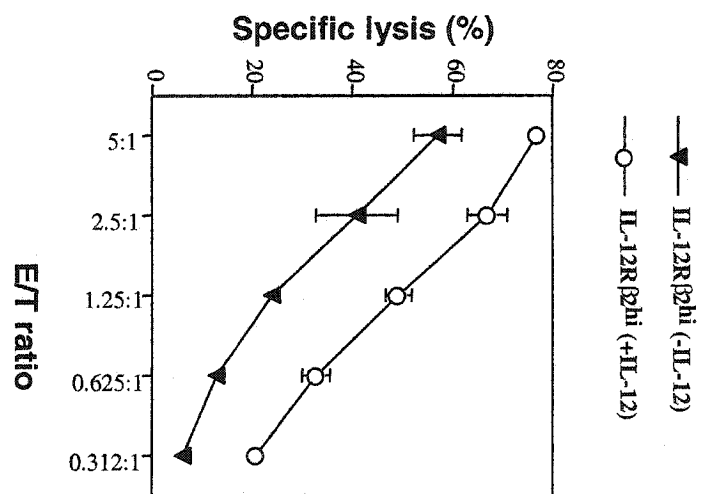
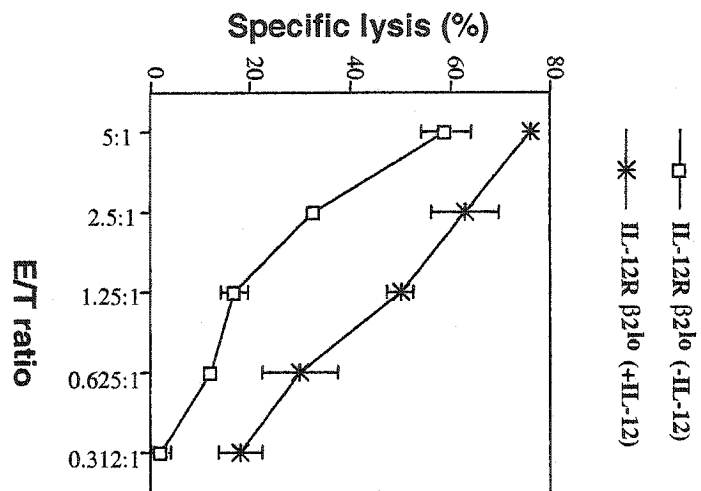
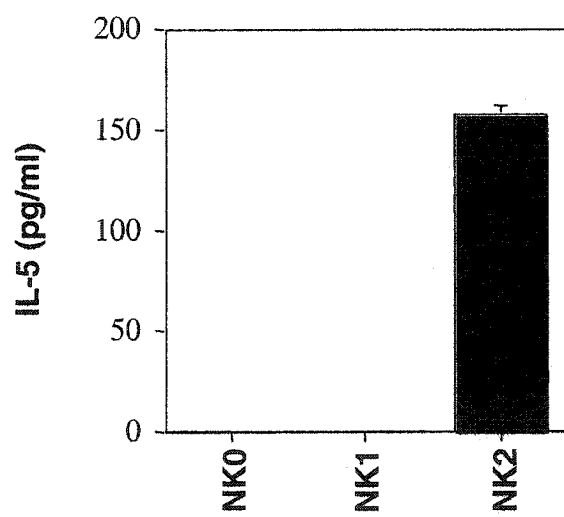
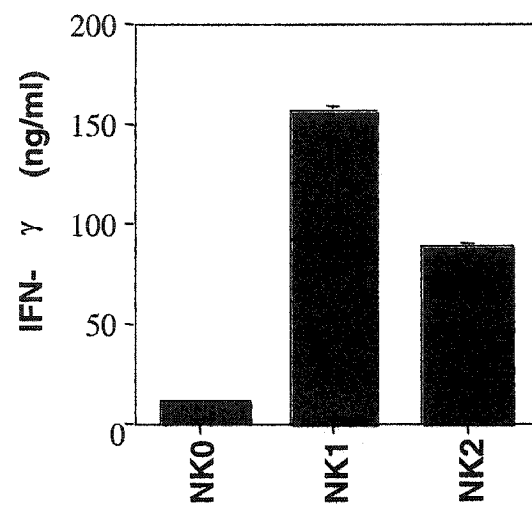


Fig. 2.16. Polarization of DX5⁺NK cells towards NK1 and NK2. DX5⁺NK cells were purified from C57BL/6 splenocytes by positive magnetic selection and cultured in duplicate at 5×10^5 cells/ml in a 12-well plate. Cells were activated with huIL-2 (1000 U/ml) only (NK0), huIL-2, IL-12 (0.1 ng/ml) and anti-IL-4 mAb (10 μ g/ml) (NK1) or huIL-2, mIL-4 (100 U/ml) and anti-IL-12 hybridoma supernatants (NK2). At d6 post-stimulation, cell supernatants were harvested and analyzed for the secretion of IFN- γ (as a T_H1-associated cytokine) and for IL-5 (as a T_H2-associated cytokine) by capture ELISA. The plotted values are the mean of replicate ELISA wells and error bars represent the SEM.



(211), when IL-2 activated DX5⁺NK cells were co-stimulated with IL-4, they produced IL-5 and lower levels of the typical T_H1 cytokine IFN- γ . These data suggest that similar to human NK cells (211), murine DX5⁺NK cells responded to T_H1/T_H2 cytokines and were able to show polarization towards distinct phenotypes when co-activated with different cytokine combinations. The generated phenotypes were termed NK1 and NK2 depending on the cytokines they produced. Again, murine IL-12R β 2^{lo} subset generated in response to IL-2 activation only, did not correspond to murine NK2 cells generated in the presence of IL-2+IL-4. Indeed, in contrast to murine NK2 cells, IL-12R β 2^{lo} DX5⁺NK cell subset showed no IL-5 transcript expression by RPA and no decrease in the level of IFN- γ mRNA in response to exogenous IL-12 when compared to IL-12R β 2^{hi} (see Fig. 2.14).

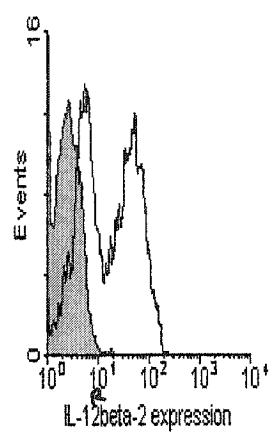
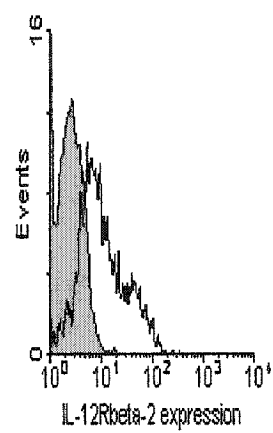
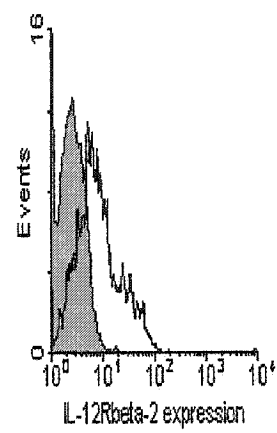
II.5.11. Regulation of IL-12R β 2 chain expression by murine NK1 and NK2 cells

Next, the IL-12R β 2 expression on murine NK1 and NK2 subsets was analyzed. DX5⁺NK cells were activated with huIL-2 only (NK0), IL-2+T_H1 cytokines (NK1) or IL-2+T_H2 cytokines (NK2) for an optimal period of 6 days and were expanded in the presence of huIL-2 only for an additional 4 days to allow the development of both IL-12R β 2^{lo} and IL-12R β 2^{hi} cell populations. At d10, the cells were analyzed for IL-12R β 2 expression by flow cytometry using the rat anti-IL-12R β 2 antiserum (217). Fig. 2.17A indicates that after 10 d expansion in the presence of IL-2, murine NK0 cells showed the typical phenotype expressing IL-12R β 2^{lo} and IL-12R β 2^{hi} cell subsets usually developed by IL-2-activated DX5⁺NK cells. However, DX5⁺NK cells stimulated under T_H2 conditions were able to respond to IL-4. In the presence of IL-4, IL-12R β 2 chain was downregulated on NK2 with a clear reduction of IL-12R β 2^{hi} cells (Fig. 2.17 C). Interestingly, when DX5⁺NK cells were activated under NK1 condition for 6 days, the expression of IL-12R β 2 chain was repressed and the number of IL-12R β 2^{hi} cells was diminished (Fig. 2.17 B).

II.6. Discussion

In this chapter, the expression of the IL-12R β 2 molecule on murine DX5⁺NK cells activated with IL-2 was investigated. In prior studies, IL-2-activated murine NK cells have been extensively used to study the affinity of IL-12/IL-12R interactions, but a

Fig. 2.17. IL-12R β 2 expression by murine NK1 and NK2 cells. DX5⁺NK cells were purified from C57BL/6 splenocytes by positive magnetic selection and cultured at 5X10⁵ cells/ml in a 12-well plate. Cells were activated with huIL-2 (1000 U/ml) only (NK0); huIL-2, mIL-12 (0.1 ng/ml) and anti-IL-4 mAb (10 μ g/ml) (NK1) or huIL-2, mIL-4 (100 U/ml) and anti-IL-12 hybridoma supernatants (NK2). At day 6 post-stimulation, cells were expanded in the presence of huIL-2 for an extra 4d. At d10, NK0 (A), NK1 (B) and NK2 (C) cells were analyzed for the expression of IL-12R β 2 by flow cytometry using the rat anti-IL-12R β 2 antiserum/ FITC-conjugated goat anti-rat Ig (clear curve). Rat pre-immune serum was used as negative control (shaded curve).

A**NK0****B****NK1****C****NK2**

detailed analysis of the regulation of IL-12 receptor expression on these cells has been hampered to date by the lack of an anti-IL-12R β 2 Ab. A rat anti-mouse IL-12R β 2 antiserum was successfully generated, which facilitated the study of the IL-12R β 2 expression and regulation on NK cells. The flow cytometric analysis using the rat anti-IL-12R β 2 antiserum revealed that in accordance with previous studies on human PHA-activated PBMC (105,145), stimulation of murine MNC with ConA for 3d induced a moderate upregulation of IL-12R β 2 subunit. NK cells also are reported to express IL-12R β 2 chain and respond to IL-12 stimulation by producing high levels of IFN- γ (12,208). The results reported herein are consistent with earlier data suggesting that both T and NK cells require activation in order to express appreciable levels of IL-12R (105,218,219). After activation, murine NK cells expressed the IL-12R β 2 at levels significantly higher than those observed on mitogen-activated T cells. However, the presence of two distinct populations of activated murine DX5⁺NK cells with differing levels of IL-12R β 2 is a novel observation. Furthermore, although the IL-2-activation of DX5⁺NK cells resulted in a dramatic expression of IL-12R β 2, no IFN- γ mRNA upregulation was observed in response to IL-2. This suggested that IL-2-activation primed DX5⁺NK cells for IL-12 responsiveness and that other cytokines are required to reach full activation and cytokine secretion. Activation of DX5⁺NK cells with IL-2 may sensitize NK cells to very low concentrations of IL-12 released *in vivo* by macrophages and DC during a preliminary innate immune response.

Specificity of rat anti-IL-12R β 2 antiserum was analyzed by IP and the results demonstrated that a 98 kDa protein was successfully immunoprecipitated from IL-2-activated DX5⁺NK cells and this is in accordance with previous studies showing that the MW of mouse IL-12R β 2 chain ranged from 95.5 to 100 kDa under reducing conditions (102,212,220).

Two human NK cell subsets have been recently described and like their T cell subset counterparts, they polarized towards NK1 and NK2 phenotypes in terms of cytokine production and IL-12R β 2 expression (211). Human NK1 and NK2 cell subsets were generated from bulk PBMC, in the presence of the strongly polarizing cytokines IL-12 or IL-4, respectively, and in the presence of irradiated feeder cells. Differentiation of

murine DX5⁺NK cells into IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets occurred in *in vitro* cultures of IL-2-blasts as well as in highly enriched DX5⁺NK cell populations, providing strong evidence that this process occurred independently of any non-NK cells that might be a source of cytokines able to regulate the expression of IL-12R β 2 chain. Because of this apparent inherent capacity to develop into IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets in the presence of IL-2, it is tempting to speculate that there are specific subsets of resting NK cells that have a predetermined fate in terms of IL-12R β 2 expression. This hypothesis may be related to the recent observation that stimulation of resting human NK cells with IL-12 results in upregulation of IL-12R on only a specific subset of CD56⁺ NK cells (39). Interestingly, this study also reported that the proportion of IL-12R⁺ NK cells could be further upregulated by the addition of IL-4 and that these latter cells were of the CD56⁺CD16⁻ subset (39).

The generation of human NK1 and NK2 cell subsets not only influenced the level of IL-12R β 2 expression but also resulted in gene expression of the T_H1-associated IFN- γ and the T_H2-associated cytokines IL-5 and IL-13 respectively (211). Neither the IL-12R β 2^{lo} nor the IL-12R β 2^{hi} murine NK cells reported herein expressed detectable levels of IL-5 or IL-13 mRNA either before or after stimulation with PMA/ ionomycin or with exogenous IL-12. These findings suggest that despite similarities in IL-12R β 2 surface expression, murine IL-12R β 2^{lo} NK cells do not correspond to the human NK2 subset described above. Indeed, RPA results showed that after stimulation with PMA/ionomycin, IFN- γ mRNA was upregulated in both IL-12R β 2^{lo} and IL-12R β 2^{hi} cell subsets. Similarly, after stimulation with IL-12, IFN- γ as well as IL-10 mRNA expression was dramatically upregulated in both cell populations. IL-12-mediated upregulation of IL-10 production in NK cells has been reported previously (211,221), and although the biological consequences of IL-10 on NK activities are not clear, it may have an autoregulatory effect on IL-12 responsiveness. IL-2 activation of DX5⁺NK cells *in vitro* resulted also in a dramatic upregulation of IL-12R β 2 at the transcriptional level. This result may have implications for LAK cell-based therapies, since these activated NK cells upregulated IL-12R β 2 chain in the presence of IL-2, but required a further stimulus to achieve their full potential in terms of cytokine production.

Flow cytometric double staining demonstrated that in accordance with previous data (147,217,222), murine DX5⁺ cells showed the presence of a low percentage of DX5⁺NK-T cells, usually ranging from 5 to 11% of total spleen MNC, after flow cytometric double staining using anti-DX5 and anti-CD3 mAbs. Also, staining with a number of NK-associated markers, including the NK cell inhibitory molecules indicated that in contrast to Ly-49A and Ly-49C NK markers, the Ly-49G2 molecule was expressed exclusively on IL-12R β 2^{hi} NK cells. The Ly-49 multigene family encodes a complex family of NK cell surface receptors, at least some of which recognize class I MHC molecules and send an inhibitory signal that represses the lytic activity of NK cells (216,223). The lytic activity of the Ly-49G2⁺ subset of NK cells is inhibited by target cells expressing H-2D^d and/or H-2L^d (224), although inhibition by H-2L^d has recently come into question (225). As a consequence of this class I-mediated inhibitory activity, Ly-49G2⁺ LAK cells from C57BL/6 mice are incapable of lysing the LAK-sensitive target P815, which is H-2D^d (224). However, as observed in the present study, both IL-12R β 2^{lo} (Ly-49G2⁻) and IL-12R β 2^{hi} (Ly-49G2⁺) NK cells from C57BL/6 mice are capable of lysing NK-sensitive YAC-1 target cells. The distinct pattern of IL-12R β 2 expression on these Ly-49G2⁻ and Ly-49G2⁺ NK subsets is a novel finding that implies the coordinated regulation of IL-12R β 2 and Ly-49G2 during the activation of these cells. Considering the role of Ly-49G2 in inhibiting the lysis of H-2D^d-expressing targets and the reported ability of IL-12 to augment LAK activity, it would be interesting to determine whether IL-12 plays a role in modulating the class I-mediated inhibition of lytic activity in these cells. Also, it may be interesting to examine whether the coordinated expression of IL-12R β 2 and Ly-49G2 is a universal phenomenon in all strains of mice. Prior analyses found that Ly-49G2 was expressed on NK cells of all strains of mice examined, but that the percentage of Ly-49G2⁺ NK cells varied considerably among different strains (216). If high level IL-12R β 2 expression on NK cells is restricted to the Ly-49G2⁺ subset, then it follows that IL-12R β 2 expression would mirror strain-dependent differences in Ly-49G2 expression. However, it is important to remember that differences in IL-12R β 2 expression were noted only in NK cells that were activated *in vitro* with high doses of IL-2; therefore, the significance of this finding to *in vivo* responses is not yet clear.

The results reported in this study indicate that despite differences in IL-12R β 2 surface expression, stimulation of IL-2-activated DX5⁺NK cells with IL-12 elicited proliferation, strong IFN- γ production and augmented cytotoxicity in both IL-12R β 2^{lo} and IL-12R β 2^{hi} populations. This finding implies that IL-12R β 2^{lo} NK cells are capable of responding to IL-12 equally as well as the IL-12R β 2^{hi} population. Previous results have shown that very low levels of IL-12R on the surface of activated human T cells renders these cells functionally responsive to IL-12 (226). In contrast to the results reported herein on murine DX5⁺NK cells, resting human CD56⁺ NK cells are reported to express IL-12R at levels that are barely detectable by flow cytometry, yet this level of receptor renders these cells responsive to IL-12 as measured by up-regulation of IL-12R (39). Since both IL-12R β 2^{lo} and IL-12R β 2^{hi} murine DX5⁺NK subsets appear to respond equally to IL-12, it is interesting to speculate about the potential role of the high levels of IL-12R β 2 in the IL-12R β 2^{hi} subset. First, it is possible that this high level expression of IL-12R β 2 is artificially driven by the high doses of IL-2 used for the *in vitro* activation of NK cells. In fact, previous studies have indicated that IL-2, at doses much lower than those used herein, has a direct influence on expression of the IL-12R on human NK cells (145). To investigate this possibility it will be interesting to look at various *in vivo* NK activation strategies, such as poly (I:C) stimulation, to determine whether these same subsets can be derived *in vivo*. Also, it will be of great interest to investigate whether stimulation of NK cells with lower concentrations of IL-2 or with IL-15, which shares many of the stimulatory characteristics, receptor molecules, and signaling pathways of IL-2, is capable of promoting the differentiation of these two subsets. Second, it would be interesting to determine the molecular configuration of the IL-12R β 2 in the IL-12R β 2^{hi} subset. For example, is the IL-12R β 2 present at the cell surface of murine DX5⁺NK cells in monomer form or is it complexed as a homodimer or a heterodimer with IL-12R β 1 or with the IL-23 cytokine receptor molecule. We did not have access to a mAb against murine IL-12R β 1, therefore, the level of surface expression of this subunit in the IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets could not be assessed. Regardless, further studies are required to determine the biological significance of IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK subsets and their potential roles during an innate immune response.

To further confirm that the murine IL-12R β 2^{lo} population does not correspond to the NK2 human phenotype reported earlier (211), DX5⁺NK cells were purified from C57BL/6 mice and were stimulated with IL-2 in the presence of polarizing T_H1 or T_H2 cytokines to generate murine NK1 and NK2 cells respectively. Previous studies reported that in human and murine T cells, IL-4 stimulation inhibits IL-12R β 2 expression and disturbs IL-12 signaling when polarizing CD4⁺T cells towards T_H2 phenotype (23,27). When murine DX5⁺NK cells were grown in the presence of IL-4 (under T_H2 conditions), DX5⁺NK cells showed a dramatic downregulation of IL-12R β 2 chain. The IL-12R β 2^{hi} population was more affected by the presence of IL-4. This finding suggested that similar to T cells, murine DX5⁺NK cells express a functional IL-4 receptor and were able to respond to IL-4 stimulation by downregulating IL-12R β 2 chain expression, by reducing IFN- γ production compared to NK1 cells and by increasing secretion of the T_H2-associated cytokine IL-5. However, in contrast to T cells, the co-signal for IL-12R β 2 downregulation on NK cells was IL-2 and not a signal via the TCR. Expression of the IL-12R β 2 chain on CD4⁺T cells has been extensively studied *in vitro* and *in vivo* and the role of IL-4 in the suppression of IL-12R β 2 expression has been established. In accordance with these reports, IL-4 was also able to downregulate IL-12R β 2 chain on murine NK2 cells as shown by flow cytometry. These data support the notion that differentiated IL-12R β 2^{lo} cells (generated in response to IL-2 only) developed a stable phenotype after IL-2 activation that is distinct from NK2 cells since they were unable to express any T_H2-associated cytokines. Interestingly, IL-12 co-activation of murine DX5⁺NK cells also resulted in an inhibition of IL-12R β 2 expression on murine NK1 cells. Indeed, unlike human NK1 that displayed an accumulation of IL-12R β 2 transcripts, murine NK1 cells grown for 6 days in the presence of IL-2+IL-12 showed a downregulation of IL-12R β 2 protein expression in response to long-term exposure to IL-12. This might be due to the presence of a negative feedback control by IL-12 on its receptor. The autocrine downregulation of IL-12R β 2 by its ligand was noticed earlier in human NK and T cells (107,145) and suggested that IL-12 modulated its own receptor. The IL-12 signaling was shown to be downregulated in human NK cells through induction of phospho-STAT4 protein degradation (107).

Analyses of cytokine and cytokine receptor profiles as well as IL-12 responsiveness of these murine NK cell subsets might be of interest to confirm definitely that the IL-12R β 2^{lo} subset generated in response to IL-2 activation corresponds to the NK1 and not the NK2 cell subsets reported in humans. Future studies are also needed to evaluate the physiological significance of these two subsets both *in vitro* and *in vivo* to establish their potential role during an innate and possibly an adaptive immune response.

In conclusion, two distinct subsets of murine NK cells that differ in terms of IL-12R β 2 surface expression after activation with IL-2 were identified. The IL-12R β 2^{lo} DX5⁺NK cell population generated in response to IL-2 activation does not present the characteristics of human or murine NK2 phenotype. In response to IL-12 stimulation, both murine IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets reported herein showed similar biological responses. Indeed, they both had the capacity to produce IFN- γ and they expressed similar proliferative response and similar cytolytic activity against NK-sensitive YAC-1 target cells. However, these two DX5⁺NK cells subsets may have different attributes, at least *in vitro*, specific for each subset. In chapter III, DX5⁺NK cell responsiveness to IL-18 cytokine was analyzed. Indeed, similar to IL-12, IL-18 was previously reported to elicit IFN- γ secretion by T and NK cells and also synergizes with IL-12 to further increase IFN- γ production, NK cytolytic activity and to polarize CD4⁺T cells towards a T_H1 phenotype (143,148,227).

CHAPTER III

Cytokine expression by murine DX5⁺ cells in response to IL-12, IL-18 or the combination of IL-12 and IL-18

III.1. Summary

IL-18 is another pro-inflammatory cytokine secreted by activated macrophages. IL-18 synergizes with IL-12 to induce high levels of IFN- γ resulting in a potent T_H1 cell development (150). Despite its capacity to promote IL-12-induced T_H1 cell development, IL-18 was reported to be the only cytokine capable of concomitantly inducing the expression of T_H1 and T_H2-associated cytokines by T and NK cells (149). Moreover, as IL-12 and IL-18 are produced by the cells of the innate immune system, analysis of the kinetics of the cytokine gene expression in innate immune cells is important to understand how and in which time frame NK cells are able to efficiently respond to IL-12 released by macrophages and DC in response to invading pathogens. Therefore, purified NK cells, activated *in vitro* with IL-2, were used to investigate NK cell responsiveness to IL-12, IL-18 and to the synergistic action of IL-12 and IL-18. Production of IFN- γ by NK cells in response to either IL-12 or IL-18 was dependent upon co-stimulation with either IL-2 or IL-15. Analyzing the kinetics of IL-12 or IL-18 responsiveness, DX5⁺NK splenocytes showed a rapid and sustained accumulation of IFN- γ transcripts. IL-18 also induced the expression of T_H2-associated IL-13 and IL-6 transcripts, whereas IL-12 induced the expression of IL-10 mRNA. Incubation of DX5⁺ splenocytes with the combination of IL-2+IL-12+IL-18 resulted in the upregulation of IFN- γ and IL-13 transcripts accompanied with a downregulation of IL-10 expression. Moreover, two distinct populations of cells producing differing levels of IFN- γ were observed by intracellular staining after stimulation with IL-2+IL-12+IL-18. Furthermore, the data demonstrated that in contrast to IL-12R β 2, IL-18R α and IL-18R β were constitutively expressed in resting murine DX5⁺NK cells. Moreover, murine NK cells responded to IL-18 independently of IL-12 signaling as cells from both wild type and IL-12R β 2KO mice produced IFN- γ and IL-13 in response to IL-2+IL-18. Finally, IL-12 or IL-18-induced IFN- γ production by DX5⁺CD3⁻ (NK cells) and DX5⁺CD3⁺ (NK-T cells) was investigated and the results indicated that in response to IL-2+IL-12 or IL-2+IL-18 NK-T cells produce high levels of IFN- γ in the absence of TCR signaling.

III.2. Hypothesis

IL-12 and/or IL-18 induce a very rapid production of IFN- γ by the innate immune cells and responsiveness of NK cells to IL-18 is dependent on IL-12R β 2 expression and consequently on IL-12 signaling.

III.3. Objectives

- To analyze the kinetics of cytokine gene expression of activated DX5⁺NK cells in response to stimulation with IL-12 and/or IL-18.
- To investigate the cytokine profile induced by the synergistic combination of IL-12+IL-18 in DX5⁺NK cells.
- To study the requirement for IL-12 signaling in the response of IL-2-activated DX5⁺NK cells to IL-18 stimulation by using mice that are deficient in the IL-12R β 2 subunit.

III.4. Materials and Methods

III.4.1. RPA analysis of cytokine gene expression in murine DX5⁺NK cells

DX5⁺NK cells were isolated from spleens of normal C57BL/6 mice by positive magnetic selection and were grown in RP-10 medium in the presence of 1000 U/ml huIL-2. At d 20, cells were plated at 5×10^6 cells/well in a 6 well-plate, left in huIL-2 (1000 U/ml) only or stimulated for 72h with huIL-2+IL-12 (1 ng/ml), huIL-2+IL-18 (100 ng/ml) or the combination of IL-2+IL-12+IL-18. Cells were harvested for RNA preparation at the indicated time points and total RNA was analyzed for cytokine gene expression using RiboQuant Multi-Probe RNase Protection Assay kit (Pharmingen) according to the protocol reported above (refer to paragraph II.4.10).

III.4.2. Analysis of IFN- γ by intracellular flow cytometric staining

IL-12 and IL-18 responsiveness was also monitored by analyzing the intracellular IFN- γ production by flow cytometry. DX5⁺NK cells, growing in RP-10 containing 1000 U/ml huIL-2 for 20 d, were seeded at 1×10^6 cells/well in a 24-well plate and stimulated

overnight with IL-12 (1 ng/ml) and/or IL-18 (100 ng/ml). The next day, IFN- γ was investigated using intracellular IFN- γ staining as explained above (paragraph II.4.7).

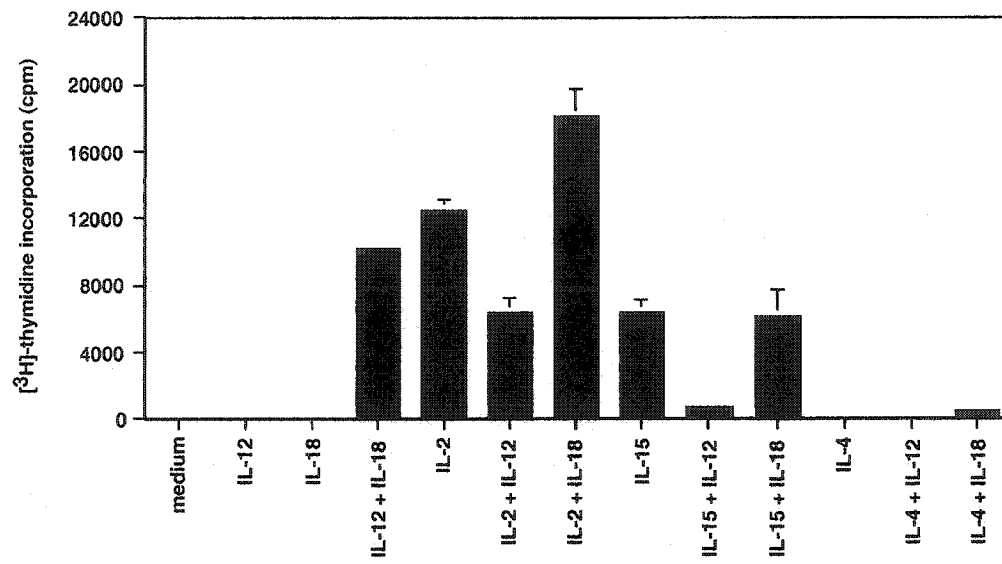
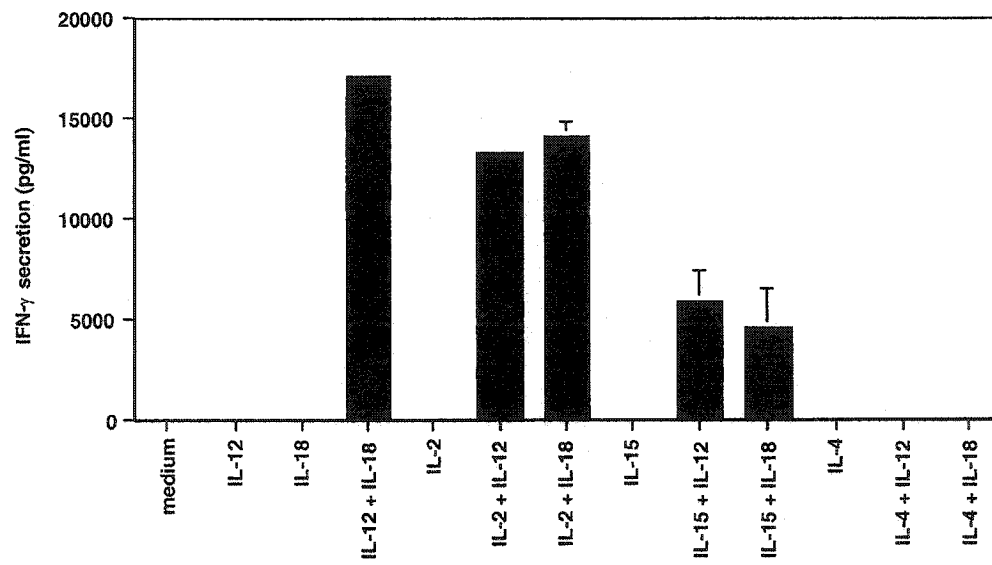
To analyze whether IL-18 responsiveness of DX5⁺NK cells was dependent upon IL-12 signaling, DX5⁺NK cells were also purified from mice deficient in the IL-12R β 2 subunit of IL-12 receptor (IL-12R β 2KO mice) (118) and were analyzed for cytokine gene expression as well as intracellular IFN- γ production following the same conditions used for normal C57BL/6 mice.

III-5- Results

III.5.1. IL-12/IL-18-induced IFN- γ expression by murine DX5⁺NK cells is IL-2/IL-15-dependent

To characterize the responses of murine DX5⁺ cells to IL-12 and IL-18 in the absence of cytokines derived from other cellular sources, DX5⁺ cells were purified from total splenocytes by positive magnetic selection using the NK marker DX5. DX5⁺ splenocytes were incubated overnight in RP-10 medium in the presence of varying combinations of cytokines and were characterized in terms of proliferation (Fig. 3.1 A) and IFN- γ secretion (Fig. 3.1 B) as reported in chapter II. IL-2 elicited vigorous proliferation of freshly purified DX5⁺ splenocytes however, cells grown in IL-2 alone did not secrete detectable amounts of IFN- γ . Incubation of DX5⁺ cells with the combination of IL-2+IL-12 or IL-2+IL-18 resulted in vigorous proliferation plus high levels of IFN- γ secretion demonstrating that concurrent activation with IL-2 was required to render the cells responsive to both IL-12 and IL-18. Addition of IL-2+IL-12 resulted in a slightly lower level of proliferation than was observed with IL-2 only whereas addition of IL-2+IL-18 resulted in an augmentation of IL-2-induced proliferation. Together, these results suggest that, IL-12 (used at 1 ng/ml) is effective in augmenting cytokine production from IL-2-activated DX5⁺ cells but not proliferation whereas IL-18 is capable of augmenting both cytokine production and proliferation. More importantly, neither IL-12 nor IL-18 could elicit IFN- γ production from 'resting' NK cells when administered alone. Interestingly, stimulation of DX5⁺ NK cells with a combination of IL-12+IL-18, in the absence of any other co-stimulus, results in strong proliferation and secretion

Fig. 3.1. Proliferation and IFN- γ production by DX5⁺NK cells (A) Proliferative response of freshly isolated DX5⁺ splenocytes separated by positive magnetic selection and incubated in the presence of the indicated cytokines or cytokine combinations. Cytokine concentrations used were: huIL-2 (1000 U/ml), IL-12 (1 ng/ml), IL-18 (100 ng/ml), IL-15 (100 ng/ml) and IL-4 (100 ng/ml). Cells were plated for 24h in the presence of the indicated cytokines then were pulsed for the last 6 h with tritiated thymidine. (B) IFN- γ secretion from cells shown in A. Supernatants were recovered from the wells just prior to addition of tritiated thymidine and IFN- γ secretion was measured by capture ELISA.

A**B**

of large amounts of IFN- γ .

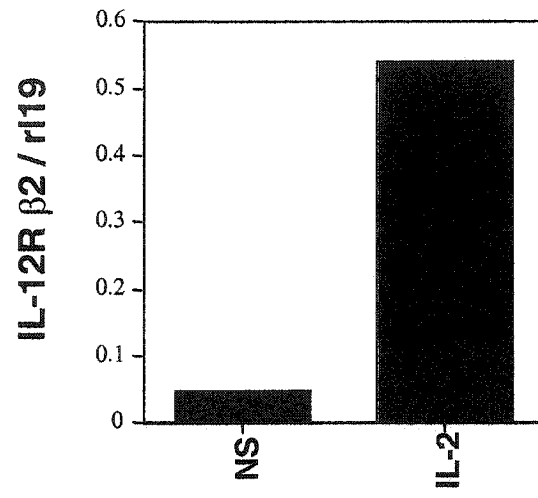
NK cells respond to IL-2 via the 'intermediate affinity' IL-2 receptor comprised of the β chain (CD122) and the common γ chain (γ) (CD132) (228). IL-15R is a trimeric complex that shares the β and γ chain of IL-2R as well as an IL-15-specific α chain (229,230). IL-15 appears to be required for the development of NK cells and has been identified as a 'survival factor' for NK cells (136). Thus, the ability of IL-15 to substitute for IL-2 in rendering NK cells responsive to IL-12 or IL-18 was tested (Fig. 3.1 A). As was observed with IL-2, DX5⁺NK cells proliferated in the presence of IL-15, albeit at a lower rate than was observed in the presence of IL-2. Despite this proliferation, cells grown in IL-15 alone did not secrete detectable amounts of IFN- γ (Fig 3.1 B), similar to what was observed with IL-2. However, DX5⁺NK cells incubated in the combination of IL-15+IL-12 or IL-15+IL-18 exhibited proliferation plus IFN- γ secretion, demonstrating that IL-2 and IL-15 share an ability to render NK cells responsive to both IL-12 and IL-18. It is also noteworthy that at the concentrations of IL-12 used (1 ng/ml), proliferation induced by either IL-2 or IL-15 was reduced after addition of IL-12 for 24h, suggesting that high concentrations of IL-12 have an inhibitory effect on proliferation but not cytokine production. NK cells are also responsive to IL-4 (211), which signals through a receptor containing the common γ chain. To determine whether signaling through the common γ chain was sufficient to render NK cells responsive to IL-12 or IL-18, DX5⁺ cells were incubated with IL-4 or IL-4+IL-12/IL-18. The data showed that DX5⁺NK cells were completely unresponsive to IL-4 or combinations thereof as measured by proliferation or IFN- γ secretion, suggesting that IL-2 and IL-15 are unique in their capacity to stimulate IL-12/IL-18 responsiveness in NK cells (Fig 3.1 A and B).

III.5.2. Effect of IL-15 on IL-12R β 2 expression in murine DX5⁺NK cells

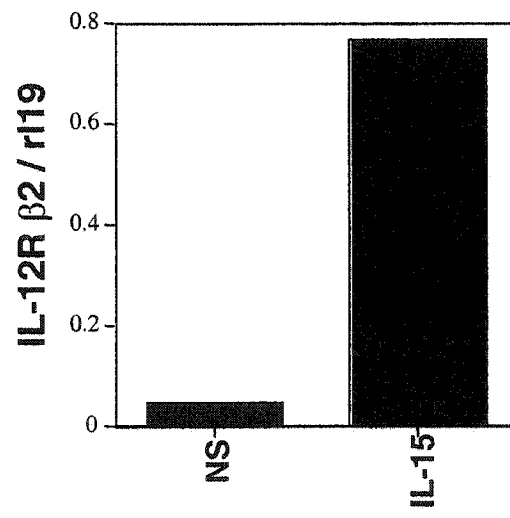
It has been reported in the previous chapter that IL-12R β 2 chain was expressed at very low levels in resting murine DX5⁺NK cells and that this expression was dramatically enhanced at the mRNA and the protein levels by IL-2 activation. However, although they express low levels of the IL-12R β 2 chain, DX5⁺NK cells were not

Fig. 3.2. IL-12R β 2 expression in response to IL-2 and IL-15 DX5⁺NK cells were purified from C57BL/6 splenocytes by positive magnetic selection and were used immediately as non-stimulated cells (NS) or activated in the presence of huIL-2 (1000 U/ml) (A) or murine IL-15 (100 ng/ml) (B). At d6, cells were harvested for RNA preparation and IL-12R β 2 gene expression was analyzed by real-time PCR. Relative copy numbers of IL-12R β 2 and control transcripts were calculated from a standard curve generated in each assay using PCR standards. The copy numbers of IL-12R β 2 transcripts were normalized to r119 levels by dividing the copy number obtained from standard curves to that obtained for r119.

A



B



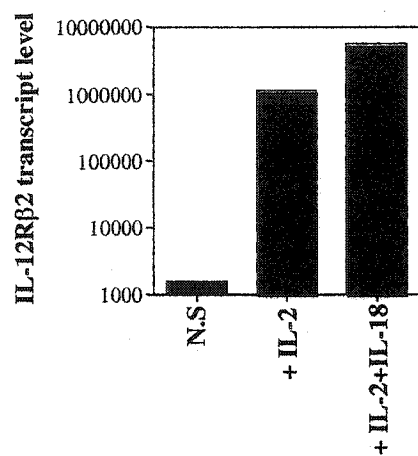
responsive to IL-12 stimulation alone and required a co-activation with IL-2 or IL-15 (Fig. 3.1). Since stimulation with IL-15 resulted in acquisition of IL-12 responsiveness by DX5⁺NK cells, the expression of IL-12R β 2 by murine DX5⁺NK cells in the presence of this cytokine was analyzed. Therefore, DX5⁺NK cells were cultured in the presence of IL-2 or IL-15 for 6 days. Total RNA was analyzed for IL-12R β 2 expression by real-time PCR and compared to freshly isolated DX5⁺NK cells (Fig.3.2 A and B). Similar to IL-2, treatment of DX5⁺NK cells with IL-15 resulted in a strong upregulation of IL-12R β 2 transcripts. The results were normalized to the housekeeping gene r19.

III.5.3. Expression of IL-18R transcripts by murine DX5⁺NK cells

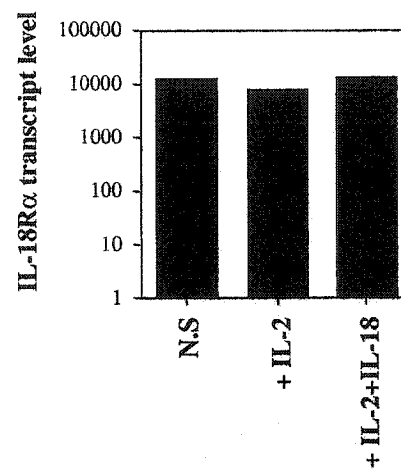
IL-12 and IL-18 cytokines act on T and NK cells via cytokine binding to their appropriate receptors. Previous studies performed on human and mouse T and NK cells were often contradictory in terms of the mechanism of action of IL-12/IL-18 and on the involvement of reciprocal induction of their receptors (134,147,160,189,194,195,198,199,231). Indeed, IL-18 has been shown to induce IFN- γ production by mouse T cells via the induction of IL-12R β 2 expression (193,194); whereas, IL-12 has been reported to be required for the induction of IL-18R (160,187,188). In a collaborative study with the laboratory of Dr. Ruth Tal-Singer at GalxoSmithKline (Collegeville, PA, USA), the expression of IL-12 and IL-18 receptor transcripts in murine DX5⁺NK cells was examined by real-time PCR. Total RNA from freshly explanted DX5⁺NK cells from C57BL/6 mice (resting cells) or after stimulation with IL-2 or IL-2+IL-18 for 6d (Fig. 3.3) was analyzed for IL-12R β 2 (A), IL-18R α (B) and IL-18R β (C) transcript levels. As reported earlier in Chapter II, resting DX5⁺NK cells express relatively low levels of IL-12R β 2 transcripts; however, DX5⁺NK cells activated with IL-2 only showed a dramatic accumulation of IL-12R β 2 mRNA confirming that IL-2 activation primed DX5⁺NK cells to respond to exogenous IL-12. IL-12R β 2 mRNA levels were further upregulated when DX5⁺NK cells were stimulated with IL-2+IL-18 providing evidence that IL-12R β 2 chain is modulated by the presence of IL-18 in the environment (Fig. 3.3 A). Interestingly, resting DX5⁺NK cells expressed IL-18R α and β transcripts (Fig. 3.3 B and C) and the mRNA levels of these IL-18R

Fig. 3.3. IL-12 and IL-18 receptor expression by DX5⁺NK cells. DX5⁺NK cells were purified from C57BL/6 splenocytes by positive magnetic selection and activated in the presence of huIL-2 (1000 U/ml). At d6, cells were left untreated (IL-2 only) or stimulated overnight with IL-2+IL-18 (100 ng/ml). IL-12R β 2, IL-18R α and IL-18R β gene expression analysis was carried out by real-time PCR and the data were compared to non-stimulated DX5⁺NK cells (N.S). Transcript levels of the ribosomal protein L19 (rl19) were used as internal gene controls. Relative copy numbers of IL-12R β 2, IL-18R α and IL-18R β transcripts were calculated from a standard curve generated in each assay using PCR standards and were normalized to the copy numbers of rl19 control transcripts.

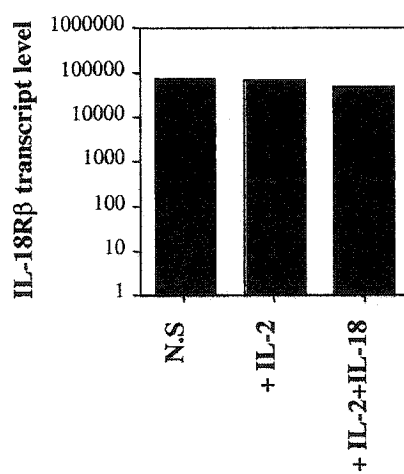
A: IL-12R β 2 mRNA expression



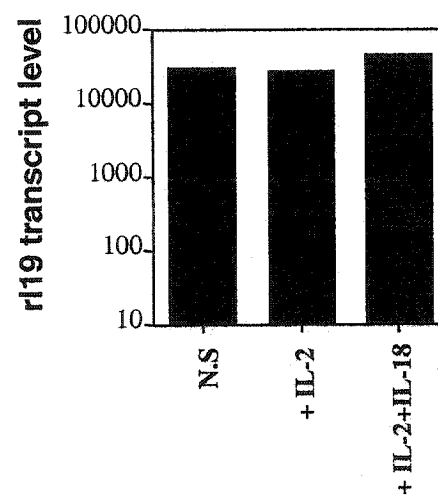
B: IL-18R- α mRNA expression



C: IL-18R β mRNA expression



D: rl19 mRNA expression



subunits did not change after activation with IL-2 or IL-2+IL-18. This suggested that IL-18R transcripts were constitutively expressed in murine DX5⁺NK cells and these levels were not affected by cell activation in the presence of exogenous cytokines. However, despite constitutive expression of IL-18R, resting DX5⁺NK cells were unable to respond to IL-18 only (Fig.3.1, A and B) suggesting that a second signal from IL-2 or IL-15 was required to render DX5⁺NK cells responsive to IL-18 stimulation. r19 transcript levels were used as internal controls to show that the differences reported in the cytokine receptor mRNA were solely due to the effect of the exogenous cytokines added to the culture (Fig. 3.3 D). It would be interesting to analyze IL-18 receptor transcript levels in DX5⁺NK cells stimulated with IL-2+IL-12 to investigate the impact of IL-12 on IL-18R expression.

III.5.4. Kinetic analysis of cytokine gene expression by DX5⁺NK cells in response to IL-12 stimulation

DX5⁺ splenocytes were stimulated with IL-2+IL-12 and harvested at different time points (T=0 to T=72h) to examine the kinetics of cytokine gene expression by RNase Protection Assay (RPA) (Fig. 3.4). DX5⁺NK cells activated with IL-2 only (T=0) did not express detectable amounts of any cytokine mRNA with the exception of a very low level of IFN- γ transcripts. In the presence of IL-2+IL-12, IFN- γ mRNA expression was dramatically up-regulated even at the earliest time point (T=6h). IFN- γ mRNA expression was maintained throughout the period of stimulation with a slight decrease at T=72h. IL-12 also induced IL-10 transcript expression beginning at T=6h and reaching a maximum at T=24h with a gradual decrease at T=72h. IL-15 mRNA also appeared to be up-regulated in response to IL-2+IL-12 but to a much lower extent, with a maximum expression at T=24h.

III.5.5. Kinetic analysis of cytokine gene expression of DX5⁺ NK cells in response to IL-18 stimulation

In the following experiments, the kinetics of cytokine gene expression of IL-2-activated DX5⁺NK cells in response to IL-18 was investigated. After 20d of expansion in

Fig. 3.4. Kinetic analysis of cytokine gene expression by purified DX5⁺ NK cells in response to IL-2+IL-12. DX5⁺ NK cells were isolated from C57BL/6 mice splenocytes by positive selection and were expanded in huIL-2 (1000 U/ml). At d 20, cells were stimulated with 1 ng/ml IL-12 and were harvested at the indicated time points. Total RNA was prepared and cytokine transcript expression was analyzed by RNase Protection Assay (RPA) using the mCK1 mouse cytokine multi-probe template set (Pharmingen).

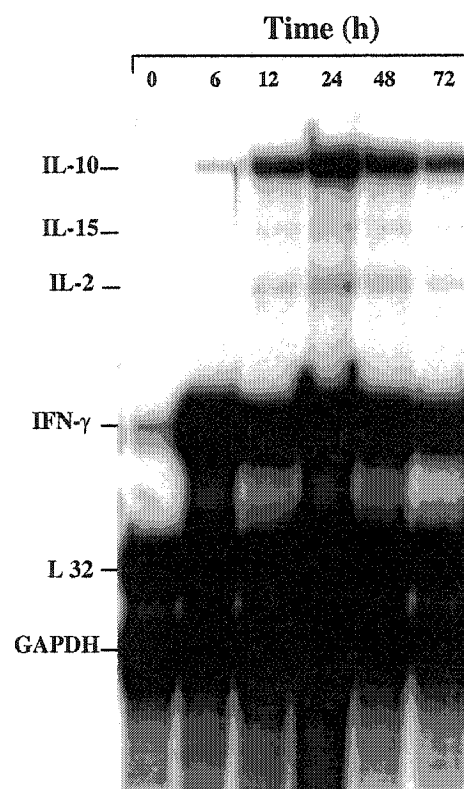
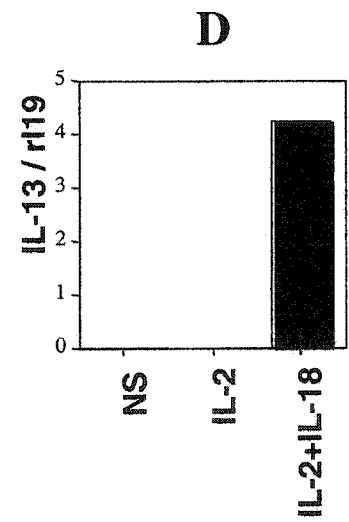
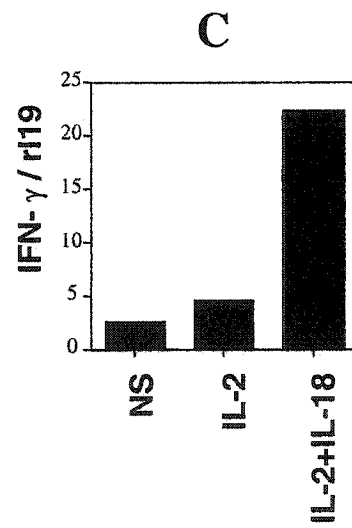
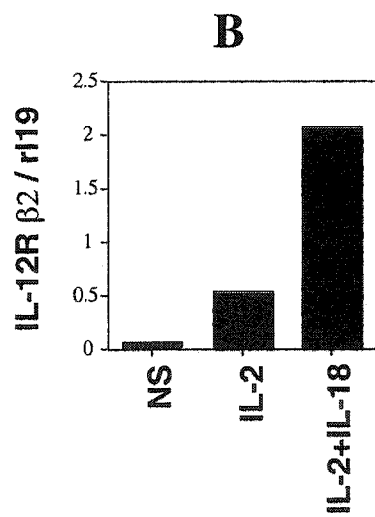
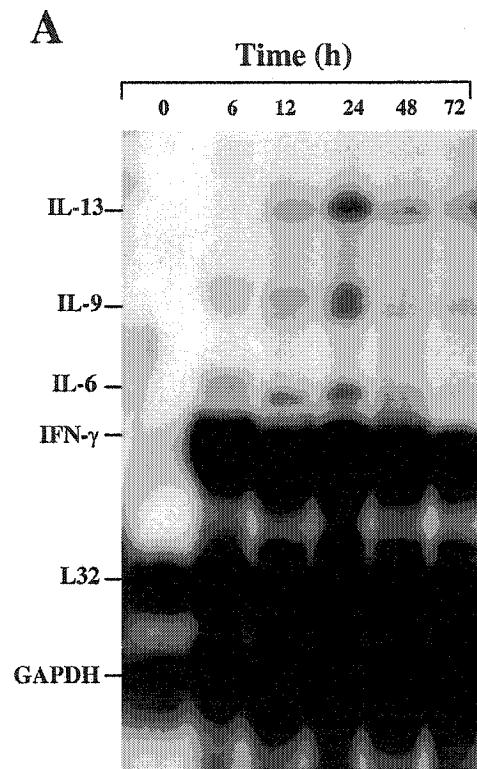


Fig. 3.5. Kinetic analysis of cytokine gene expression by purified DX5⁺NK cells in response to IL-2+IL-18. **A**, DX5⁺NK cells were isolated from C57BL/6 mice splenocytes by positive selection and were expanded for 20d in huIL-2 (1000 U/ml). Cells were then stimulated with IL-2+IL-18 (100 ng/ml) and were harvested at the indicated time points. Total RNA was prepared and cytokine transcript expression was analyzed by RNase Protection Assay (RPA) using the mCK1 mouse cytokine multi-probe template set (Pharmingen). **B-D**, DX5⁺NK cells were isolated from C57BL/6 mice splenocytes by positive magnetic selection and stimulated 6d with huIL-2 (1000 U/ml). At d6, cells were incubated overnight in the presence of IL-2 or IL-2+IL-18. Cells were compared to non-stimulated DX5⁺NK cells (N.S). Total RNA was analyzed for IL-12R β 2 (**B**), IFN- γ (**C**) or IL-13 (**D**) gene expression by real-time PCR. Relative copy numbers of different transcripts analyzed were calculated from a standard curve generated in each assay using PCR standards. The copy numbers of different transcripts were normalized to r119 levels by dividing the copy number obtained from standard curves to that obtained for r119.

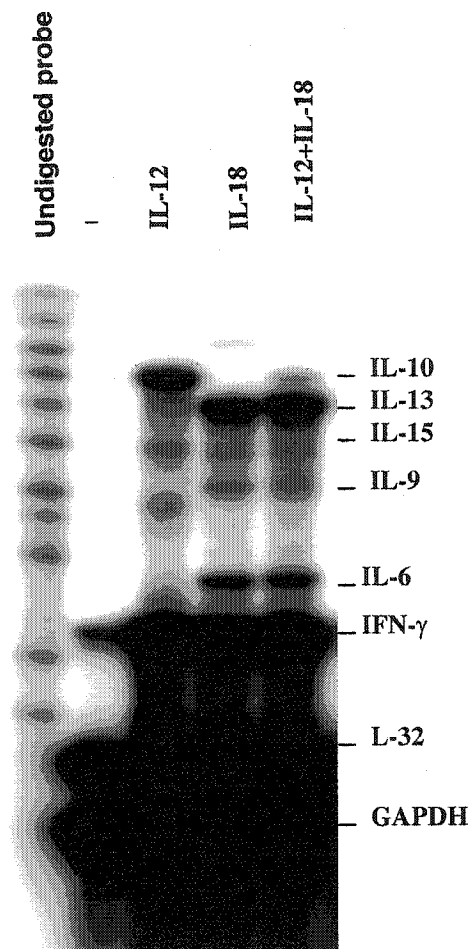


IL-2, DX5⁺NK cells were stimulated with IL-2+IL-18. Cells were harvested at the indicated time points and total RNA was analyzed by RPA for cytokine transcript profile. As observed in Fig. 3.5 A, DX5⁺NK cells activated with IL-2 only (T=0) exhibited very low levels of IFN- γ gene expression. Upon addition of IL-18, IFN- γ gene expression was significantly upregulated by T=6h. The IFN- γ mRNA expression was maintained during IL-18 stimulation with a very minor decrease at T=72h. Similar to IFN- γ , IL-13 transcripts were also induced in response to IL-18. DX5⁺NK cells showed an up-regulation of IL-13 mRNA starting at T=12h with a maximum induction at T=24h. IL-9 and IL-6 mRNAs were also expressed but at lower levels compared to IFN- γ transcripts. Like IFN- γ , IL-6 mRNA showed an early up regulation at T=6h. IL-6 transcript expression was maintained over-time and exhibited a slight decrease at T=72h. The IL-18-induced expression of IL-12R β 2, IFN- γ and IL-13 transcripts was also analyzed by real-time PCR and the data are reported in Fig. 3.5 B-D. These results confirmed that resting DX5⁺NK cells express a very small amount of IFN- γ transcripts (Fig. 3.5 C). Activation in the presence of IL-2 induced a slight upregulation of IFN- γ mRNA. However, addition of IL-18 to the cell culture resulted in a strong expression of IFN- γ transcripts by these cells. In contrast to IFN- γ , no IL-13 mRNA was detected in resting and IL-2-activated DX5⁺NK cells suggesting that the expression of IL-13 was a direct effect of IL-18 on DX5⁺NK cells (Fig. 3.5 D). Again, as shown in Fig. 3.3, IL-2+IL-18 also induced a strong upregulation in IL-12R β 2 gene expression in murine DX5⁺NK cells.

III.5.6. The combination of IL-2+IL-12+IL-18 induces T_H1 and T_H2 cytokine transcript expression

The observation that IL-2-activated murine DX5⁺NK cells produced two different patterns of cytokine transcripts in response to either IL-12 or IL-18 prompted us to examine the profile of cytokine mRNAs induced in response to the combination of IL-12 and IL-18. Therefore, IL-2-activated DX5⁺NK cells (d20) were stimulated in the presence of IL-2+IL-12, IL-2+IL-18 or IL-2+IL-12+IL-18. At T=24h, total RNA was prepared and

Fig. 3.6. Analysis of cytokine gene expression by DX5⁺NK cells in response to IL-2+IL-12+IL-18. DX5⁺NK cells were isolated from C57BL/6 mice splenocytes by positive selection. They were expanded for 20 d in huIL-2 (1000 U/ml) then were kept in IL-2 only (-) or co-stimulated with 1 ng/ml IL-12 and/or 100 ng/ml IL-18 for 24h. Total RNA was analyzed for cytokine transcript expression by RNase Protection Assay (RPA) using the mCK1 mouse cytokine multi-probe template set (Pharmingen). Undigested multi-probe was used as markers.

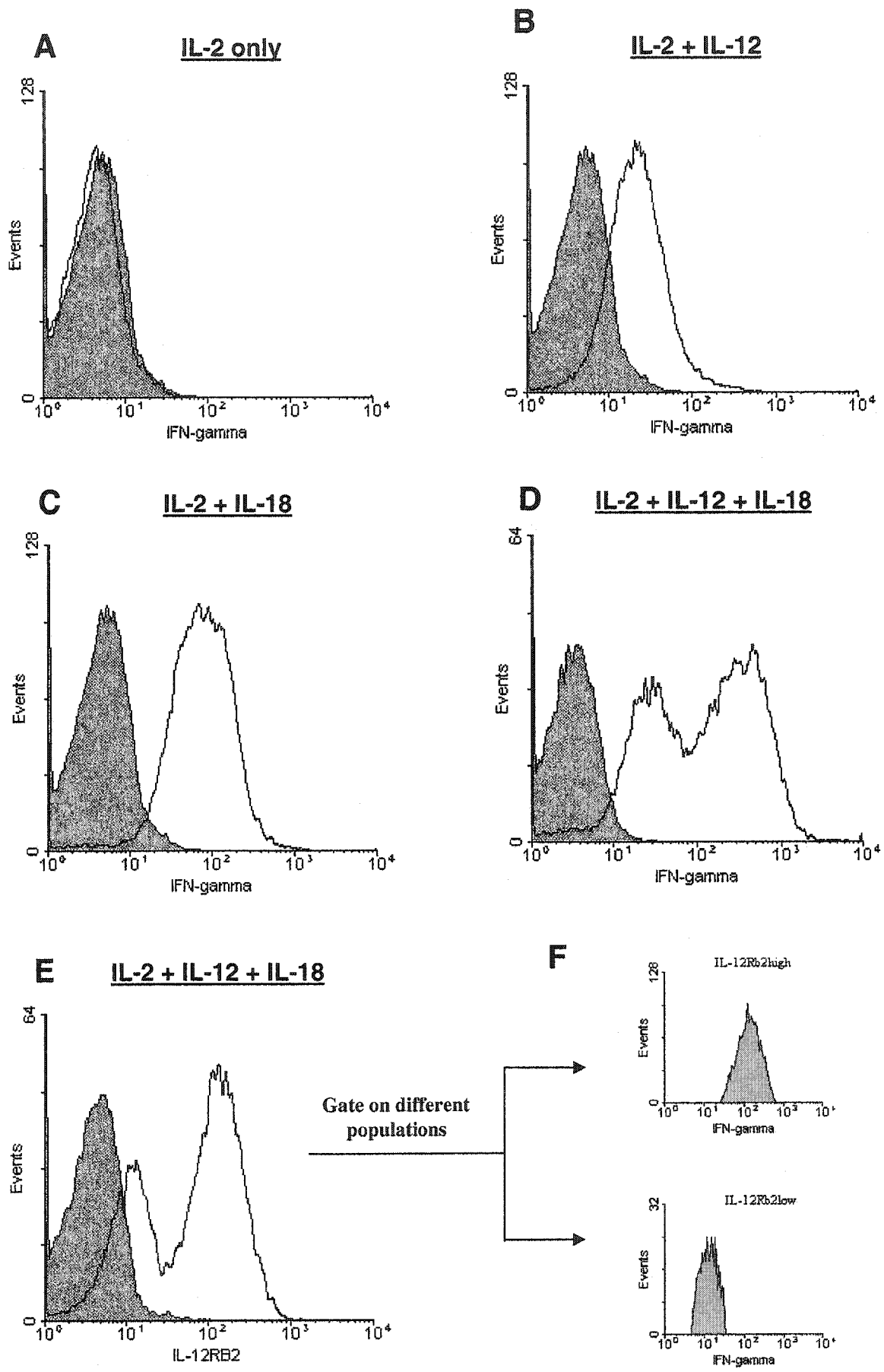


analyzed by RPA for cytokine gene expression (Fig. 3.6). As previously observed, IL-2+IL-12 stimulation induced an upregulation of IFN- γ , IL-10 and IL-15 transcripts, whereas, IL-2+IL-18 stimulation resulted in an increased expression of IFN- γ , IL-13, IL-9 and IL-6 mRNAs. The combination of IL-2+IL-12+IL-18 also increased the IFN- γ mRNA levels and IL-13, IL-6, IL-9 and IL-15 transcripts were also co-expressed. However, IL-12-induced IL-10 mRNA was dramatically downregulated by the concurrent addition of IL-12+IL-18.

III.5.7. Murine DX5⁺NK blasts stimulated with the combination of IL-2+IL-12+IL-18 developed into two distinct NK populations producing different levels of IFN- γ protein

Next, IFN- γ protein expression was analyzed using IFN- γ -intracellular staining of DX5⁺ NK cells stimulated with IL-2+IL-12, IL-2+IL-18 or IL-2+IL-12+IL-18. IL-2-activated DX5⁺ splenocytes (d20) were incubated overnight in the absence or presence of the cytokines mentioned above, and intracellular IFN- γ was analyzed by flow cytometry (Fig. 3.7). Cells activated with IL-2 only showed no IFN- γ protein production (Fig. 3.7 A), whereas cells incubated with IL-2+IL-12 or IL-2+IL-18 showed a significant upregulation of IFN- γ protein (Fig. 3.7 B and C). However, the combination of IL-2+IL-12+IL-18 induced the formation of two distinct populations of DX5⁺NK cells based upon intracellular IFN- γ staining (Fig. 3.7 D). The results demonstrated earlier that murine NK cells differentiate into IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets when stimulated *in vitro* with IL-2 ((217) and chapter II). Therefore, the surface expression of IL-12R β 2 was analyzed on these two IFN- γ producing DX5⁺NK cell populations stimulated with the combination of IL-2+IL-12+IL-18 using the rat anti-IL-12R β 2 antiserum. As previously observed, DX5⁺NK cells differentiated into IL-12R β 2^{lo} and IL-12R β 2^{hi} NK subsets after 20 days culture in the presence of IL-2 (Fig. 3.7 E). Double staining for IL-12R β 2 surface expression and intracellular IFN- γ protein production showed that the two populations of IFN- γ producing DX5⁺NK cells developed in response to IL-2+IL-12+IL-18 stimulation corresponded to IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK subsets (Fig. 3.7 F).

Fig. 3.7. Murine DX5⁺NK cells stimulated with the combination of IL-2+IL-12+IL-18 form two distinct cell populations producing different levels of IFN- γ . DX5⁺NK cells were isolated from C57BL/6 mice splenocytes by positive selection. Cells were expanded for 20 d in huIL-2 (1000 U/ml) then were left untreated (IL-2 only) (A) or stimulated overnight with IL-2+IL-12 (1 ng/ml) (B), IL-2+IL-18 (100 ng/ml) (C), or IL-2+IL-12+IL-18 (D-F). The intracellular IFN- γ protein expression was analyzed by flow cytometry. Antibodies used for intracellular staining were PE-conjugated anti-mouse IFN- γ mAb (clear curve) or PE-conjugated isotype control antibodies (shaded curve). DX5⁺NK cells stimulated with IL-2+IL-12+IL-18 were also stained for IL-12R β 2 surface expression (E), using the rat anti-IL-12R β 2 antiserum and FITC-conjugated anti-rat Ig. Two parameters analysis of cells stained for IL-12R β 2 surface expression and intracellular IFN- γ revealed that the two different IFN- γ -producing DX5⁺ populations (F), emerging in response to stimulation with IL-2+IL-12+IL-18, correspond to IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets.



III.5.8. Murine DX5⁺NK cells responded to IL-18 independently of IL-12

To investigate whether IL-18 can act independently of endogenous IL-12, the kinetics of cytokine gene expression of DX5⁺NK cells isolated from C57BL/6 lacking the IL-12R β 2 gene (IL-12R β 2KO mice) were analyzed. IL-2-activated DX5⁺NK cells (d20) from these mice were stimulated for 72h with IL-2+IL-18. Cells were harvested at the indicated time points and total RNA was analyzed for cytokine gene expression using RPA (Fig. 3.8). Similar to DX5⁺ splenocytes isolated from wild type C57BL/6 mice, IL-12R β 2KO-DX5⁺NK cells showed a significant and rapid up-regulation of IFN- γ mRNA in response to IL-2+IL-18. IFN- γ transcript induction was maintained for 48h and decreased slightly at T=72h. IL-13 transcripts were also expressed starting at T=12h with maximal induction at T=24h. IL-13 mRNA decreased at 48h and showed no expression at 72h. IL-6 transcripts were also rapidly induced (T=6h), but in contrast to wild type mice, IL-6 mRNA was not detected at 24h post-stimulation. These results give clear evidence that, at the transcriptional level, IL-18 stimulation up-regulates IFN- γ gene expression in murine DX5⁺NK cells independently of IL-12 signaling. For further confirmation, IL-18-induced IFN- γ and IL-13 gene expression was analyzed by real-time PCR in DX5⁺NK cells from IL-12R β 2KO mice. The results displayed in Fig. 3.9 demonstrates that in response to IL-2+IL-18, murine DX5⁺NK cells were able to increase IFN- γ (B) and IL-13 (C) transcript levels independently of IL-12R β 2 signaling. Deficiency in IL-12R β 2 expression in the IL-12R β 2KO mice was also confirmed by real-time PCR (A).

Next, IFN- γ protein expression was examined in IL-2-activated splenic DX5⁺NK cells (d20) isolated from IL-12R β 2KO mice in response to IL-18. Using flow cytometry, the intracellular IFN- γ produced by these cells in response to IL-12, IL-18 and the combination of IL-12+IL-18 was assayed (Fig.3.10). Flow cytometric results showed that similar to wild type, no IFN- γ protein was produced by DX5⁺NK cells from IL-12R β 2KO in response to IL-2 only (Fig. 3.10 A). Furthermore, no IFN- γ protein was detected in response to IL-2+IL-12, confirming that IL-12 signaling is completely abrogated in these cells (Fig. 3.10 B). In contrast, high levels of IFN- γ were produced when the cells were incubated overnight in the presence of IL-2+IL-18 (Fig. 3.10 C). The combination of

Fig. 3.8. IL-18-induced IFN- γ expression in murine DX5⁺NK cells is IL-12 independent. DX5⁺NK cells were isolated from IL-12R β 2KO mouse splenocytes by positive magnetic selection and were expanded for 20 d in huIL-2 (1000 U/ml). Cells were then stimulated with IL-2+IL-18 (100 ng/ml) and were harvested at the indicated time points. Total RNA was prepared and cytokine transcript expression was analyzed by RNase Protection Assay (RPA) using the mCK1 mouse cytokine multi-probe template set (Pharmingen). DX5⁺NK cells lacking IL-12-signaling responded to IL-18 by expressing of IFN- γ , IL-13 and IL-6 transcripts.

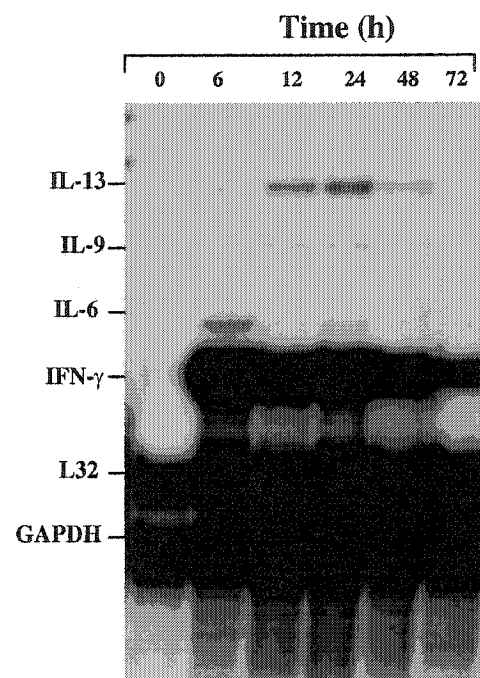
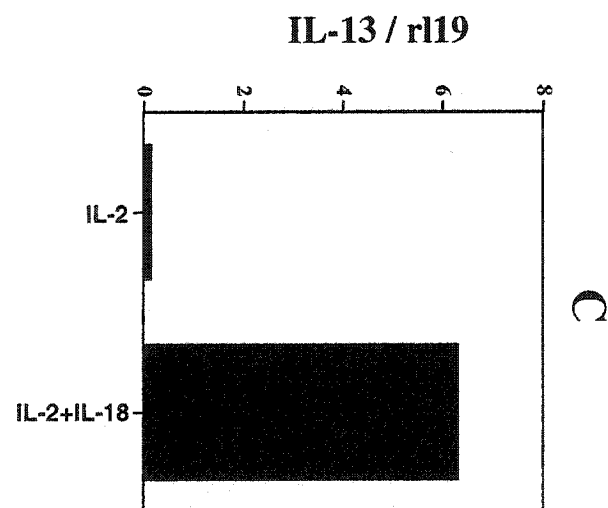
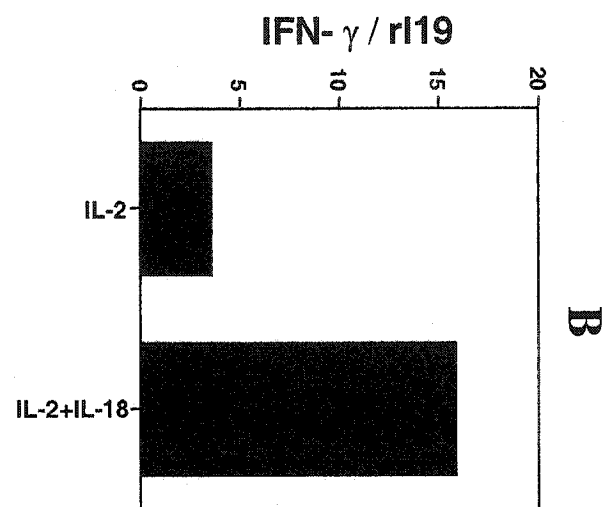
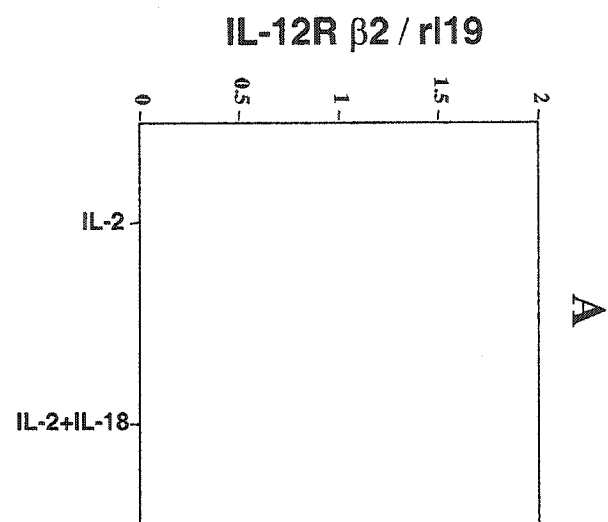


Fig. 3.9. In the absence of IL-12 signaling, IL-2+IL-18 induced IL-13 mRNA expression in DX5⁺NK cells. DX5⁺NK cells were isolated from IL-12R β 2KO mouse splenocytes by positive magnetic selection and were expanded in huIL-2 (1000 U/ml). At d6, cells were stimulated overnight with IL-2+IL-18 (100 ng/ml) and were harvested for RNA preparation. IL-12R β 2 (A), IFN- γ (B) and IL-13 (C) transcript levels were determined by real-time PCR using specific mouse probes and primers. Relative copy numbers of the indicated transcripts were calculated from a standard curve generated in each assay using PCR standards. The copy numbers of these transcripts were normalized to r119 levels by dividing the copy number obtained from standard curves to that obtained for r119. The data confirmed that DX5⁺NK cells from C57BL/6 mice lacking IL-12 signaling were able to upregulate IFN- γ and IL-13 mRNA in response to IL-2+IL-18.



IL-2+IL-12+IL-18 did not show any synergistic effect on IFN- γ protein expression providing further evidence that the IL-12R is not functional in these mice (Fig. 3.10 D). Together, these data provide definitive evidence that in murine DX5⁺ NK cells, IL-18 stimulation induces IFN- γ expression independently of IL-12 signaling activity.

III.5.9. Murine CD3⁺DX5⁺ (NK-T) cell responsiveness to IL-2+IL-12 or IL-2+IL-18 stimulations

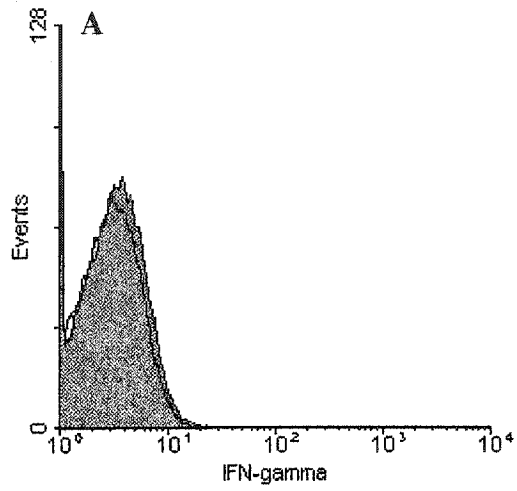
As stated earlier, splenocytes purified on the basis of surface expression of the DX5 marker comprise a mixture of DX5⁺CD3⁻ NK cells and a small number of DX5⁺CD3⁺ NK-T cells [Fig. 2.9, (222)]. Both DX5⁺CD3⁻ NK cells and DX5⁺CD3⁺ NK-T cells were maintained after *in vitro* expansion in huIL-2 and the NK/NK T composition of these cultures (85-95% NK and 5-15% NK-T) did not change appreciably over time. To establish whether the IL-2-activated DX5⁺CD3⁻ NK cells, DX5⁺CD3⁺ NK-T cells or both produced IFN- γ in response to IL-12 or IL-18, cells were stimulated overnight with IL-2+IL-12 or IL-2+IL-18 and levels of intracellular IFN- γ were analyzed by flow cytometry. The results reported in Fig. 3.11 indicate that DX5⁺CD3⁺ NK-T cells responded to cytokine stimulation in the absence of any TCR cross-linking. Moreover, both DX5⁺CD3⁻ NK and DX5⁺CD3⁺ NK-T cells produced substantial amounts of IFN- γ in response to IL-2+IL-12 or IL-2+IL-18, however, the level of intracellular IFN- γ was somewhat higher in the DX5⁺CD3⁺ NK-T population.

III.6. Discussion

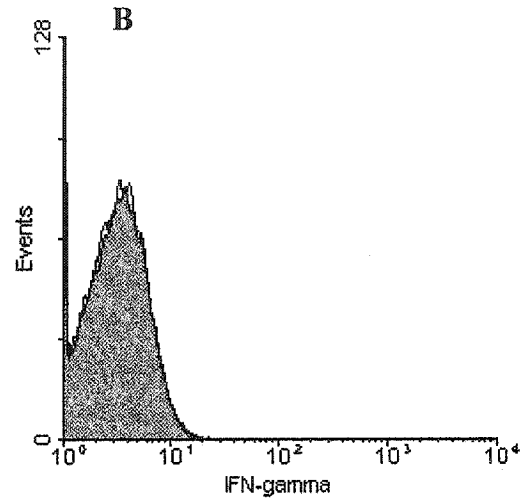
IL-12 and IL-18 are two powerful cytokines able to induce IFN- γ production by T cells (174,178,192,194) and NK cells (147,175,198,222). However, each of these pro-inflammatory cytokines induces a specific cytokine profile in addition to IFN- γ . More specifically, IL-10 expression is induced in response to IL-12 (211,221), whereas IL-13 and IL-6 expression is induced in response to IL-18 (147,174,175,232). Although numerous studies have identified the critical role of IL-12 and IL-18 as strong inducers of cytokine gene expression, to our own knowledge, no study has specifically

Fig. 3.10. IL-2+IL-18 stimulation induced strong IFN- γ protein production in the absence of IL-12 signaling. DX5⁺NK cells were isolated from IL-12R β 2KO mouse splenocytes by positive selection and were expanded in huIL-2 (1000 U/ml). At d20, cells were incubated overnight in the presence of **A**, IL-2 only (1000 U/ml); **B**, IL-2+IL-12 (1 ng/ml); **C**, IL-2+IL-18 (100 ng/ml) or **D**, IL-2+IL-12+IL-18. Cells were treated with monensin for the last 4 h of culture to block IFN- γ secretion. Cells were fixed, permeabilized then stained with PE-conjugated anti-IFN- γ mAb (clear curve) or PE-conjugated isotype control antibodies (shaded curve). DX5⁺NK cells from IL-12R β 2KO mice responded to IL-2+IL-18 by increasing IFN- γ production. The cells did not show any response to IL-2+IL-12 confirming that they were defective in IL-12 signaling.

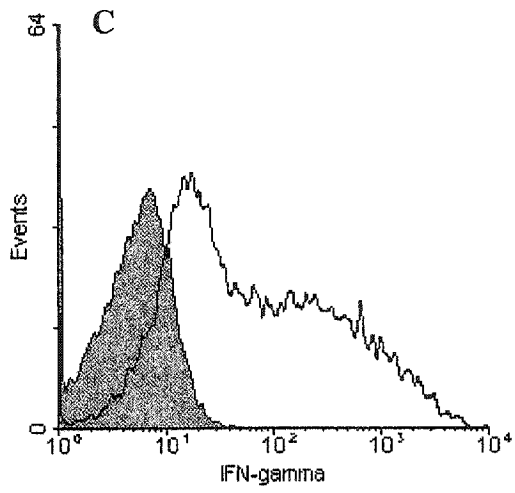
+IL-2



+IL-2+IL-12



+IL-2+IL-18



+IL-2+IL-12+IL-18

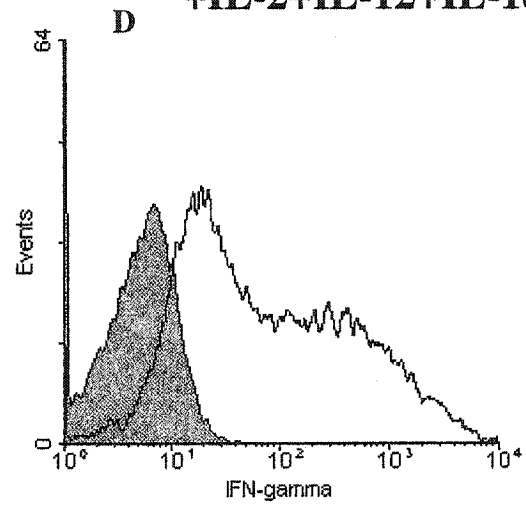
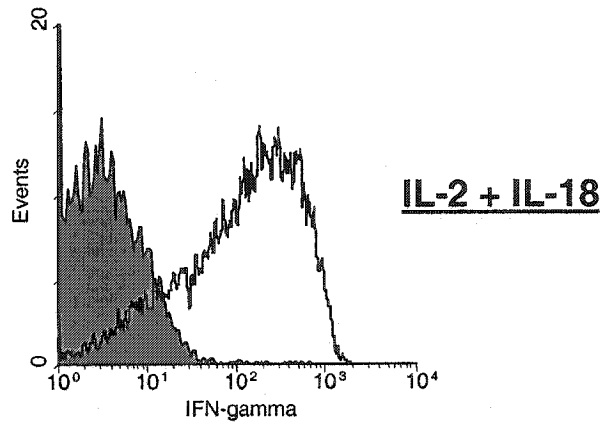
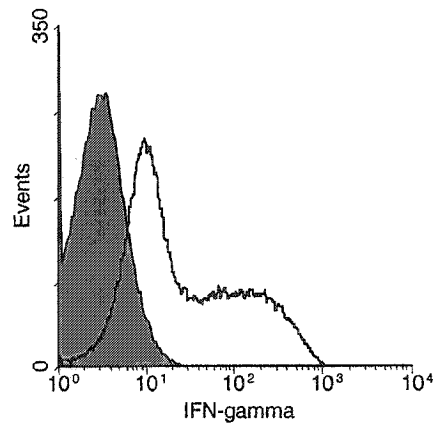
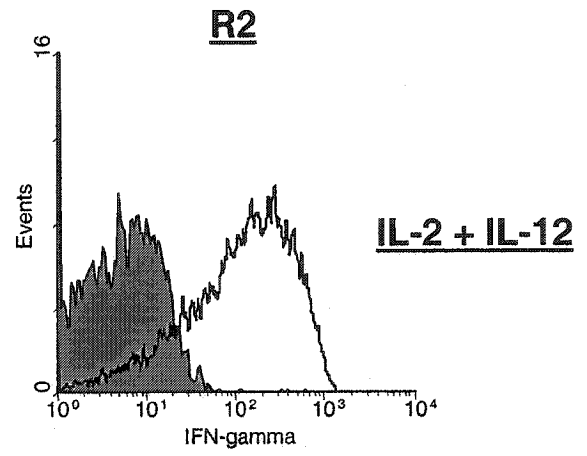
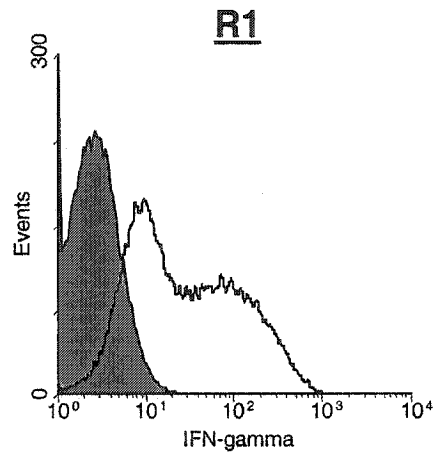
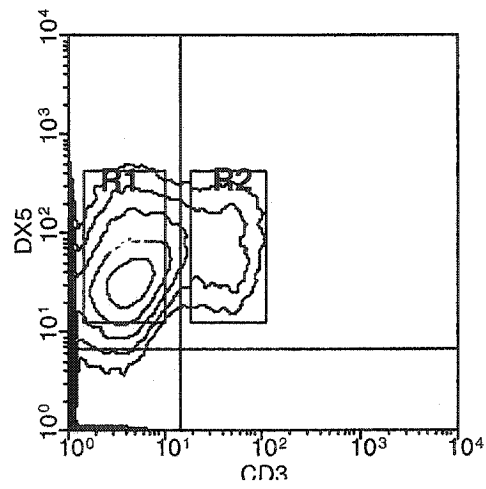


Fig. 3.11. Murine DX5⁺CD3⁺NK-T cells produced IFN- γ in response to cytokines in a TCR-independent manner DX5⁺NK cells were purified from C57BL/6 splenocytes using positive selection and were expanded in huIL-2 (1000 U/ml) for 8 d. Cells were then stimulated overnight with IL-2+IL-12 (1 ng/ml) or IL-2+IL-18 (100 ng/ml) and treated with monensin for the last 4 h of culture to block IFN- γ secretion. Cells were surface stained to detect the expression of CD3 and DX5, fixed, and permeabilized. Cells were then stained with either anti-IFN- γ (clear curve) or isotype control Ab (shaded curve) to detect intracellular levels of IFN- γ . Gated regions indicated as R1 and R2 in upper panel represent DX5⁺CD3⁻ (NK) cells and DX5⁺CD3⁺ (NK-T) cells respectively.



addressed the kinetics of cytokine gene expression by NK cells in response to either IL-2+IL-12 or IL-2+IL-18. In agreement with earlier studies (141,147,175,184,198,233-236), the present findings show that murine DX5⁺NK cells manifested significant upregulation of IFN- γ in response to stimulation with IL-2+IL-12 or IL-2+IL-18. Induction of IFN- γ expression was rapid and maximal levels of IFN- γ transcript were reached within 6h of cytokine treatment. High levels of IFN- γ transcripts were detected earlier at T=1 to T=3h after stimulation (data not shown). This rapid response is consistent with NK cells constituting the first line of defense of the innate immune system. However, it is interesting to note that, despite the constitutive expression of IL-12 and IL-18 receptors at the transcriptional level, NK and NK-T cells require concurrent activation with other cytokines such as IL-2 in order to acquire responsiveness to IL-12 or IL-18. This would be paradoxical with a role for NK cells as an immediate source of IFN- γ since it infers a requirement for activation of antigen-specific T cells as a source of IL-2, a process that normally requires several days to develop. Recent studies showed that dendritic cells constitute an important source of IL-2 (237) and the availability of this source might partially explain the rapid expansion and IFN- γ production by NK cells observed *in vivo* in response to any external infection. However, the conditions and the triggers able to induce sufficient IL-2 production by dendritic cells are not yet well understood. IL-2 has long been recognized as an important co-stimulus for IL-12-elicited responses in NK cells (238-241); but the exact mechanism of this activity is not yet clear. Recent studies (145) indicated that IL-2 upregulates the IL-12 signaling molecule STAT4 (111,112), yet freshly purified NK cells express constitutive amounts of STAT4 that is presumably available for signaling (145). IL-2 itself has also been shown to directly activate STAT4 in NK cells (in the absence of IL-12) via tyrosine phosphorylation (242) but the relationship between this event and IL-12-induced responses in NK cells is not clear.

IL-2 has also been shown to function as an important co-stimulus for IL-18-induced responses (243-245), nevertheless, the majority of these studies have focused upon the stimulation of bulk cultures of PBMC or primary T cells. The results presented herein support the data of Tomura et al (245) who showed that purified murine NK cells are essentially unresponsive to IL-18 alone but respond to the combination of IL-2+IL-18

with high level proliferation and IFN- γ secretion. Furthermore, a recent study by Son et al (142) more systematically analyzed the responses of distinct subsets of human cells to the combination of IL-2+IL-18 and found that CD4, CD8 and NK populations all responded to this cytokine combination; however, NK cells represented the primary source of IL-2/IL-18-induced cytotoxicity and were the dominant cell population after *in vitro* expansion in this cytokine combination. In addition, treatment of NK cells with IL-2+IL-18 resulted in a dramatic up-regulation of the IL-2R α chain (CD25), which may account for the synergistic activity of these two cytokines (142,192). Alternatively, the results presented herein demonstrated that the NK survival factor IL-15 could substitute for IL-2 as an activator of IL-12/IL-18 responsiveness. IL-15 has previously been reported to act as an important co-stimulus for IL-12-induced IFN- γ secretion (133,199) and IL-18-induced GM-CSF secretion by human CD56⁺NK cells (199). This is consistent with the ability of IL-15 to share many of the activities of IL-2 via signaling through the IL-2 receptor β chain (CD122) and the common γ chain (CD132). Indeed IL-15 has been shown to play a crucial role during the priming phase of the generalized Shwartzman reaction (246) likely by rendering NK cells responsive to LPS-induced IL-12. Thus, IL-15 likely plays an important role during the innate phase of an *in vivo* immune response since it is a pleiotropic cytokine that is more universally expressed than IL-2 (229). However, it is unlikely that IL-15 is expressed in sufficient amounts to render NK cells constitutively responsive to IL-12 or IL-18 since studies have shown that *in vivo* administration of IL-12 or IL-18 alone have little effect on levels of serum IFN- γ (181, 309).

In addition, the ability of IL-12 and IL-18 to enhance cytotoxic activity of NK cells and CD8⁺T cells might play a pivotal role in the therapeutic potential of these cytokines in cancer treatment (93,137). However, previous study has shown that co-stimulation with IL-2 is a pre-requisite for IFN- γ induction by NK cells (245). Given that the co-injection of mice with IL-2 and IL-18 induces high toxicity (181), the use of IL-15 as a possible substitute for IL-2 might be a possibility to take in consideration as a therapeutic strategy for diseases requiring suitable T_H1 responses.

Although sharing many characteristics, IL-12 and IL-18 did not induce similar cytokine patterns. In agreement with earlier studies, the results presented herein

demonstrated that the T_H2-type cytokines IL-10 and IL-13 were induced in response to IL-2+IL-12 and IL-2+IL-18, respectively, but at much lower levels than IFN- γ . In addition, induction of these T_H2 cytokines occurred subsequent to the initial burst of IFN- γ production, typically reaching a maximum at 24 to 48 h post cytokine exposure. IL-10 has been previously reported to have both negative and positive effects on IL-12/IL-18 responses such as IFN- γ production and cytotoxicity (247,248). However, the present data demonstrate that at the transcript level, IL-12-induced IFN- γ expression remained relatively constant throughout the time period analyzed (0 to 72 h) despite the concurrent induction of IL-10 expression. Similarly, IL-18-induced upregulation of IL-13 expression had no impact on IL-18-induced IFN- γ expression despite the fact that IL-13 is normally considered to be a T_H2-associated cytokine (249). IL-18-induced upregulation of IL-13 is also consistent with a recent report describing the IL-18-induced switching of an antigen-specific antibody response to a predominantly IgE isotype (179). Interestingly, despite the synergy reported between IL-18 and IL-10 in inducing IFN- γ and increasing the cytolytic activity of human NK cells (182,183), it was observed that when murine DX5⁺ NK cells were incubated in the combination of IL-2+IL-12+IL-18, the IL-12-induced expression of IL-10 was significantly decreased suggesting that either IL-12 or IL-18 induced factors had a negative impact on IL-10 expression. In summary, although IL-18 is considered a strong inducer of IFN- γ expression, the biological effects of this pro-inflammatory cytokine *in vivo* is likely to be complex since IL-18 concomitantly induces expression of T_H1 and T_H2-associated cytokines.

It has been reported elsewhere that activation of human and murine NK cells with IL-2 alone resulted in an up-regulation of IFN- γ and IL-13 mRNA (175,222,232,250). In contrast, the data presented here showed that murine DX5⁺NK cells stimulated with IL-2 only expressed a very low level of IFN- γ mRNA and no other cytokine transcripts were detected. At the protein level, intracellular IFN- γ was below the level of detection in the presence of IL-2 alone. The discrepancy between the present data and these earlier reports could possibly be explained by the different methods used to isolate and stimulate NK cells. In the paper by Lauwerys et al. (222), total splenocytes were stimulated *in vitro* with IL-2 for 3d before sorting NK cells, followed by a second stimulation of NK cells

with IL-2. In the paper of Hoshino et al (175), mice were pre-treated *in vivo* with IL-2 for 3d before NK cell enrichment. In both instances, the IL-2-activated cells would be exposed to multiple cytokines derived from the mixed splenocyte populations *in vivo* and the IFN- γ and IL-13 mRNA detected after IL-2 stimulation may reflect the residual effects of these endogenous cytokines. In contrast, the present studies were performed using highly enriched DX5⁺NK cells; therefore, the effects of cytokines derived from mixed cell populations can be discounted.

The results presented in this chapter also showed that IL-18 induced a strong upregulation of IL-12R β 2 gene expression in IL-2-activated DX5⁺NK cells. However, the IL-18-induced upregulation of IL-12R β 2 expression was not required for IL-12 responsiveness of these DX5⁺NK cells. Furthermore, the exact mechanism underlying the induction of IL-12R β 2 expression by IL-18 is not clear and the present data cannot confirm whether the effect of IL-18 on IL-12R β 2 was direct or was induced by IFN- γ produced in response to IL-2+IL-18 stimulation or by other reagents released in the culture medium.

The DX5⁺NK cells studied herein actually comprise a mixture of both DX5⁺CD3⁻ NK cells and a smaller population of DX5⁺CD3⁺ NK-T cells ((147,217,222) and Fig. 3.11). NK-T cells form an unusual cell subset that comprise both CD4⁻CD8⁻ and CD4⁺ lymphocytes and is characterized by the expression of the NK1.1 surface marker and the usage of an invariant V α 14-J β 281 chain preferentially associated with a V β 8.2 chain. NK-T cells are positively selected by the non-polymorphic MHC-class I-like molecule CD1d and recognize CD1d-bound lipid ligands (251). The results reported here show that although both populations (NK and NK-T cells) were responsive to stimulation with IL-2+IL-12 or IL-2+IL-18, DX5⁺CD3⁺ NK-T cells were more powerful in terms of IL-12/IL-18-induced IFN- γ production than DX5⁺CD3⁻NK cells. To our knowledge, the present data were the first to demonstrate that a population of IL-2-activated NK-T cells responded to IL-12 or IL-18 stimulation independently of TCR-stimulation.

In addition, these results demonstrated that DX5⁺ splenic cells incubated in the combination of IL-2+IL-12+IL-18 formed two distinct cell populations producing different levels of IFN- γ . Analysis of these populations by flow cytometry suggested that the two discrete populations corresponded to the IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets

reported earlier in chapter II. The IFN- γ^{lo} and IFN- γ^{hi} populations do not correspond to DX5⁺CD3⁻NK and DX5⁺CD3⁺NK-T cells since both IL-12R β 2^{lo} and IL-12R β 2^{hi} subsets are comprised of similar number of DX5⁺CD3⁻ (NK) cells and DX5⁺CD3⁺ (NK-T) (see Fig. 2.10). Although this finding provides evidence of another biological difference between the IL-12R β 2^{lo} and IL-12R β 2^{hi} DX5⁺NK subsets, the physiological significance of these different populations *in vivo* remains unknown.

Previous studies have yielded confusing and sometimes contradictory results regarding the interdependence between IL-12 and IL-18 for each other's activity. This may be due, in part, to the use of bulk human PBMC or total mouse splenocytes for many studies as it is becoming increasingly apparent that the regulation of IL-12 and IL-18-mediated responses is distinct between NK and T cells. For example, several studies have indicated that the ability of T cells to respond to IL-18 is strictly IL-12 dependent (160,187,188,194). This is reportedly due to a lack of expression of the IL-18R prior to stimulation via the TCR. Activation of the TCR leads to the transient up-regulation of the two components of the IL-12R on T cells (12,28) and binding of IL-12 by the IL-12R results, amongst other activities, in the up-regulation of IL-18R (160,188). NK cells on the other hand are reported to constitutively express receptors for both IL-12 [(145,147,162,217) and chapter II] and IL-18 [(147,161,162,189) and chapter III] on their cell surface. Although the present results showed that IL-12 and IL-18 receptor transcripts were constitutively expressed in resting DX5⁺NK cells, NK cells do not respond to either IL-12 or IL-18 alone unless they are stimulated concurrently with IL-2 or IL-15. Furthermore, the data using IL-12R β 2KO mice provide definitive evidence that murine DX5⁺NK cells respond to IL-18 in the absence of IL-12 signaling. Indeed RPA and flow cytometric analyses revealed that although IL-12R β 2KO mice were completely deficient in their response to IL-12, wild type and IL-12R β 2KO mice were indistinguishable in terms of the spectrum of cytokine genes induced by IL-2+IL-18. Thus, although IL-12 and IL-18 are synergistic in many of their activities, in murine DX5⁺NK cells, at least, they also appear to be capable of functioning independently of one another.

In Summary, the results presented in this chapter indicate that in response to IL-2+IL-12 or IL-2+IL-18, DX5⁺NK cells showed a rapid and dramatic up-regulation of

IFN- γ followed shortly thereafter by up-regulation of other T_H2 cytokine transcripts. When DX5⁺NK cells were stimulated with the combination of IL-2+IL-12+IL-18, the IL-12-induced IL-10 mRNA was significantly downregulated and two distinct populations producing different levels of IFN- γ protein emerged. Furthermore, the experiments utilizing IL-12R β 2KO mice provide definitive evidence that murine DX5⁺NK cells respond to IL-18 independently of IL-12 signaling. Finally, these data showed that DX5⁺NK cells were comprised of 85-95% DX5⁺CD3⁻ corresponding to NK cells and a small subpopulation of DX5⁺CD3⁺ (5-11%) corresponding to NK-T cells and that NK-T cells were able to respond to IL-2+IL-12 or IL-2+IL-18 independently of any TCR cross-linking. Moreover, both NK and NK-T subpopulations were able to respond to IL-2+IL-12 and to IL-2+IL-18 by a high expression of IL-12R β 2 and a strong production of IFN- γ . Since NK-T cells expressed responsiveness to cytokine stimulation in the absence of TCR signals, the data prompted us to investigate whether conventional CD4⁺T cells would be able to respond to cytokines in the absence of any TCR ligation. This study constitutes the theme of the following chapter.

CHAPTER IV

‘Bystander Polarization’ of CD4⁺T cells: activation with high-dose of IL-2 renders naive T cells responsive to IL-12 and/or IL-18 in the absence of TCR ligation

IV.1. Summary

The study reported in the previous chapter on NK cells demonstrated that despite the constitutive expression of IL-12 and IL-18 receptors, responsiveness to IL-12 or IL-18 stimulation required a concurrent activation with a second stimulus, in particular IL-2 or IL-15. NK cells express the intermediate affinity IL-2 receptor composed of the β chain (shared by IL-15) and the γ chain (shared by IL-4, IL-7, IL-9 and IL-15). Earlier studies also demonstrated that the capacity of NK cells to respond to IL-12 or IL-18 is a direct consequence of IL-2 signaling via the intermediate affinity receptor without prior activation (252,253). Furthermore, the results presented here indicated that DX5⁺NK cells were comprised of CD3⁻DX5⁺ (NK) cells and CD3⁺DX5⁺ (NK-T) cells and that NK-T cells responded to IL-2+IL-12 or IL-2+IL-18 independently of TCR signaling. Naïve conventional T cells also express the intermediate affinity IL-2 receptor (228) but in contrast to NK cells, naïve T cells are reported to lack the expression of IL-12 and IL-18 receptors (145,160). T cell activation via the TCR has been reported to be required to render these cells responsive to IL-12 or IL-18, however, recent studies have suggested that TCR-dependent upregulation of IL-12R may actually be IL-2 dependent (194,204). In this chapter, responsiveness of conventional naïve CD4⁺T cells to stimulation with high dose IL-2 was analyzed in the absence of any antigenic stimulation. Naïve CD4⁺CD62L⁺T cells exhibited proliferation and IFN- γ production in response to IL-2+IL-12 or IL-2+IL-18. In addition, they upregulated the expression of the IL-12R gene. In response to cytokine activation, naïve CD4⁺T cells from OVA-specific TCR transgenic mice (D011.10/Rag2^{-/-}) adopted an activated phenotype characterized by the upregulation of CD25/CD44 and downregulation of CD45/CD62L cell surface activation markers. Furthermore, similar to the IL-12+IL-18 innate pathway (254), cytokine-induced IFN- γ production was cyclosporin A (CsA) resistant. Interestingly, naïve CD4⁺T cells stimulated with IL-2+IL-12 showed signs of polarization towards the T_H1 phenotype. Finally, the results demonstrated that similar to NK cells, CD4⁺T cells respond to IL-18 independently of IL-12 signaling.

IV.2. Hypothesis

Naïve CD4⁺T cells, expressing intermediate affinity IL-2 receptor, are activated by IL-2 only and are rendered responsive to IL-12 and/or IL-18. This activation plays a role in the polarization of naïve CD4⁺T cells in the absence of any TCR ligation.

IV.3. Objectives

- To investigate whether IL-2-activated CD4⁺T cells are able to respond to IL-12 or IL-18 stimulation in the absence of any specific TCR ligation.
- To analyze whether IL-12/IL-18 responsiveness induces T cell polarization in the absence of TCR signaling.

IV.4. Materials and Methods

IV.4.1. Enrichment of murine CD4⁺ and naïve CD4⁺CD62L⁺T cells

Murine CD4⁺T cells were enriched from mouse splenic MNC by positive magnetic selection using FITC-conjugated anti-CD4 mAb and anti-FITC microbeads (Miltenyi). Briefly, MNC were stained for 20 min at 4°C with 2 µl/10⁷ cells of 0.5mg/ml FITC-conjugated anti-CD4 mAb in MACS buffer at a final volume of 10⁷cells/100 µl. Stained cells were washed using 10 to 20 x the labeling volume of MACS buffer. Cells were then incubated in the presence of 10 µl/10⁷ cells of anti-FITC microbeads for 20 min at 4°C following the manufacturer's protocol. Cells were washed twice from anti-FITC microbeads using 10 to 20 x the labeling volume of MACS buffer. Stained cells were resuspended in 500 µl/10⁸ cells of degassed MACS buffer and passed through the LS⁺ midi-MACS magnetic columns. The columns were washed three times with 3 ml of MACS buffer and the flow-through fractions were recovered in new 15 ml centrifuge tubes. Magnetic columns were removed from the separator and positively selected cells were flushed out in new 15 ml centrifuge tube with 1 ml MACS buffer. A cell fraction (50 µl) from different purification steps was resuspended in 500 µl FACS buffer and directly analyzed for CD4⁺T cell separation by flow cytometry analysis of FITC-conjugated anti-CD4 staining. Before anti-CD62L staining, the anti-FITC microbeads

were removed from the CD4⁺T cells by incubating the cells with Macs Release Reagent for 10 min at 4°C. The release reaction is stopped by mixing the cells with Macs Stop Reagent. CD4⁺CD62L⁺T cells were further isolated from the CD4⁺T cell fraction of mice splenocytes by positive selection using 10 μ l/10⁷ cells of anti-CD62L microbeads (Miltenyi) in a final volume of 100 μ l/10⁷ cells of MACS buffer. After 20 min incubation at 4°C, the cells were extensively washed using 10 to 20 x the labeling volume of MACS buffer and run through MS⁺ mini-MACS magnetic columns. A 50 μ l fraction from separated cell fractions (CD4⁺CD62L⁻ and CD4⁺CD62L⁺T cells) were stained 25 min with 1:100 v/v of 100 μ g/ml PE-conjugated anti-CD62L mAb in 100 μ l FACS buffer at 4°C and the yield of CD4⁺CD62L⁺T cell separation was analyzed by two-color flow cytometry (CD4-FITC/ CD62L-PE).

Murine naïve CD4⁺T cells were used in different experiment setups reported in the different chapters of the present dissertation. Therefore, enriched CD4⁺ or CD4⁺CD62L⁺ T cells were resuspended in RP-10 medium and cultured in the presence of different stimuli following the requirements of the experiments. Cells were either stimulated with cytokines, plate-bound anti-CD3 plus soluble anti-CD28, soluble anti-CD3 plus irradiated syngeneic APCs or with Ag plus irradiated syngeneic APCs when CD4⁺T cells from OVA-specific TCR transgenic mice (D011.10/Rag2^{-/-}) were used. These latter mice are commonly called DO11.10/Rag2^{-/-} mice. Syngeneic APCs were irradiated at 3000 rads using Pentak 12.5 mA 250 kVP irradiator.

IV.4.2. RPA analysis of cytokine and cytokine receptor gene expression.

CD4⁺CD62L⁺T cells from normal C57BL/6 mice (2.5 x 10⁶ cells/well in 24 well-plate) were stimulated with 1000 U/ml huIL-2 in the presence or absence of IL-12 (1ng/nl), IL-18 (100 ng/ml) or IL-12+IL-18 in the absence of any TCR stimulation. At d3, cells were lysed using Trizol reagent and total RNA was used for analysis of common cytokine receptor and cytokine mRNA expression by RPA using the mCR3 or mCK1 template sets (Appendix A.3), respectively, according the manufacturer's protocol (RiboQuant, Pharmingen). Day 3 was the optimal time point for the expression of different cytokines and cytokine receptor transcripts in naïve CD4⁺T cells in response to cytokines only. Radiolabeled RNase-protected probes were resolved on 5% denaturing

polyacrylamide gels and were detected by exposure to film (XAR, Eastman Kodak) for 24h at -70°C .

IV.4.3. Activation of murine naïve $\text{CD4}^{+}\text{T}$ cells with cytokines only

To analyze the activation and polarization of naïve $\text{CD4}^{+}\text{T}$ cells using cytokines only in the absence of any antigenic stimulation, $\text{CD4}^{+}\text{CD62L}^{+}\text{T}$ cells from normal C57BL/6 mice or $\text{CD4}^{+}\text{T}$ cells from DO11.10/Rag2^{-/-} mice (2×10^4 cells/well in 96 well-plate), enriched as explained above, were resuspended in RP-10 medium and stimulated with different cytokines for 5d. The indicated cytokines were used at the following concentrations: huIL-2 (1000U/ml), IL-12 (1ng/ml) and IL-18 (100 ng/ml). As a positive control, cells were stimulated with plate-bound anti-CD3 (5 $\mu\text{g/ml}$), soluble anti-CD28 (1 $\mu\text{g/ml}$) and huIL-2 (10 U/ml). At d5 of culture, half of culture supernatant was removed for analysis of cytokine secretion by ELISA and the remaining cells were pulsed overnight with [³H]-thymidine (1 $\mu\text{Ci/well}$) (Amersham Pharmacia Biotech) to measure cytokine-induced proliferation.

IV.4.4. Polarization of naïve $\text{CD4}^{+}\text{T}$ cells with cytokines only

Naïve $\text{CD4}^{+}\text{T}$ cells from DO11.10/Rag2^{-/-} mice were stimulated at 1×10^6 cells/ml in 24 well-plates (2 ml) in the presence of IL-2 (1000 U/ml), IL-2+IL-12 (1 ng/ml) or IL-2+IL-18 (100 ng/ml) cytokines. As a control, T cells were also stimulated with 1 μM specific antigen (OVA₃₂₃₋₃₃₉ peptide, H-ISQAVHAAHAEINEAGR-OH; a gift from Dr. Peter Probst; Corixa Corporation, Seattle, WA) plus 5×10^6 irradiated (3000 rads) syngeneic CD4^{+} T cell-depleted APCs in the absence of exogenous cytokines. At d7, cells were washed extensively and rested in the absence of cytokines for 1d. Rested cells were then restimulated with plate-bound anti-CD3 (5 $\mu\text{g/ml}$) and huIL-2 (10 U/ml) for 3d and cell culture supernatants were analyzed for IFN- γ and IL-4 secretion by ELISA. For IL-2 titration experiments, naïve $\text{CD4}^{+}\text{T}$ cells from DO11.10/Rag2^{-/-} mice (2×10^4 cells/well in 96 well-plate), were stimulated with varying concentrations of huIL-2 in the presence or absence of a fixed concentration of IL-12 (1 ng/ml) or IL-18 (100 ng/ml). At d5, cell culture supernatants were analyzed for IFN- γ production by ELISA.

IV.4.5. Characterization of cell activation phenotype by flow cytometry

Naïve CD4⁺T cells isolated from spleens of DO11.10/Rag2^{-/-} mice by positive magnetic selection were cultured at 2×10^5 cells/well (in a 96-well plate) and used immediately (naïve T cells) or stimulated with 1000 U/ml huIL-2, IL-2+IL-12 (1 ng/ml), IL-2+IL-18 (100 ng/ml) or OVA peptide plus irradiated syngeneic T-cell depleted APCs. At d5 post-stimulation, cells were recovered and surface stained with antibodies specific for CD25, CD44, CD45 and CD62L then analyzed by flow cytometry. For OVA-stimulation, cells were first gated on the CD4⁺T cell population.

IV.4.6. Effect of cyclosporin A on cytokine-activated naïve CD4⁺T cells

Cyclosporin A (CsA) is an immunosuppressant that blocks the calcium-dependent signal transduction pathway emanating from the TCR thereby preventing the activation of T lymphocytes via the antigenic receptor (255) and inhibiting endogenous IL-2 production through the suppression of the phosphatase calcineurin (310). To investigate whether the activation of conventional CD4⁺T cells by exogenous IL-2 was solely due to the high dose of IL-2 and not to any signal from the TCR, naïve CD4⁺T cells were enriched from DO11.10/Rag2^{-/-} spleens by positive magnetic selection as described above. Cells (5×10^4 cells/well) were plated in triplicate in a 96-well plate. For TCR stimulation, cells were cultured in a 96-well plate coated with plate-bound anti-CD3 (5 µg/ml) and soluble anti-CD28 mAb (1 µg/ml). For cytokine stimulation, cells were incubated in the presence of IL-2 (1000 U/ml), IL-2+IL-12 (1 ng/ml) or IL-2+IL-18 (100 ng/ml). Different cell stimulations were performed in the presence and absence of 100 ng/ml of CsA (Sigma). At d7, cell supernatants were harvested and analyzed for IFN-γ production by capture ELISA.

IV.4.7. CD4⁺T cell responsiveness to IL-18 in the absence of IL-12 signaling

Splenic CD4⁺T cells enriched from C57BL/6 wild-type or mice deficient in the IL-12Rβ2 gene (IL-12Rβ2KO) by positive magnetic selection were cultured as duplicates in a 96-well plate (2×10^5 cells/well). Cells were stimulated with 5 µg/ml plate-bound anti-CD3 and 1 µg/ml soluble anti-CD28 mAb in the presence or absence of 1 ng/ml IL-

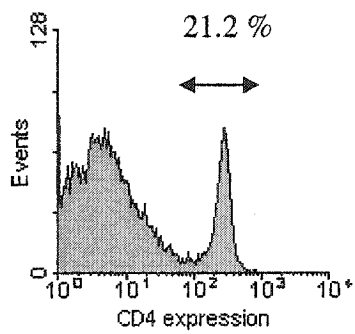
12 and/or 100 ng/ml IL-18. At d3, cell supernatants were harvested and analyzed for IFN- γ production by capture ELISA.

IV.5. Results

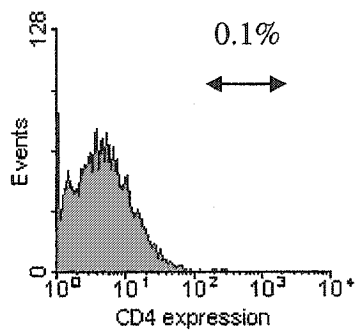
IV.5.1. Responsiveness of CD4⁺CD62L⁺T cells to IL-2+IL-12 and/or IL-2+IL-18

In the current study, the principal aim was to determine whether IL-2 could substitute for TCR stimulation as an activator of IL-12/IL-18 responsiveness in conventional naïve CD4⁺T cells. Therefore, CD4⁺CD62L⁺T cells were obtained from spleens of normal C57BL/6 mice by positive magnetic selection and were stimulated *in vitro* with IL-2 or various combinations of IL-2, IL-12 and IL-18. Fig. 4.1 shows naïve CD4⁺CD62L⁺T cell enrichment from normal C57BL/6 mice by positive magnetic selection. The CD4⁺T cell population constitutes approximately 20% of the total splenic MNC (Fig. 4.1A) and was routinely enriched at 96 to 99.5% purity using MACS columns (Fig. 4.1C). MACS columns efficiently bind almost all the CD4⁺T cells from total MNC (Fig. 4.1B). The purified CD4⁺T cell pool comprised naïve CD4⁺T cells (expressing high level of CD62L cell surface marker) and memory CD4⁺T cells (expressing low or no CD62L). Naïve CD4⁺CD62L⁺ cells were usually representing about 40% of enriched CD4⁺T cell population and were enriched at 92 to 96% purity (Fig. 4.1D). As has been previously observed (254), naïve CD4⁺CD62L⁺T cells were unresponsive to stimulation with IL-12 or IL-18 only as measured by proliferation or IFN- γ secretion (Fig. 4.2). These same cells exhibited proliferation (Fig. 4.2 A) and blast formation when incubated in the presence of high dose IL-2 (1000 U/ml); however, incubation in IL-2 alone did not elicit secretion of detectable levels of IFN- γ (Fig. 4.2 B). In contrast, when CD4⁺CD62L⁺T cells were incubated concurrently with either IL-2+IL-12 or IL-2+IL-18, they proliferated and secreted substantial amounts of IFN- γ into the culture media. Importantly, the IL-2+IL-12/IL-2+IL-18-induced production of IFN- γ occurred in the **absence** of TCR stimulation suggesting that signaling through the intermediate affinity IL-2R was sufficient to render naïve T cells responsive to either IL-12 or IL-18. In addition, the response of CD4⁺CD62L⁺T cells to a combination of IL-2+IL-12+IL-18 in the absence of TCR ligation was assessed. IL-2+IL-12+IL-18 augmented the amount of IFN- γ secreted in comparison to either IL-2+IL-12 or IL-2+IL-18 but at the same time had a slightly negative effect on proliferation. Indeed, stimulation under these conditions

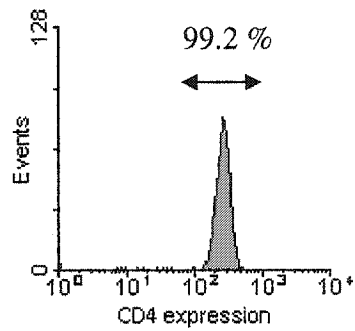
Fig.4.1. CD4⁺CD62L⁺T cell enrichment from C57BL/6 MNC. Naïve CD4⁺ T cells were isolated from C57BL/6 spleen MNC by positive magnetic selection. Total MNC were stained with FITC-conjugated anti-CD4 mAb followed by anti-FITC microbeads then separated on LS⁺ midi-MACS magnetic columns. Anti-FITC microbeads were released from the positively selected cells then CD4⁺T cell population was re-stained with anti-CD62L microbeads to isolate CD62L^{hi} (naïve CD4⁺T cells) and CD62L^{lo} (memory CD4⁺T cells) cells on MS⁺ mini-MACS magnetic columns. A cell aliquot from different CD4⁺CD62L⁺T cell isolation steps was then stained with biotin-conjugated anti-mouse CD62L mAb followed by PE-conjugated-streptavidin and was analyzed by flow cytometry to evaluate cell separation efficiency.



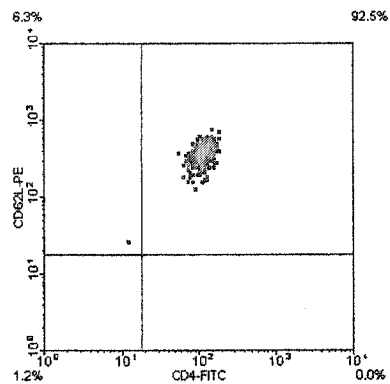
A) Total MNC



B) Macs unbound CD4⁺T cells

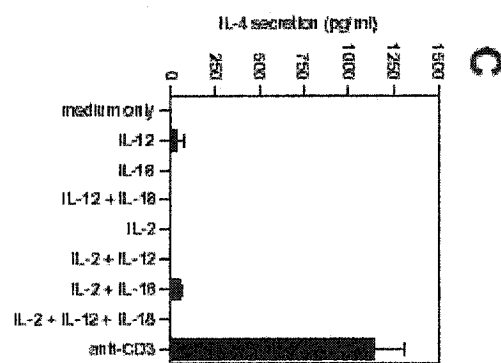
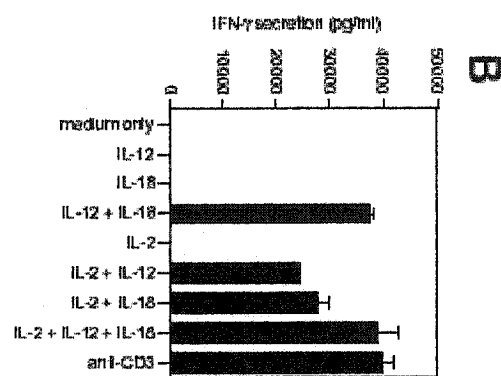
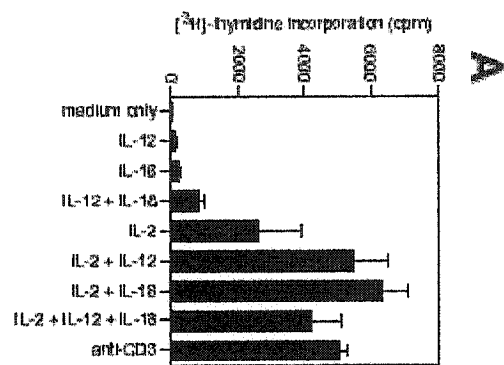


C) Macs bound CD4⁺T cells



D) Macs bound CD4⁺CD62⁺T cells

Fig. 4.2. Response of naïve CD4⁺ T cells stimulated with IL-2, IL-12, IL-18 or combinations thereof. (A) CD4⁺CD62L⁺T cells were enriched from the spleens of naïve C57BL/6 female mice and were plated in 96-well plates (2x10⁴ cells/well) in the presence of the indicated cytokines. Cells stimulated with plate-bound anti-CD3 (5 µg/ml) and soluble anti-CD28 (1 µg/ml) were used as positive controls. After 5 days of culture, half (100 µl) of the culture supernatant was removed for analysis of cytokine secretion and the remaining cells were pulsed overnight with [³H]-thymidine to measure cytokine-induced proliferation. The plotted values are the mean of triplicate experiments and error bars represent the SEM. Secretion of IFN-γ (B) and IL-4 (C) by the cells shown in panel (A) were measured by ELISA. The plotted values are the mean of replicate ELISA wells and error bars represent the SEM.



induced an “over-activation” observed by light microscopy. Also, using trypan blue exclusion, it was noticed that 25 to 30 % of cells do not survive to 5d stimulation with IL-2+IL-12+IL-18. The mechanism involved in cell death in response to IL-2+IL-12+IL-18 cytokine combination was not investigated in this study. In addition, CD4⁺CD62L⁺T cells incubated in the presence of IL-12+IL-18 (in the **absence** of IL-2) secreted large amounts of IFN- γ and proliferated albeit rather weakly. This TCR-independent up-regulation of IFN- γ synthesis in the presence of IL-12+IL-18 has been previously referred to as the ‘innate pathway’ of T cell activation (254,256).

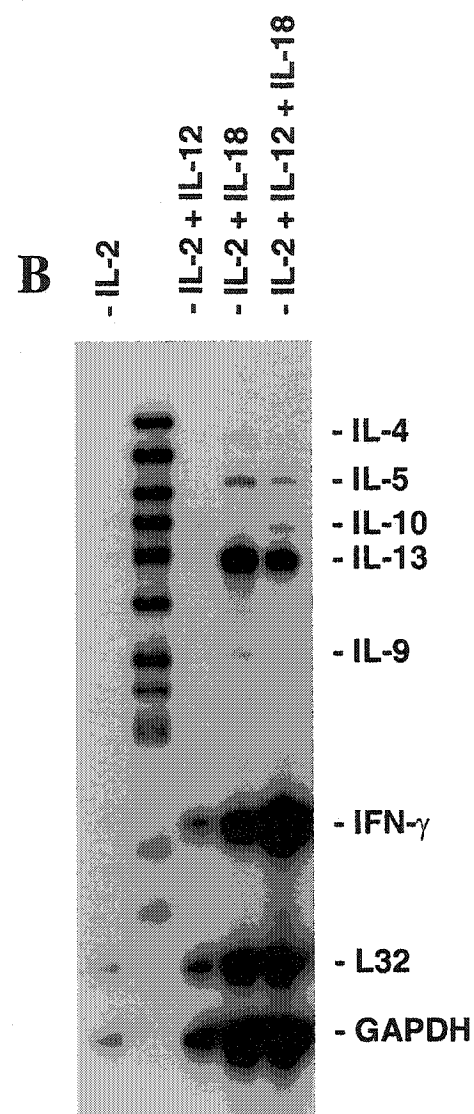
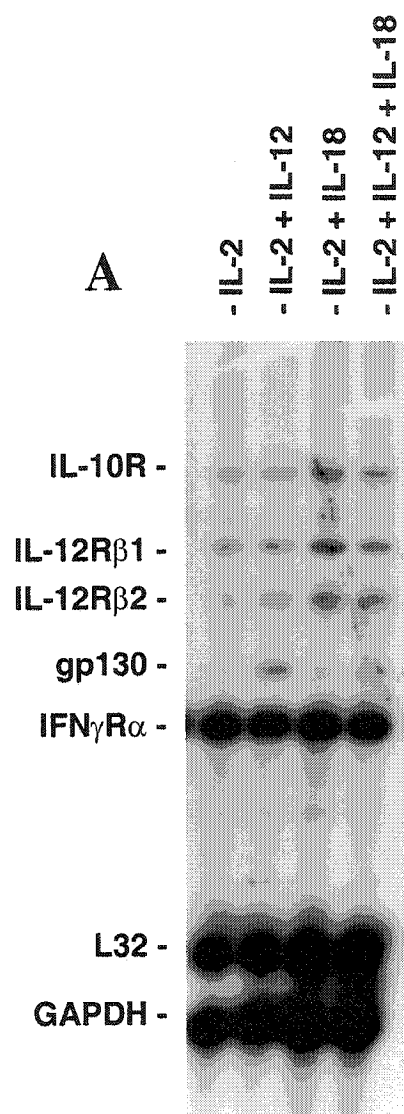
IL-4 secretion by naïve CD4⁺CD62L⁺T cells activated with the various cytokine combinations versus cells stimulated with anti-CD3 was also assessed. T cells stimulated with IL-2 alone or with any combination of IL-2, IL-12 or IL-18 did not secrete detectable amounts of IL-4 (Fig. 4.2 C). This was in contrast to stimulation of T cells through the TCR using plate-bound anti-CD3, which resulted in secretion of both IFN- γ and IL-4 indicative of a Th0 phenotype. Lastly, it is important to emphasize that although the CD4⁺CD62L⁺ T cells utilized for these studies were obtained by positive magnetic selection, the same experiments were performed using cells obtained by negative magnetic selection (untouched cells) and they showed a similar pattern of proliferation and cytokine secretion (data not shown).

IV.5.2. Analysis of cytokine receptor and cytokine gene expression

Analysis of mRNA from naïve CD4⁺CD62L⁺ T cells by RNase Protection Assay confirmed that cells incubated in high-dose IL-2 expressed both the β 1 and β 2 chains of the dimeric IL-12R (Fig. 4.3 A). Concurrent stimulation with IL-2+IL-12, IL-2+IL-18 or IL-2+IL-12+IL-18 led to further increases in levels of IL-12R β 2 mRNA expression. We were unable to obtain sufficient RNA from unstimulated naïve CD4⁺CD62L⁺T cells to perform RPA analysis, however, a large number of studies have shown that neither IL-12R β 1 nor IL-12R β 2 are expressed on non-activated T cells (12,13,257).

Analysis of the pattern of cytokine gene expression in these same cells (Fig 4.3 B) revealed that stimulation of naïve CD4⁺CD62L⁺T cells with IL-2+IL-12 induced expression of IFN- γ mRNA and to a lesser extent IL-10 mRNA. However, CD4⁺CD62L⁺

Fig. 4.3. Cytokine receptor (A) and cytokine (B) mRNA expression by naïve CD4⁺T cells in response to stimulation with the indicated cytokines or cytokine combinations. CD4⁺CD62L⁺T cells of normal C57BL/6 mice were stimulated (2.5×10^6 cells/well in 24 well plates) according to the parameters described in Fig. 4.2. At d3 post-stimulation, cells were lysed and the total RNA was used for analysis of cytokine receptor and cytokine gene expression by RNase protection assay (RPA) using the mCR3 (A) or mCK1 (B) template sets (Pharmingen).



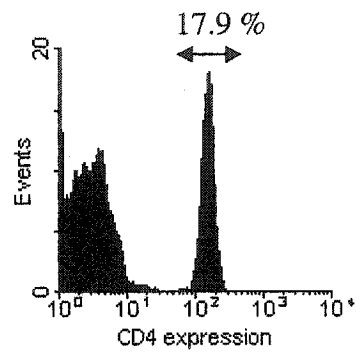
T cells incubated with IL-2+IL-18 up-regulated IFN- γ as well as IL-13 mRNA expression. Cells incubated with IL-2+IL-18 also synthesized a small amount of IL-4 and IL-5 mRNA. The combination of IL-2+IL-12+IL-18 was synergistic in terms of the IFN- γ mRNA expression; however, the combination of IL-2+IL-12+IL-18 also resulted in a slight decrease in the expression of IL-4, IL-5 and IL-13 mRNA. None of the cytokines assessed using the mCK1 template set were expressed in the presence of IL-2 only.

IV.5.3. Naïve CD4⁺T cell responsiveness to varying concentrations of IL-2

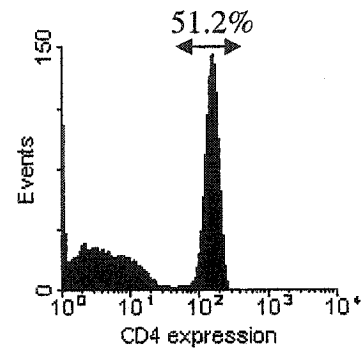
The present data using naïve CD4⁺CD62L⁺T cells from C57BL/6 mice suggested that naïve CD4⁺ T cells can be activated via the intermediate affinity IL-2R to become IL-12/IL-18 responsive. To address the possibility that the IL-12/IL-18-induced IFN- γ observed might be derived from a small population of contaminating *in vivo*-activated T cells that might be hyper-responsive to IL-2, the above experiments using CD4⁺ cells from DO11.10/Rag2^{-/-} mice were repeated. The T cell compartment of these mice has been shown to be comprised exclusively of a homogeneous population of CD4⁺CD25⁻, CD44^{lo}, CD62L^{hi} naïve T cells (258). These mice are of B10.D2 background and are homozygous for a T cell receptor gene that encodes a TCR specific for the ovalbumin (OVA) peptide 323-339 presented by the MHC class II molecule I-A^d. These mice are also deficient in the Rag 2 gene (recombinase activating gene 2) and are thus unable to rearrange their endogenous TCR (259). Furthermore, DO11.10/Rag2^{-/-} mice are deficient in NK-T cells, which normally express V α 14 and V β 8 TCR genes (260) and in CD8⁺T cells (259). Thus, the contribution of any contaminating NK-T cells to IL-12/IL-18-induced IFN- γ can also be discounted. CD4⁺T cells from DO11.10/Rag2^{-/-} mice were enriched by positive magnetic selection. These mice are characterized by the presence of a relatively higher population of splenic CD4⁺T cells in the MNC pool compared to normal C57BL/6 mice. Indeed, splenic MNC cells were comprised of 45 to 65% CD4⁺T cells (Fig. 4.4). However, the spleen has a very small size compared to C57BL/6 mice and was usually composed of approximately 6 to 10 x 10⁶ splenocytes in total.

First, we wished to determine the minimal concentration of IL-2 necessary to

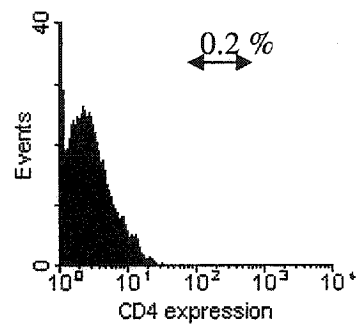
Fig. 4.4. CD4⁺T cell isolation from DO11.10 OVA-TCR transgenic mice. Naïve CD4⁺T cells were purified from splenic MNC of DO11.10/Rag2^{-/-} mice by positive magnetic selection using FITC-conjugated anti-CD4 mAb and anti-FITC microbeads (Miltenyi). Cell separation steps were analyzed by flow-cytometry. DO11.10/Rag2^{-/-} mice are reported to lack NK-T and CD8⁺T cells (259,260); however, these mice are characterized by the presence of a rich population of CD4⁺T cells in the spleen.



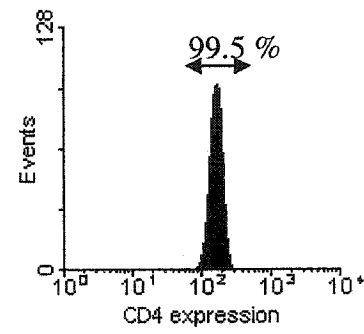
A) MNC before RBC lysis



B) MNC after RBC lysis

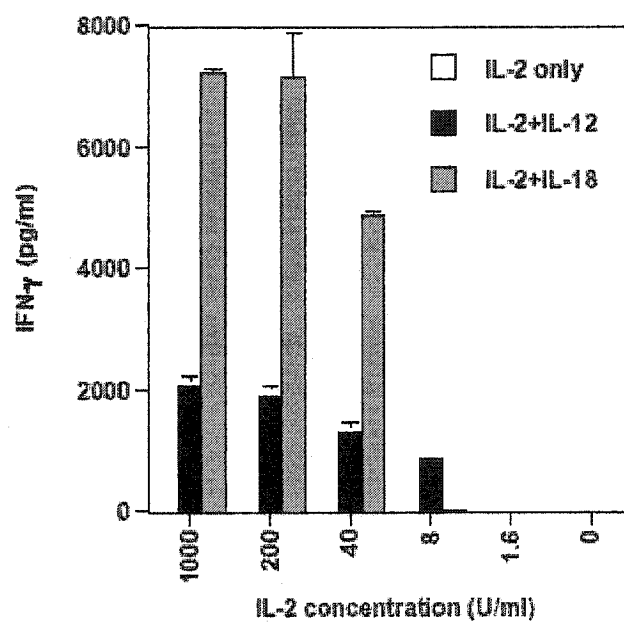


C) Macs unbound CD4⁺T cells



D) Macs bound CD4⁺T cells

Fig. 4.5. Responsiveness of naïve CD4⁺ T cells to IL-12 and IL-18 stimulation at varying concentrations of IL-2. CD4⁺ T cells purified from the spleens of DO11.10/Rag2^{-/-} mice (2x10⁴ cells/well in 96 well-plate) were stimulated with the indicated concentrations of IL-2 in the presence or absence of fixed concentrations of IL-12 (1 ng/ml) or IL-18 (100 ng/ml). At d5, culture supernatants were harvested and assessed for IFN-γ production by ELISA. The plotted values are the mean of replicate ELISA wells and error bars represent the SEM.

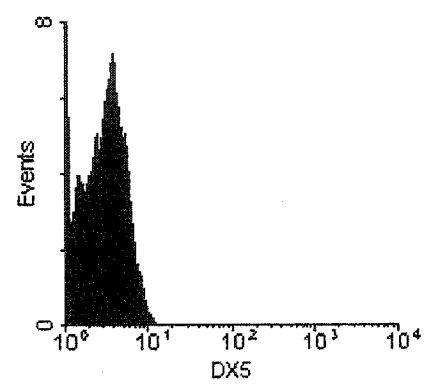
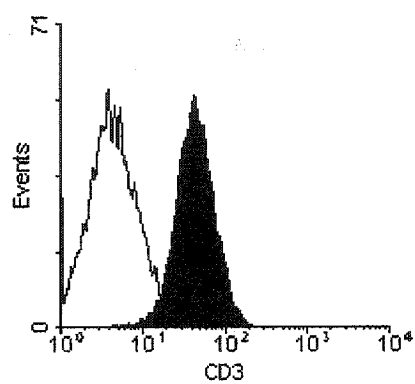


render naïve T cells responsive to IL-12 or IL-18. Thus, CD4⁺ T cells were isolated from the spleens of DO11.10/Rag2^{-/-} mice (>99.5 % purity) and were incubated in varying amounts of IL-2 in the presence of a fixed concentration of IL-12 or IL-18 (Fig. 4.5). IFN- γ was produced in response to both IL-12 and IL-18 in an IL-2 dose-dependent manner. Detectable amounts of IFN- γ were still secreted by cells when stimulated with as little as 40 U/ml IL-2 (+IL-18) or 8 U/ml IL-2 (+IL-12). Thus, although the current *in vitro* study utilized stimulation with high doses of exogenous IL-2 (1000 U/ml) as optimal conditions, it is entirely possible that, during an *in vivo* immune response, local IL-2 concentrations could reach levels sufficient to render naïve T cells responsive to IL-12 or IL-18 via an antigen-independent mechanism. To address the possibility of NK cell contamination, CD4⁺T cells from DO11.10/Rag2^{-/-} mice obtained by positive magnetic selection, were cultured in the presence of IL-2 for 6d and cells were analyzed by flow cytometry for the expression of CD3 and for the expression of the NK marker DX5. As shown in Fig. 4.6, all cells present in the cell culture expressed the CD3 marker confirming their T cell status; however, no cells expressing the DX5 marker were detected excluding the possibility of NK cell contamination.

IV.5.4. *In vitro* polarization of naïve CD4⁺T cells in the absence of TCR ligation

Given that high-dose IL-2 had rendered naïve CD4⁺T cells responsive to IL-12/IL-18, the next question was whether stimulation of cells with IL-2+IL-12/IL-18 in the absence of antigen would have any capacity to polarize the cells towards a T_H1 phenotype. Naïve CD4⁺T cells from DO11.10/Rag2^{-/-} mice were incubated for 7 d in the presence of IL-2 only, IL-2+IL-12, IL-2+IL-18 or IL-2+IL-12+IL-18. Cells were then washed, rested for one day in the absence of cytokines, and then restimulated with plate-bound anti-CD3 and soluble anti-CD28 (Fig.4.7). As a control, cells were also primed with OVA₃₂₃₋₃₃₉ peptide plus irradiated syngeneic T cell-depleted splenocytes in the absence of exogenous cytokines (neutral conditions). As previously reported (7), DO11.10/Rag2^{-/-}CD4⁺T cells primed with APC plus OVA₃₂₃₋₃₃₉ in the absence of exogenous cytokines secreted both IFN- γ and IL-4 upon secondary stimulation with plate-bound anti-CD3 consistent with a mixed or non-polarized (T_H0) state.

Fig. 4.6. Phenotypic characterization of naïve CD4⁺T cells stimulated *in vitro* with huIL-2 for 6d. CD4⁺T cells purified from the spleens of DO11.10/Rag2^{-/-} mice (2x10⁵ cells/well in 96 well-plate) were stimulated with 1000 U/ml of huIL-2. At d6, cells were analyzed by flow cytometry to investigate the phenotype of IL-2-activated CD4⁺T cells. Cells were stained with biotin-conjugated anti-mouse CD3/PE-conjugated streptavidin or with PE-conjugated anti-DX5 mAb. Flow cytometric results show that all the cells activated with IL-2 for 6 days expressed CD3 cell surface marker but no DX5 cell marker expression was detected confirming the absence of any NK cell contamination.



-  Non stained cells: Autofluorescence
-  Cells stained for the indicated cell marker

DO11.10/Rag2^{-/-} CD4⁺T cells incubated with high-dose IL-2 during the primary stimulation (no antigen) also secreted both IFN- γ and IL-4 upon anti-CD3 stimulation, albeit at lower levels than those cells stimulated with specific antigen during the primary stimulation. This is again consistent with a non-polarized or T_H0 phenotype of these cells. In contrast, cells incubated with IL-2+IL-12 or IL-2+IL-12+IL-18 during the primary stimulation secreted increased levels of IFN- γ and undetectable levels of IL-4 after anti-CD3 stimulation. This latter finding implies that exposure of naïve CD4⁺T cells to the combination of IL-2+IL-12 or IL-2+IL-12+IL18 is capable of initiating polarization towards the T_H1 phenotype in the absence of stimulation via specific antigen. In agreement with previous data showing that IL-18 induces secretion of both T_H1 and T_H2 cytokines (158,177), cells incubated with the combination of IL-2+IL-18 during primary stimulation produced even higher levels of IFN- γ but also significant amounts of IL-4 after anti-CD3 stimulation. Together, these data imply that IL-2 in the presence of IL-12 or IL-18 mimicked TCR signaling and induced IFN- γ production in response to these cytokines.

IV.5.5. Characterization of the phenotype of cytokine-stimulated naïve CD4⁺T cells

In order to compare the activation state of DO11.10/Rag2^{-/-} naïve T cells stimulated with various combinations of IL-2, IL-12 and IL-18 versus cells stimulated with specific antigen plus irradiated APC, cells were surface stained with antibodies specific for CD25, CD44, CD45 and CD62L before and after stimulation and were analyzed by flow cytometry. As has been previously observed (261), purification of CD4⁺ cells by positive magnetic selection did not in and of itself result in activation of T cells as measured by any of these specific markers. Thus, after cell enrichment, cells incubated in medium only retained a phenotype of CD25⁻CD44^{lo}CD45^{hi}CD62L^{hi} (Fig 4.8). After incubation in the various cytokine combinations, most cells converted to a CD25⁺CD44^{hi}CD45^{lo}CD62L^{lo} phenotype comparable to what was observed after Ag stimulation using OVA₃₂₃₋₃₃₉ peptide. In the present study, incubation of naïve T cells with the combination of IL-2+IL-12+IL-18 lead to an accentuated expression of CD25 and CD44 markers in comparison to cells incubated with single or double cytokine combinations. Indeed the levels of CD25 and CD44 expression induced in response to IL-2+IL-12+IL-18 were greater than those observed after stimulation with specific antigen

Fig. 4.7. *In vitro* stimulation with IL-2+IL-12 polarizes naïve CD4⁺ T cells towards a T_H1 phenotype in the absence of TCR ligation. Naïve CD4⁺T cells purified from the spleens of DO11.10/Rag2^{-/-} mice (1x10⁶ cells/ml in 24 well-plate) were stimulated with the indicated cytokines. As a control, T cells were also stimulated with specific antigen (OVA₃₂₃₋₃₃₉ peptide) plus irradiated syngeneic T cell-depleted splenocytes in the absence of exogenous cytokines. After 7d of culture, the cells were recovered, washed extensively and cultured 1d in the absence of cytokines. The rested cells were then restimulated with plate-bound anti-CD3 and culture supernatants were collected at d3 post-stimulation. Levels of IFN-γ and IL-4 secretion were measured by capture ELISA. The plotted values are the mean of replicate ELISA wells and error bars represent the SEM.

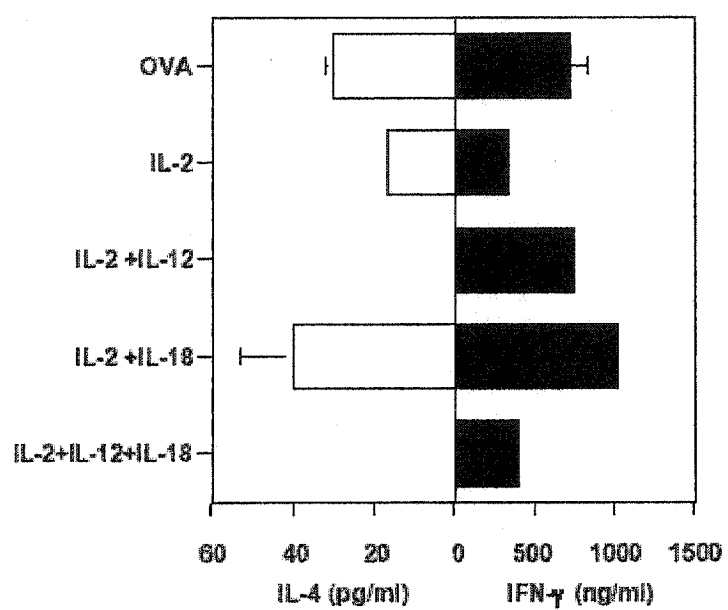
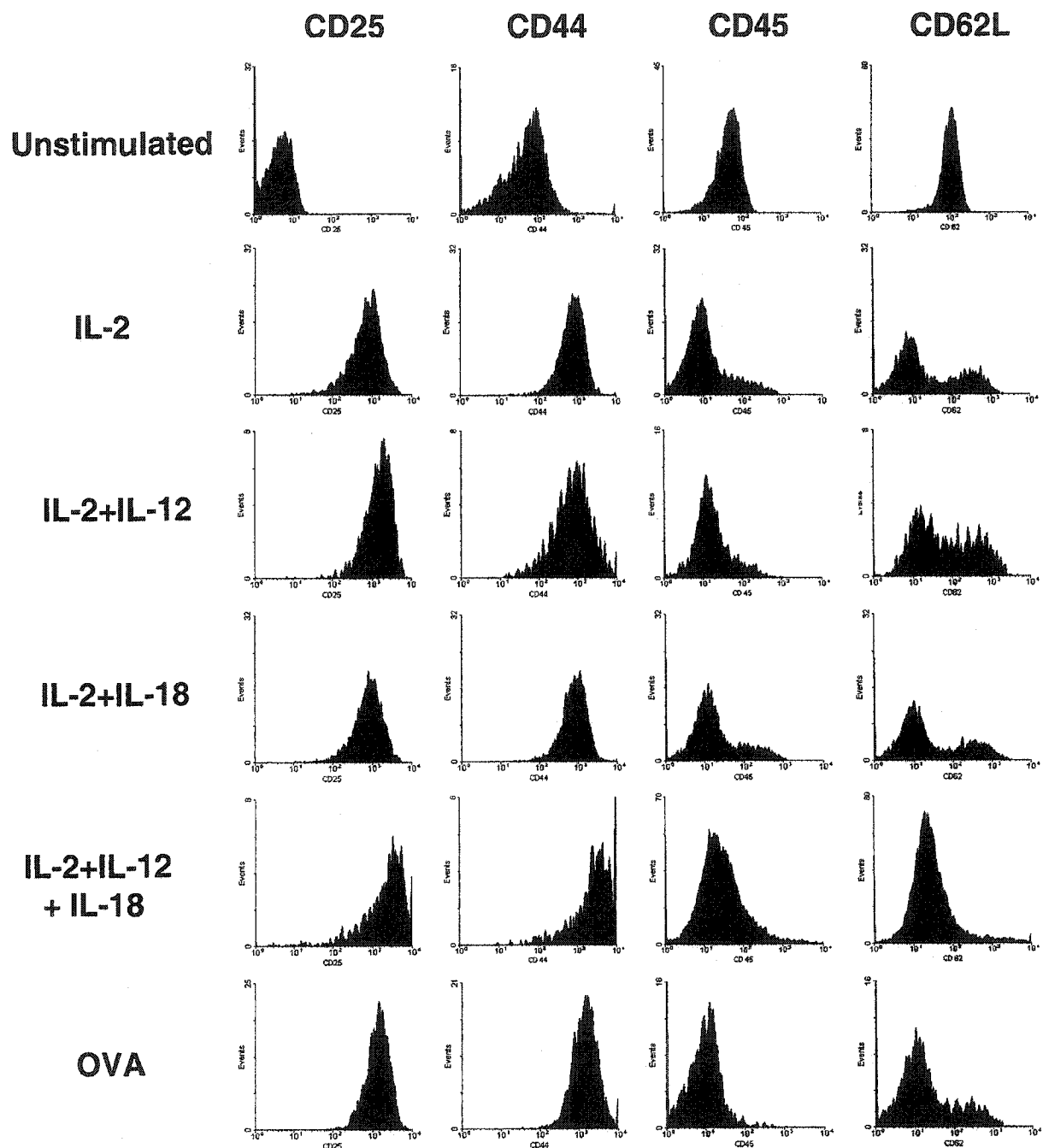


Fig. 4.8. Cell surface activation marker expression by naïve T cells after stimulation with cytokines. Naïve CD4⁺T cells from the spleens of DO11.10/Rag2^{-/-} mice were stimulated with the indicated cytokine combinations or OVA₃₂₃₋₃₃₉ peptide plus irradiated APC as positive control. At d5 post-stimulation, cells were recovered, surface stained with antibodies specific for CD25, CD44, CD45 and CD62L and analyzed by flow cytometry. For OVA peptide stimulation, cells were first gated on the CD4⁺T population.



suggesting that this cytokine combination was more efficient than TCR ligation in terms of inducing expression of the high affinity IL-2R. Likewise, the percentage of cells converting to CD62L^{lo} was actually greater in the presence of IL-2+IL-12+IL-18 than in the presence of specific Ag stimulation. Together these findings indicate that naïve T cells exposed to IL-2 or combinations of IL-2/IL-12/IL-18, in the absence of antigen, exhibited a cell surface phenotype that was consistent with that of activated T cells (CD25⁺,CD44^{hi},CD45^{lo},CD62L^{lo}). In addition, the data imply that signaling via intermediate IL-2R activated naïve T cells to render them responsive to IL-12/IL-18 stimulation.

IV.5.6. IFN- γ secretion by cytokine-activated naïve CD4⁺T cells was CsA-resistant

Since co-activation of naïve CD4⁺T cells with IL-2+IL-12 or IL-2+IL-18 resulted in cell proliferation and IFN- γ production independently of TCR signaling, we were tempted to investigate IL-12/IL-18 responsiveness of IL-2-activated CD4⁺T cells in the presence and absence of CsA. As mentioned above, CsA inhibits any activation of T lymphocytes through TCR. As expected, in naïve CD4⁺T cells stimulated with different cytokines in the presence of anti-CD3 stimulation, IFN- γ production was completely blocked by the presence of CsA (Table-1). In contrast, IL-2+IL-12 or IL-2+IL-18-induced IFN- γ was only partially inhibited by CsA treatment. IL-12+IL-18-induced IFN- γ in the absence of IL-2 and TCR signaling, reported earlier as the “innate pathway” and that was previously shown to be resistant to the presence of CsA in the medium (254), also exhibited partial inhibition by CsA in the culture (Table-1). These data suggest the presence of a TCR-independent pathway able to stimulate CD4⁺T cells with cytokines (IL-12 and/or IL-18) in the absence of any specific antigen but which required a concurrent activation of CD4⁺T cells with IL-2.

IV.5.7. CD4⁺T cells responded to IL-18 independently of IL-12 signaling

Different reports have provided controversial data regarding T cell responsiveness to IL-18 and its dependency upon IL-12 signaling (188,192,193,195). In an attempt to understand the interrelationship between IL-18 and IL-12 signaling, CD4⁺T cells from

	anti- CD3 ^a	anti-CD3 + IL-12 ^b	anti-CD3 + IL-18 ^c	IL-2 ^d	IL-2 + IL-12	IL-2 + IL-18	IL-12 + IL-18
- CsA	42 ± 7 ^f	964 ± 20	121 ± 12	N.D.	2.4 ± 0.6	110 ± 10	>4,000
+ CsA ^e	N.D.	N.D.	N.D.	N.D.	1.1 ± 0.1	44 ± 0.3	948 ± 47

Table 1: IL-2-induced acquisition of IL-12/IL-18 responsiveness (IFN- γ secretion) in CD4⁺ T cells is Cyclosporin A resistant.

CD4⁺ T cells were purified from spleens of naive D011.10 (Rag2^{-/-}) T cell receptor transgenic mice by positive magnetic selection (MACS) and plated in triplicate in 96 well plates at a density of 5x10⁴ cells/well. Cells were stimulated for 7 days in the presence or absence of cyclosporin A using immobilized anti-CD3 and/or cytokines as indicated. At day 7 supernatants were collected and levels of secreted IFN- γ were measured by cytokine capture ELISA. Cyclosporin A completely inhibited the acquisition of IL-12/IL-18 responsiveness through anti-CD3 activation but only partially inhibited IL-2-induced acquisition of IL-12/IL-18 responsiveness.

^a immobilized anti-CD3 (5 μ g/ml)

^b IL-12 used at 1 ng/ml

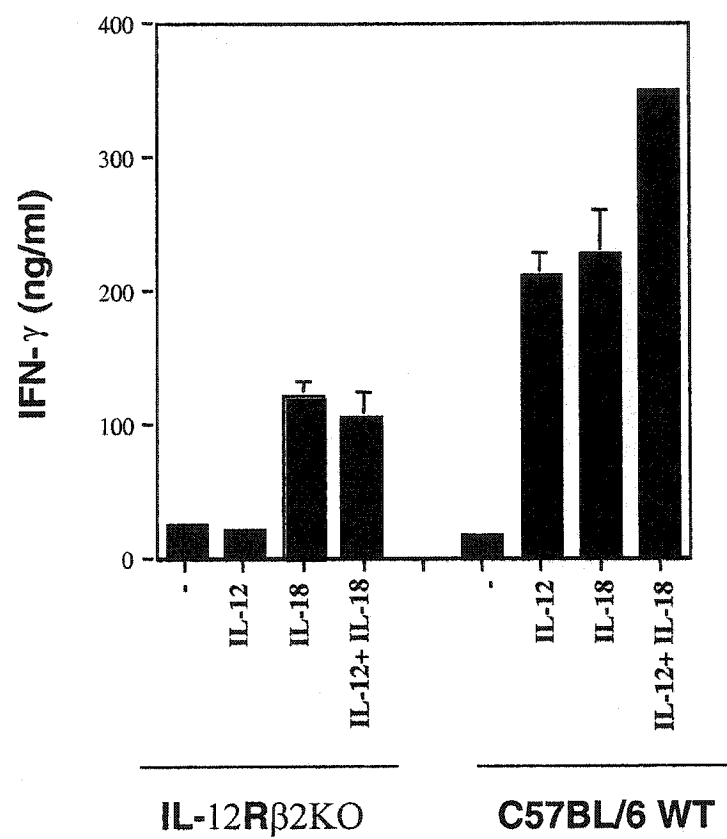
^c IL-18 used at 100 ng/ml

^d IL-2 used at 1000 U/ml

^e CsA (cyclosporin A) used at 100 ng/ml

^f Values represent the mean level of secreted IFN- γ (ng/ml) of triplicate wells $\pm$ the standard error of the mean (N.D. = not detected)

Fig. 4.9. Murine CD4⁺T cells respond to IL-18 stimulation independently of IL-12 signaling. Splenic CD4⁺T cells were enriched from C57BL/6 wild-type mice or from IL-12R β 2KO mice and were plated as duplicates in 96-well-plate (2×10^5 cells/well). Cells were stimulated with 5 μ g/ml plate-bound anti-CD3 and 1 μ g/ml soluble anti-CD28 in the absence (-) or presence of IL-12 (1ng/ml) and/or IL-18 (100 ng/ml). At d3, supernatants were harvested and analyzed for IFN- γ production by ELISA. The plotted values are the mean of replicate ELISA wells and error bars represent the SEM. The figure shows that although lacking IL-12 signaling, CD4⁺T cells from IL-12R β 2KO responded to IL-18 by inducing IFN- γ secretion.



C57BL/6 wild-type and mice lacking the IL-12R β 2 gene (IL-12R β 2KO) were stimulated with anti-CD3/anti-CD28 in the presence or absence of IL-12 and/or IL-18 cytokines (Fig. 4.9). In response to anti-CD3 only (no exogenous cytokines), CD4⁺T cells from both strains of mice secreted low levels of IFN- γ . However, in the presence of IL-12 or IL-18, CD4⁺T cells from C57BL/6 mice exhibited a significant upregulation of IFN- γ . Anti-CD3 stimulation in the presence of IL-12 and IL-18 displayed a synergistic effect on IFN- γ secretion. In contrast, CD4⁺T cells from IL-12R β 2KO mice stimulated with anti-CD3+IL-12 did not show any IFN- γ induction confirming that these cells were defective in IL-12 signaling. However, in response to anti-CD3+IL-18, IL-12R β 2KO-CD4⁺T cells significantly increased IFN- γ production and showed their ability to respond to IL-18 independently of IL-12 activity. In response to anti-CD3+IL-12+IL-18, IL-12R β 2KO CD4⁺T cells did not display any synergy providing further confirmation of their deficiency in IL-12-transduced signals. Similar to the findings for DX5⁺NK cells, these data constitute strong evidence that IL-18 is capable of acting on murine T cells independently of IL-12.

IV.6. Discussion

IL-12 and IL-18 are considered as powerful cytokines bridging the innate and adaptive immune system. IFN- γ -induced by these two cytokines has an important role in the activation of cell-mediated immunity. During the early phases of intracellular infection, IFN- γ produced by NK cells (innate immune system) activates macrophages that increase IL-12 production, which will promote the T_H1 immune response by T cells (adaptive immune system). Naive CD4⁺T cells are reported to be unresponsive to IL-12 or IL-18 and this unresponsiveness has been attributed to the lack of expression of IL-12 and IL-18 receptors prior to activation (145,160). Receptors for both IL-12 and IL-18 are transiently expressed on the surface of CD4⁺T cells after activation via the TCR, rendering these cells responsive to the effects of these two powerful IFN- γ inducers. In agreement with previous reports (145), the results presented in the previous chapter showed that DX5⁺CD3⁻ NK cells (which lack conventional Ag receptors) acquire responsiveness to IL-12 and/or IL-18 after activation with IL-2. More importantly,

DX5⁺CD3⁺NK-T cells were stimulated independently of TCR signaling and were more powerful in terms of IFN- γ production than conventional NK cells when activated with IL-2+IL-12 or IL-2+IL-18. In the present chapter, the results demonstrated that naïve conventional CD4⁺T cells were able to produce IFN- γ in response to high dose IL-2+IL-12 or IL-2+IL-18 in the absence of TCR cross-linking. Consistent with earlier data showing that signaling through the intermediate affinity IL-2R expressed on resting T cells (228) was sufficient to induce cell proliferation in response to either high dose IL-2 (262) or IL-15 (263), the results demonstrated that activation of naïve CD4⁺T cells with high dose IL-2 rendered these cells responsive to IL-12 or IL-18. A similar response has been recently observed in human T cells where signaling via either the IL-2R or IL-15R was sufficient to sensitize CD4⁺T cells to IL-12 in the absence of any antigen stimulation (264). Also, murine T cells have been shown to produce both IL-13 and IFN- γ in response to the combination of IL-2+IL-18 in the absence of any TCR stimulation (175). However, these later experiments utilized bulk CD3⁺ T cells from mice that had been treated *in vivo* with IL-2 thus it was unclear whether the IFN- γ was derived from NK-T, CD4⁺ or CD8⁺ cells or whether it represented *in vivo* activation of a memory T cell pool.

We also observed that CD4⁺CD62L⁺T cells incubated in the presence of IL-12+IL-18 (in the absence of IL-2) proliferated and secreted large amounts of IFN- γ as has been previously reported (254). Although IL-12+IL-18 induced low cell proliferation of CD4⁺T cells in comparison to IL-2+IL-12 or IL-2+IL-18 reported herein, the three combinations of cytokines induced similar effects on CD4⁺T cells in terms of IFN- γ production. These results are also compatible with previous data showing that in the absence of TCR signaling, IL-2 induces IL-12 production via CD40/CD40L interaction between T cells and autologous monocytes and that the resulting IL-12 synergizes with IL-2 to instruct human T cells to produce IFN- γ in a TCR-independent manner (265). Moreover, the results presented here are in agreement with a recent study reporting that murine naïve CD4⁺T cells upregulate CD40L in response to stimulation with IL-18 alone and induce IL-4 and IL-13 cytokines in response to IL-18+IL-2 in an antigen-independent manner (177). The resulting T_H2-specific IL-4 expression was also able to induce IgE-isotype switching in B cells (177).

Phenotypic characterization of murine naïve CD4⁺T cells after activation with high dose IL-2 in the presence or absence of different cytokines indicated that most cells acquired an activated phenotype (CD25⁺CD44^{hi}CD45^{lo}CD62L^{lo}) comparable to what was observed after antigenic activation. This is in agreement with a recent report demonstrating that stimulation of human PBMC with IL-2 results in up-regulation of the IL-2R α chain (CD25) in the absence of antigenic stimulation (262).

The fact that high-dose IL-2 had rendered naïve T cells responsive to IL-12/IL-18 prompted us to examine the polarization of CD4⁺T cells after priming with cytokines alone. As previously reported, control DO11.10/Rag2^{-/-}CD4⁺T cells primed with APC plus OVA₃₂₃₋₃₃₉ specific antigen in the absence of exogenous cytokines secreted both IFN- γ and IL-4 upon secondary stimulation with plate-bound anti-CD3, consistent with a mixed or non-polarized (T_H0) state (7). DO11.10/Rag2^{-/-} CD4⁺T cells incubated with high-dose IL-2 alone also exhibited a similar Th0 phenotype with cells producing both IFN- γ and IL-4 upon anti-CD3 re-stimulation. In accordance with previous data demonstrating that DO11.10/Rag2^{-/-} mice on a B10.D2 background are weak producers of IL-4 (266), the amounts of IL-4 secreted by DO11.10/Rag2^{-/-} CD4⁺T cultured with IL-2 or OVA peptide/APCs in the absence of exogenous cytokines (neutral conditions) were relatively low compared to IL-4 secreted by normal C57BL/6 CD4⁺CD62L⁺T cells. In contrast, cells primed with IL-2+IL-12 or IL-2+IL-12+IL-18 secreted increased levels of IFN- γ and undetectable levels of IL-4 after anti-CD3 stimulation. This later finding implies that exposure of naïve CD4⁺T cells to the combination of IL-2+IL-12 was capable of inducing polarization towards the T_H1 phenotype in the absence of TCR signaling. These findings are remarkable in that they suggest that the ultimate phenotype of CD4⁺T cells may be influenced prior to antigen exposure. Although we have yet to analyze whether bystander polarization is transient or whether it can be maintained for longer periods of time, the results potentially have profound implications for the immune status of individuals suffering from acute or chronic immune stimulation. We have thus coined the term 'bystander polarization' to describe this phenomenon. If 'bystander polarization' is a biologically relevant phenomenon that occurs both *in vitro* and *in vivo*, it will be necessary to develop a means of discriminating between the activated/memory pool that forms as a result of antigen exposure and the activated/memory pool that forms

as a result of 'bystander polarization'. Furthermore, if cells subjected to bystander polarization are maintained *in vivo* for extended periods of time then the T cell pool which is considered 'memory' in terms of phenotypic markers may actually be comprised of both antigen-experienced and antigen-naïve (bystander polarized) T cells.

Although the mechanisms behind up-regulation of IL-12R on T cells remain poorly understood, a number of recent studies have suggested that TCR-dependent up-regulation of IL-12R may actually be IL-2 dependent (204,257). Furthermore, IL-12-dependent up-regulation of IFN- γ transcription can be blocked by addition of cyclohexamide (267) suggesting that new protein synthesis in general and IL-2 in particular is required for IL-12-induced responses. Together these data and the data presented herein suggest that signaling via the IL-2R may in fact be the primary mechanism for rendering T cells IL-12/IL-18 responsive. Thus TCR ligation simply elicits the production of IL-2 which itself up-regulates the surface expression of IL-12R and IL-18R and IL-12/IL-18 responsiveness.

Previously, it was thought that naïve CD4⁺T cells only expressed functional IL-12/18 receptors during TCR-dependent activation via antigen, mitogen or anti-TCR antibodies. In this manner T_H1 skewing or polarization would be restricted to periods of TCR stimulation. However, RPA data reported herein demonstrated that in response to IL-2, naïve CD4⁺T cells proliferated, produced cytokines and upregulated β 1 and β 2 chains of IL-12R. This suggested that, in the peripheral lymphoid tissue, naïve CD4⁺T cells may be influenced by an ongoing immune response to an unrelated antigen, if that response is strong enough to elevate local IL-2 concentrations to levels sufficient to render bystander T cells responsive to IL-12 and/or IL-18. Similarly, in accordance with recent data using human T cells (264), exposure to IL-15, *in vivo*, may also render naïve T cells responsive to the polarizing effects of IL-12/IL-18. Also, in agreement with the fact that IL-18 is the only cytokine capable of inducing simultaneously both T_H1 and T_H2 cytokine production (149,158), the data reported that in response to IL-2+IL-18, naïve CD4⁺T cells increased cytokine gene expression of type 1 (IFN- γ) as well as type 2 (IL-5, IL-13) cytokines. However, naïve T cells incubated in the presence of IL-2+IL-12 only exhibited accumulation of IFN- γ transcripts with barely detectable levels of IL-10 mRNA.

IL-12/IL-18-induced IFN- γ (in the absence of IL-2 or TCR signaling) has been previously reported as the “innate pathway” of T cell activation and it differs from conventional TCR-mediated IFN- γ induction in that it is unaffected by the presence of cyclosporin A (CsA) (256). IFN- γ induced by this “innate pathway” involves STAT4 and NF- κ B activation (254). Whether this pathway modifies TCR signaling or involves a different pathway is still unclear. Experiments involving CsA treatment showed herein, suggested that similar to the “innate pathway” of T cell activation, IL-2+IL-12-induced IFN- γ generated during “bystander polarization” of naïve CD4⁺T cells towards a T_H1 phenotype was CsA-resistant. In contrast, IFN- γ induced in response to TCR activation (anti-CD3) was completely blocked by CsA confirming that IL-2 rendered CD4⁺T cells responsive to IL-12 independently of TCR cross-linking.

Finally, it is worth emphasizing that the cytokine driven production of IFN- γ by T cells reported herein is more reminiscent of the innate responses of NK cells rather than adaptive immunity since it occurs in an Ag-independent manner. Indeed both T cells and NK-T cells respond to the combination of IL-12+IL-18 in the absence of specific Ag by synthesizing large amounts of IFN- γ (168). These antigen-independent responses, together with the bystander responses described herein, have the potential to contribute to host protection during the early phases of an immune response against infection, prior to the successful clonal expansion of antigen-specific T cells. Despite the obvious potential benefits of having a capability for widespread and rapid responsiveness to pro-inflammatory cytokines such as IL-12 and IL-18, such responsiveness also represents a double-edged sword for the host. This is particularly evident in studies where co-delivery of IL-2+IL-12 results in rapid onset of septic shock-like symptoms and mortality within a few days (268). Together these results confirmed the powerful and profound effects that IL-2, IL-12 and IL-18 have on both the innate and adaptive immune system. Bystander polarization can now be added to the list of these effects and we propose that bystander polarization may have far reaching implications, both positive and negative, in terms of health and disease. For example, significant bystander polarization early in life due to a chronic infectious disease might result in the accrual of a peripheral T cell pool with a pre-disposition for T_H1 polarization and thus an increased potential to develop T_H1-associated diseases later in life. Alternatively, a lack of significant bystander polarization

due to successful vaccination against infectious diseases in combination with a highly sterile environment early in life might contribute to an increased disposition to develop future T_H2 -mediated diseases such as allergy and asthma. Certainly, the mechanisms and potential consequences of bystander polarization warrant further investigation.

CHAPTER V

**Constitutive expression of IL-12R β 2 subunit does not alter T_H2
cell stability and does not confer resistance to *L. major* -
susceptible mice**

V.1. Summary

T helper cells differentiate towards a T_H1 phenotype in response to IL-12 and towards a T_H2 phenotype in response to IL-4 stimulation. T_H1 cells respond to IL-12 via signaling through the IL-12R β 2 chain. This results in IFN- γ production that has been shown to block the effects induced by IL-4 and to help T_H1 cells maintain the IL-12R β 2 chain expression upregulated (294). In contrast to T_H1 cells, previous studies have shown that in T_H2 cells, the IL-12R β 2 chain is downregulated by the presence of IL-4 (269). Downregulation of IL-12R β 2 expression results in the extinction of IL-12 signaling to then allow CD4⁺T cell differentiation towards a T_H2 phenotype. This suggests that IL-12R β 2 chain is regulated both positively and negatively. The best functional model to study such T_H1/T_H2 differentiation is infection of mice with the intracellular parasite *L. major* (91) where the outcome of infection depends on the strain of mice infected. C57BL/6 mice for example (known as a resistant strain) develop a T_H1 immune response characterized by high levels of IFN- γ required to kill the parasites (270). This results in the clearance of the infection and healing [(91) and Fig. 5.1]. In contrast, when BALB/c mice (susceptible strain) are infected with *L. major*, they develop a chronic and fatal T_H2 response a few weeks after infection (Fig. 5.1). The basis of T_H2 cell development in these susceptible mice has been extensively studied but is yet not well understood. Infected BALB/c mice exhibit a burst of IL-4 in the draining lymph nodes early during infection. This source of IL-4 is thought to be derived from a particular CD4⁺T cell population in response to *L. major* antigen. These CD4⁺T cells express a restricted TCR repertoire (V β 4/V α 8) and expand in response to the *Leishmania* protein (LACK antigen) homologue of mammalian intracellular receptors for activated protein kinase C (RACK1) (30,32,57). Taken together, these different studies reported above suggest that regulation of IL-12R β 2 chain expression might be critical for the development of a T_H1 phenotype and might be necessary for the development and stability of a T_H2 phenotype. In an attempt to understand the mechanism governing T_H1/T_H2 cell differentiation, BALB/c mice that constitutively express the IL-12R β 2 subunit were generated and the role played by IL-12R β 2 expression *in vitro* and *in vivo* was analyzed. The results reported in the present chapter demonstrate that IL-12R β 2 expression was not necessary for the

development and stability of a T_H2 phenotype and that although constitutively expressing IL-12Rβ2 chain, T_H2 cells from transgenic mice did not revert to a T_H1 phenotype in response to exogenous IL-12. Furthermore, BALB/c mice transgenic for the IL-12Rβ2 gene remained susceptible to *L. major* infection and did not develop the adequate T_H1 immune response necessary to heal the infection.

V.2. Hypothesis

Downregulation of the IL-12Rβ2 chain is critical for the stability of a T_H2 cell phenotype. Generation of BALB/c mice that constitutively express IL-12Rβ2 chain under the control of the human CD2 promoter will exhibit T_H2 to T_H1 reversal in response to IL-12. This reversibility will have consequences on the pattern of susceptibility developed by BALB/c mice in response to *L. major* infection.

V.3. Objectives

- Generate transgenic BALB/c mice constitutively expressing IL-12Rβ2 under a promoter that is not inhibited by the early production of IL-4 upon T-cell activation. The promoter proposed in this project is the CD2 promoter that is constitutively expressed in T cells.
- Analyze the *in vitro* role of IL-12Rβ2 constitutive expression in T_H2 cell development.
- Study reversibility of T_H2 to T_H1 phenotype *in vitro* in IL-12Rβ2-transgenic BALB/c mice.
- Define the role played by the constitutive expression of IL-12Rβ2 *in vivo* using *L. major* infection model.

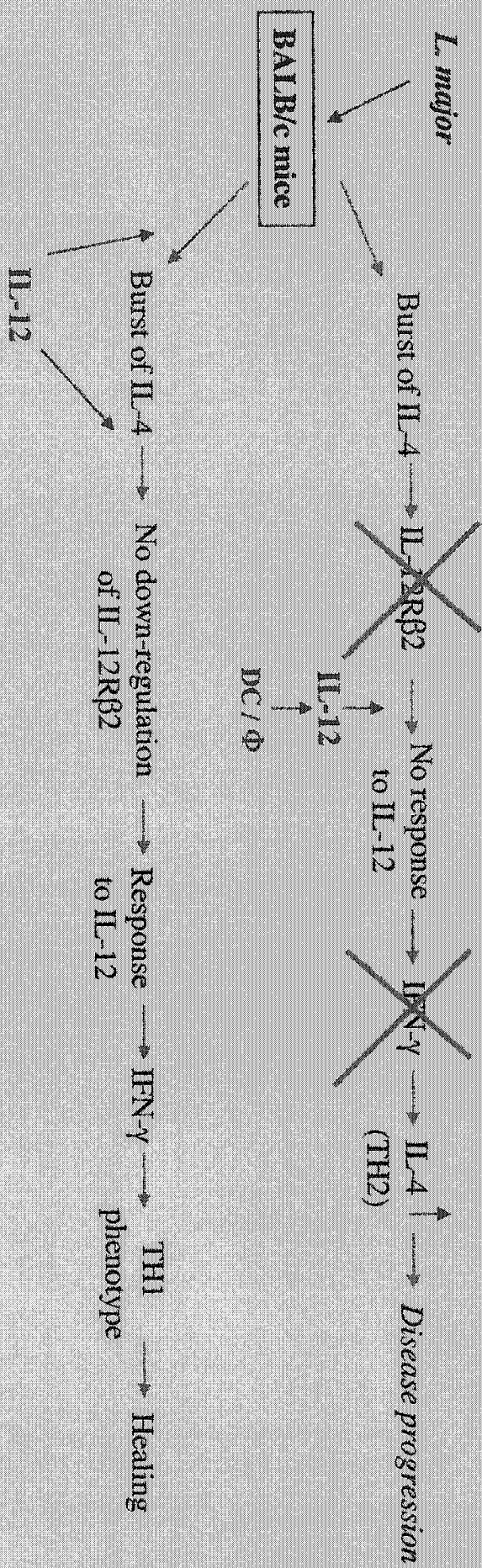
V.4. Materials and Methods

V.4.1. Generation of a plasmid DNA construct containing the murine IL-12Rβ2 gene

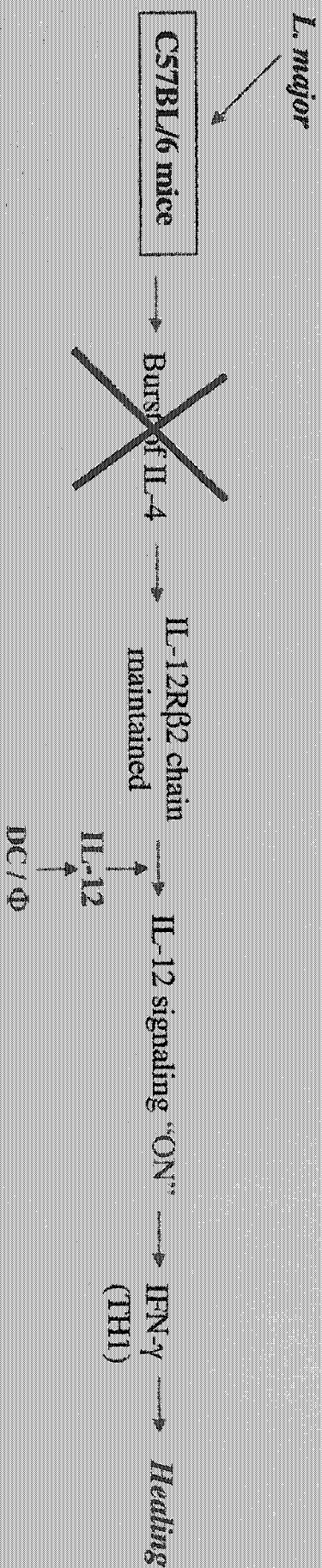
In order to generate transgenic BALB/c mice that constitutively express IL-12Rβ2 chain, a DNA construct, which included the complete murine IL-12Rβ2 gene (102) inserted in the VACD2 minigene vector (271) was prepared. This construct expresses the IL-12Rβ2 gene under the control of the CD2 promoter. The choice of this

Fig. 5.1. T_H1/T_H2 development in the model of murine leishmaniasis. Mice infection with the intracellular parasite *L. major* constitutes the best model to study T_H1/T_H2 cell differentiation depending on the mice strains. (A) BALB/c mice, known as susceptible strain, develop a non-healing T_H2-immune response. BALB/c mice express a burst of IL-4 in draining LN early during infection, which inhibits T_H1 cell development by extinction of the IL-12-signaling. IL-12 given to BALB/c mice early during infection, can rescue them from the infection by inducing an appropriate T_H1 immune response. (B) In contrast, C57BL/6 mice, known as resistant strain, do not exhibit any burst of IL-4 after *L. major* infection and maintain responsiveness to IL-12 released late by parasite-infected DC and macrophages (Φ). Thus, C57BL/6 mice develop a healing T_H1 immune response and resolve the infection. (C) To investigate whether IL-12Rβ2 downregulation was required for T_H2 cell development and stability, transgenic BALB/c mice constitutively expressing the IL-12Rβ2 gene were generated. IL-12Rβ2 gene was expressed under the human CD2 promoter that is not downregulated by the presence of IL-4 in the environment. The objective was to answer some of the raised questions about reversibility of the T_H2 cells from VAIL-12Rβ2-transgenic mice to T_H1 phenotype in response to IL-12 stimulation by investigating the *L. major* infection outcome developed by these transgenic BALB/c mice constitutively expressing IL-12Rβ2 gene

A) *Leishmania major* infection of BALB/c mice: Susceptible mice



B) *Leishmania major* infection of C57BL/6 mice: Resistant mice



C) *Leishmania major* infection of VAIL-12R β 2-transgenic BALB/c mice

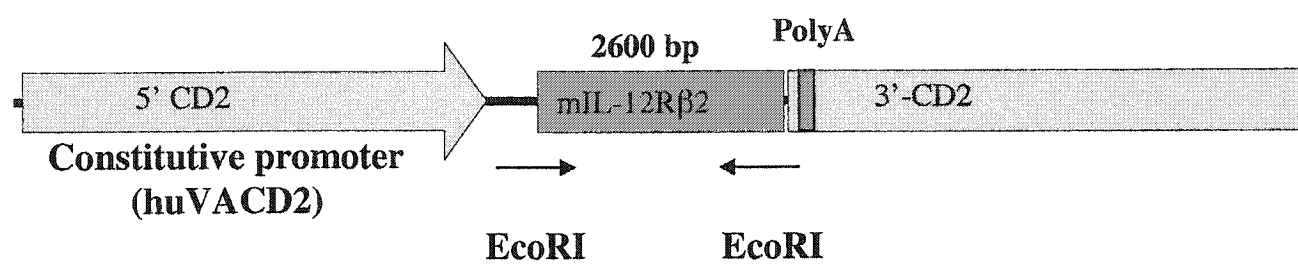


promoter is based on the fact that it controls the constitutive expression of the cell surface molecule CD2 (LFA-2) on cells of T cell lineage. VACD2, obtained from Dr. Dimitris Kioussis (MRC, UK), contains 5' and 3' flanking regions of human the CD2 gene and a multiple cloning site (MCS) to insert the DNA of interest (271). A cDNA clone, containing the complete murine IL-12R β 2 chain (102), was kindly provided by Dr. Kenneth Murphy (Howard Hughes Institute, Washington University). The IL-12R β 2 ORF (2600 bp) was amplified from the cDNA clone by PCR to allow the addition of EcoR1 restriction sites at either ends using specific primers (Appendix A.5.2). The PCR product was then subcloned into the pCR2.1-TOPO vector (Invitrogen) using the TA cloning protocol following the manufacturer's instructions. After plasmid amplification using TOP10 competent *E. coli*, and purification using a standard kit for plasmid purification (Qiagen), the insert was digested with EcoR1 restriction enzyme and subcloned into EcoR1-digested-VACD2 using the Rapid DNA Ligation kit (Boehringer Mannheim). The plasmid obtained was subsequently called VAIL-12R β 2 (Fig. 5.2). The presence of the insert and its correct orientation were confirmed by digestion of the plasmid with EcoRI and BamHI restriction enzymes (Life Technologies) respectively. The accuracy of the PCR-generated DNA and the construct VAIL-12R β 2 were confirmed by DNA-sequencing on 373 STRECH Automated Sequencer (Applied Biosystems). Sequencing was performed by the staff at the Biotechnology Research Institute at the faculty of Medicine (University of Ottawa). The insert of VAIL-12R β 2 construct was also used for *in vitro* transfection studies using the Jurkat cell line.

V.4.2. Generation of a plasmid DNA construct containing the murine IL-12R β 1-gene

5 x 10⁶ BALB/c splenocytes were cultured in 6 well-plates and activated *in vitro* with 2.5 μ g/ml ConA in the presence and absence of 20 U/ml huIL-2. At day 3, cells were harvested for total RNA extraction using Trizol reagent and cDNA was produced by RT-PCR. The IL-12R β 1 ORF was amplified by PCR using murine IL-12R β 1 primers

Fig. 5.2. Cloning of mIL-12R β 2 gene in VACD2 vector. To generate transgenic BALB/c mice constitutively expressing the IL-12R β 2 gene, a 2600 bp EcoR1-fragment containing the complete mouse IL-12R β 2 cDNA was inserted into the VACD2 minigene cassette digested with the same EcoR1 restriction enzyme. The construct obtained was termed VAIL-12R β 2 and was digested with Sall and NotI restriction enzymes before injection into mouse hosts to eliminate the bacterial DNA.



containing 5' and 3' EcoR1 restriction sites (refer to Appendix A.5.1 for murine IL-12R β 1 specific primer sequences). The PCR product was subsequently subcloned into the pcDNA3.1/neo plasmid vector (Novagen) under the control of cytomegalovirus-enhancer-promoter digested with EcoR1 restriction enzyme. This vector encodes for resistance to neomycin (G418). The construct was named pcIL-12R β 1 and was used for *in vitro* transfection of the Jurkat cell line.

V.4.3. Generation of VAIL-12R β 2-transgenic BALB/c mice

VAIL-12R β 2 plasmid, containing the insert in the correct orientation, was amplified by transformation of Max Efficiency DH5 α Competent Cells (see protocol in Appendix A.6.6). Amplified plasmid was then purified using Qiafilter Maxi Prep Tip 500 (Qiagen) following the manufacturer's instructions. 100 μ g of VAIL-12R β 2 construct were linearized with EcoR1/ and digested using NotI/SalI restriction enzymes (Life Technologies) to remove all bacterial DNA sequences. The resulting 14 kbp construct was purified and used for mouse oocyte microinjection.

VAIL-12R β 2-transgenic (tg) founder mice were generated by the staff at the Transgenic Mouse Facility (Ottawa Regional Cancer Center) under the supervision of Dr. Barbara Vanderhyden (Fig. 5.2). Because the microinjection of VAIL-12R β 2 cDNA into oocytes from female BALB/c mice was difficult to achieve, the staff of the Transgenic Facility chose to microinject oocytes obtained from (FVB/N x BALB/c) F1 females. A backcross of five generations of (FVB/N x BALB/c) screened as positive VAIL-12R β 2-tg mice to BALB/c strain was suitable to obtain IL-12R β 2-tg mice of pure BALB/c background (272,273).

V.4.4. Screening for potential IL-12R β 2-transgenic (tg) founders

V.4.4.1. Preparation of genomic DNA (gDNA) from tail clip tissue

About 1 cm of tail clip tissue was obtained from three week old potential VAIL-12R β 2-tg mice and used to prepare gDNA using DNeasy Tissue Kit (Qiagen) following the manufacturer's instructions.

V.4.4.2. Generation of specific VAIL-12R β 2 probe

The DNA probe used for Southern blot analysis of VAIL-12R β 2-tg mice is schematically reported in Fig. 5.3. A ^{32}P -radioactive probe was synthesized as follows: 10 μg VAIL-12R β 2 DNA plasmid was digested with BamHI restriction enzyme for 2 h at 37°C to generate two bands of 1600 and 5000 bp. The DNA digest was run on a 0.8% agarose gel and the 1600 bp band was purified from the gel. The purified DNA fragment was used as template to synthesize the ^{32}P -radioactive DNA probe. Therefore, 25 ng of 1600 bp-DNA fragment was resuspended in ddH₂O in an Eppendorf tube to a final volume of 13.5 μl and was denatured by boiling the tube 3 min at 100°C. The tube was transferred to 37°C water bath for 10 min then kept on ice until use. The following reagents were added to the tube for radioactive probe synthesis: 5 μl of the Oligo-labeling (OLB) buffer (see composition in Appendix A.4.4), 1 μl of 5 $\mu\text{g}/\text{ml}$ DNase-free BSA (Sigma), 5 μl of [α - ^{32}P]-dCTP at 10 $\mu\text{Ci}/\mu\text{l}$ (Amersham Pharmacia Biotech) and 0.5 μl Klenow (DNA polymerase I at 7 U/ml) (Life Technologies). The mixture was incubated 3 h at RT. The radioactive probe DNA was then purified from unincorporated nucleotides using QIAquick Nucleotide Removal Kit (Qiagen) following the manufacturer's instructions. 25 ng of purified radioactive probe was denatured 3 min in boiling water before hybridization. The radioactive probe was used to detect the VAIL-12R β 2 transgene in mice genomes by Southern blot analysis. The generated probe would be able to hybridize with gDNA fragments of 2000 and 2400 bp in VAIL-12R β 2-tg gDNA digested with NcoI restriction enzyme (Fig. 5.3).

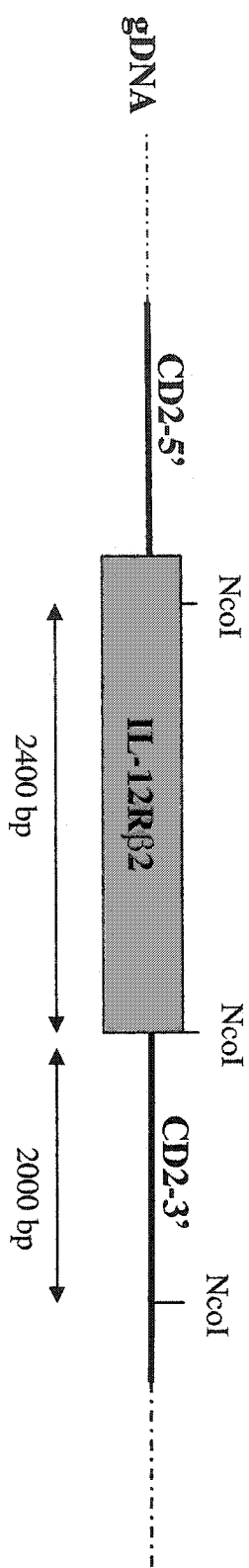
V.4.4.3. Southern blot analysis of potential VAIL-12R β 2-tg founders

Screening for potential transgenic founder mice that incorporated the VAIL-12R β 2-transgene was performed using Southern blot analysis of gDNA purified from tail clip tissue of fourteen mice aged three weeks. A Southern blot experiment was performed as follows: 6 μg gDNA from each mouse were digested with NcoI restriction enzyme (Life Technologies) for 4h at 37°C and the digests were kept at -20°C until the next day. Digested gDNA was extracted using phenol/chlorophorm precipitation (Appendix A.6.2) and was separated by electrophoresis on a 0.8 % agarose gel at 60 V. The gel was then rinsed with distilled H₂O before denaturation.

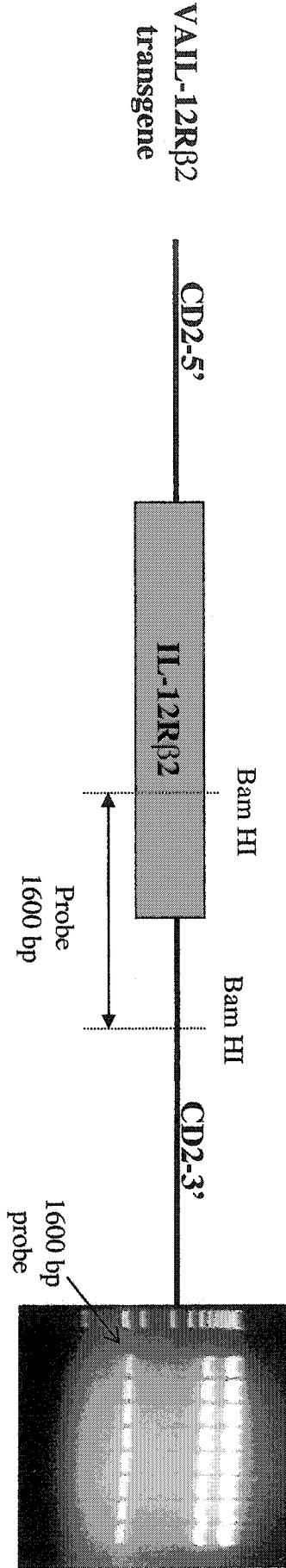
a. Denaturation of gDNA gel

Fig. 5.3. Design of the VAIL-12R β 2 probe for Southern-blot analysis. [32 P]-radiolabeled cDNA probe used in Southern blot screening of potential VAIL-12R β 2-tg founders was designed based on the restriction map of the VAIL-12R β 2 construct. The generated probe has the capacity to detect two DNA fragments of 2000 and 2400 bp in NcoI-digested gDNA from positive VAIL-12R β 2-tg mice.

Representation of genomic DNA from potential positive VAIL-12R β 2-transgenic mice:



Representation of DNA probe used for transgenic mice screening:



The different steps of DNA gel denaturation were carried out at RT with slow shaking. The gel was placed in denaturing buffer (0.5 M NaOH and 1.5 M NaCl in ddH₂O) for 30 min, rinsed with ddH₂O then transferred to neutralizing buffer (0.5 M Tris-HCl, pH 7.0 and 1.5 M NaCl in ddH₂O) for 30 min at RT. The gel was ultimately soaked in 20 X SSC transfer buffer (3 M NaCl and 0.3 M Na-citrate in ddH₂O, pH 7.0) for 30 min.

b. DNA transfer to nylon membrane

Nytran SPC Nylon transfer membrane (Schleicher & Schuell) was immersed in ddH₂O then equilibrated 5 min in 20 X SSC transfer buffer. DNA transfer was performed using Turboblotter Rapid Downward Transfer system (Schleicher & Schuell) following the manufacturer's instructions. At the end of the transfer, the agarose gel was stained for 30 min with 0.1% ethidium bromide in the Tris-Acetate-EDTA (TAE) buffer (for a 10 X stock solution, 0.4 M Tris-acetate (Sigma); 0.2 M glacial acetic acid (BDH) and 0.01M EDTA (Sigma) in ddH₂O, pH 8). The DNA transfer was assessed by UV transillumination. The membrane was then dried at RT, was wrapped in a plastic wrap and placed DNA-side-down on Fotodyne 3-3000 UV transilluminator for 5 min to cross-link the gDNA to the membrane.

c. Southern-blot analysis of potential VAIL-12Rβ2-tg founders

* Pre-hybridization

Pre-hybridization was carried out at 65°C for 3h in heat-sealed plastic bags using 5 ml of pre-hybridization solution (6 x SSC buffer, 0.5 % SDS and 5 x Denhardt's solution (Appendix A.4.2) in ddH₂O) per 100 cm² of membrane. 0.5 ml of a 1 mg/ml solution of sonicated, heat denatured salmon sperm DNA (Life Technologies) was boiled for 5 min at 100°C, chilled on ice then added to the pre-hybridization to block the nonspecific attachment of the probe DNA to the surface of the membrane.

* DNA blot hybridization

Membrane hybridization was performed overnight at 65°C. The membrane was incubated with 25 ng of radioactive probe (25 µl) in 5 ml of pre-hybridization solution per 100 cm² of membrane. The next day, the membrane was washed from non-bound probe using different washing solutions. Washing of the hybridized membrane was performed with increasing stringency of the washing solution, which is obtained by decreasing the salt concentration of the washing solution. The wash steps were as follows: 1) 15 min at 65°C

in 250 ml of 2 x SSC solution with shaking. 2) 30 min at 65°C in 250 ml of 2 x SSC solution containing 0.1% SDS. 3) 10 min at 65°C in 250 ml of 0.2 x SSC solution. 4) the membrane was soaked in 6 x SSC solution at RT. Finally, the membrane was wrapped in Saran Wrap and exposed to X-ray film (XAR, Eastman Kodak) for 3 to 7 days at - 80°C. Two mice exhibited low copy number of the transgene (tg 9379 and 9316) and one mouse displayed relatively higher copy number (tg 9380). The mouse # tg 9380 was mated to pure BALB/c mice at the Animal Care Facility of the University of Ottawa to generate positive VAIL-12R β 2-tg mice of BALB/c background. Tg mice were mated to pure BALB/c mice for five generations and were kept heterozygous for the VAIL-12R β 2 transgene.

V.4.5. Screening for positive carriers of VAIL-12R β 2-tg by PCR

Each generation of (FVB/N x BALB/c) backcrossed to BALB/c mice was screened for the presence of the transgene by PCR amplification of VAIL-12R β 2 from 100 ng gDNA purified from mouse tail clip tissue. IL-12R β 2-5'-1600 and CD2-3'-UTR were used as sense and anti-sense primers (Appendix A.5.4.1) respectively. PCR reactions were carried out as follows: for each PCR reaction, a master mix containing 5 μ l of 10 X PCR buffer (Amersham Pharmacia Biotech), 8 μ l of 1.25 mM dNTPs mixture (dATP, dTTP, dGTP, dCTP) (Amersham Pharmacia Biotech), 2.5 μ l of 20 μ M IL-12R β 2-5'-1600 and CD2-3'-UTR primers, 5 μ l DMSO, 0.5 μ l Taq DNA polymerase at 5000 U/ml was prepared and completed to 50 μ l with ddH₂O. A volume of 45 μ l of master mix were dispensed in each 200 μ l reaction tubes. 5 μ l templates containing 100 ng gDNA were added to the master mix in duplicates. No template (5 μ l H₂O) or 1 ng of VAIL-12R β 2 plasmid were used as negative and positive controls, respectively. PCR reaction tubes were vortexed and briefly spun to collect the samples. Using the Programmable Thermal Controller PTC-100 (MJ Research), the PCR reactions were amplified as follows: 5 min in 94°C, 35 cycles of 1 min at 94°C (denaturation), 1 min at 50-55°C (annealing) and 3 min at 72°C (elongation) followed by 10 min at 72°C to allow complete elongation of all product DNA. The PCR reactions were analyzed for the

presence of the transgene (1000 bp) by electrophoresis on 0.8% agarose gels. Mice bearing VAIL-12R β 2 in their genome were backcrossed to pure BALB/c mice.

V.4.6. Analysis of VAIL-12R β 2 gene expression in the VAIL-12R β 2-tg mice

V.4.6.1. cDNA synthesis

VAIL-12R β 2 transgene expression was analyzed by RT-PCR in different generations of tg mice using total RNA isolated from tail clip tissue or isolated from the spleen of one representative mouse. The expression of the IL-12R β 2 gene was also assayed *in vitro* in Jurkat cells transfected using the IL-12R β 2 insert from the VAIL-12R β 2 plasmid. Total RNA samples were processed for RT-PCR using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Life Technologies). In a 200 μ l nuclease-free tube, 1 to 5 μ g total RNA were combined with 1 μ l of 0.5 mg/ml Oligo-dT (Amersham Pharmacia Biotech) and RNase free H₂O to a final volume of 12 μ l. The mixture was heated 10 min at 70°C and chilled quickly on ice. The mixture was spun briefly at 10,000 x g and 4 μ L of 5 x 1st Strand buffer, 2 μ L of 0.1 M DTT plus 1 μ L of 10 mM dNTP mix (dATP, dGTP, dCTP and dTTP) were added followed by 2 min incubation at 37°C to equilibrate the mixture. 1 μ L M-MLV reverse transcriptase (at 200 U/ml) was then pipetted into the mixture and the reaction was extended with 50 min incubation at 37°C. In the final step, heating 15 min at 70°C inactivated the reaction and the cDNA was ready to be used as a template for PCR amplification of the transgene or stored at -20°C.

V.4.6.2. PCR amplification of VAIL-12R β 2 from generated cDNA

Reverse transcription was followed by PCR amplification of the cDNA template using IL-12R β 2-2000 and CD2-UTR as sense and anti-sense primers respectively (Appendix A.5.4.2). PCR master mix and running conditions were as reported above (section V.4.5).

V.4.7. *In vitro* expression of VAIL-12R β 2 gene

V.4.7.1. Cell line culture and maintenance

The human leukemia Jurkat cell line was obtained from Dr. Mark Freedman (Division of Neurology at the Ottawa Hospital, General Campus). Jurkat cells were grown in RP-10 and expanded in fresh medium every 2-3 d of culture.

V.4.7.2. Jurkat cell transfection with IL-12R β 2 gene

In order to ensure the expression of the VAIL-12R β 2 construct used to generate the IL-12R β 2-tg mice, a stable transfection of the Jurkat cells was performed using Eugene-6 reagent (Roche). For this purpose, 2 μ g of VAIL-12R β 2 as well as 2 μ g of pcDNA3.1/zeocin vector (Novagen) were digested with EcoRI restriction enzyme. The digests were separated on a 0.8% agarose gel. The IL-12R β 2 insert of 2600 bp and the vector pcDNA3.1/zeo/EcoRI were cut from the agarose gel, purified using Prep-A-Gene DNA Purification System (BioRad) and ligated using the Rapid DNA Ligation kit (Boehringer Mannheim). The ligation reactions were used to transform Max Efficiency DH5 α Competent Cells (see protocol in Appendix A.6.6). Amplified pCDNA3.1/zeo containing the IL-12R β 2 gene (termed pcIL-12R β 2/zeo) was purified using Qiafilter Maxi Prep Tip 500 (Qiagen) following the manufacturer's protocol. The plasmid was analyzed for the presence and for the correct orientation of the IL-12R β 2 insert by digestion with EcoRI and BamHI restriction enzymes, respectively. The correct plasmid was confirmed by sequencing.

One day before transfection experiments, exponentially growing Jurkat cells were seeded at a density of 5 to 8 x 10⁵ cells per ml in β -2ME-starved fresh RP-10 medium. The next day, 2 μ g of pcIL-12R β 1/geneticin or pcIL-12R β 2/zeocin were used to prepare the DNA/Eugene-6 reagent complex in OptiMEM medium (Life Technologies) following the manufacturer's protocol (Boehringer Mannheim). As control, 2 μ g of Green fluorescent protein (GFP) plasmid (pML-8), obtained from Dr. M.W. McBurney (Ottawa Regional Cancer Center), were used. 1 ml aliquots of growing Jurkat cells (8 x 10⁵ cells) were placed in 6 well-plates and the transfection was performed by distributing the DNA/Eugene-6 reagent complexes around the wells. The cells were returned to the incubator at 37°C. At d2, transfection yield was determined by analyzing GFP-transfected cells by microscopy using an inverted microscope (Axioverts S100TV, Zeiss) at the laboratory of Dr. Stephen Lee (Cellular Molecular Medicine Department, University of

Ottawa). Transfected-Jurkat cells were then washed and seeded in RP-10 containing 0.75mg/ml geneticin (neomycin, G418) (Life Technologies) or 0.5mg/ml zeocin to select the Jurkat cells stably transfected with pcIL-12R β 1 or pcIL-12R β 2 plasmids, respectively. At d15 of culture, cells showing resistance to antibiotics were harvested for gDNA and RNA preparations. A second stable transfection of IL-12R β 2-transfected Jurkat cells using pcIL-12R β 1 plasmid was performed to obtain a Jurkat cell line that co-expresses both IL-12R β 1 and IL-12R β 2 subunits. These double transfected cells were grown in RP-10 medium and selected against 0.5mg/ml zeocin plus 0.75 mg/ml neomycin. The integration of pcIL-12R β 1 and/or IL-12R β 2 constructs into the Jurkat cell genome was investigated by PCR amplification of the IL-12R β 1 and IL-12R β 2 from gDNA using specific primers (Appendix A.5.1 and A.5.2).

V.4.7.3. Expression of IL-12R β 2 in transfected Jurkat cells

IL-12R β 2 and/or IL-12R β 1-transfected Jurkat cells were harvested for RNA preparation. cDNA was generated from total RNA by RT-PCR and IL-12R β 2 transgene was amplified by PCR using IL-12R β 2-2000-5' (sense) and IL-12R β 2-3'UTR (anti-sense) primers (Appendix A.5.4.4).

V.4.8. Functionality of VAIL-12R β 2 transgene *in vivo*

V.4.8.1. Generation of IL-12R β 2KO mice expressing VAIL-12R β 2 transgene

In order to test the functionality of the VAIL-12R β 2 transgene *in vivo*, VAIL-12R β 2-tg mice were bred to IL-12R β 2KO mice (118). gDNA from tail clip tissue of the F1 generation was screened for the expression of VAIL-12R β 2 transgene by PCR. The VAIL-12R β 2⁺ F1 mice were backcrossed to pure IL-12R β 2KO mice. gDNA from the F2 generation was screened by PCR for the expression of the VAIL-12R β 2 transgene using IL-12R β 2-1600-5' and IL-12R β 2-3'UTR primers and for the endogenous IL-12R β 2 using IL-12R β 2-5' and IL-12R β 2-3'-70 primers (Appendix A.5.4.3). IL-12R β 2KO mice not expressing the endogenous IL-12R β 2 gene but which integrated the VAIL-12R β 2 transgene only (VAIL-12R β 2⁺/IL-12R β 2KO mice) were then used for CD4⁺ T cell separation.

V.4.8.2. Functionality of VAIL-12R β 2 transgene in IL-12R β 2KO mice

CD4⁺T cells were isolated from VAIL-12Rβ2⁺/IL-12Rβ2KO and from normal IL-12Rβ2KO splenocytes by positive magnetic selection using MACS columns (Miltenyi). Cells were cultured in triplicate in RP-10 medium at 5 x 10⁴ cells/well in a 96-well plate and stimulated with soluble anti-CD3 (1 µg/ml) and 5 x 10⁵ irradiated syngeneic APCs in the presence or absence of 100 U/ml mIL-12. At d3, cell supernatants were harvested and analyzed for IFN-γ production by capture ELISA.

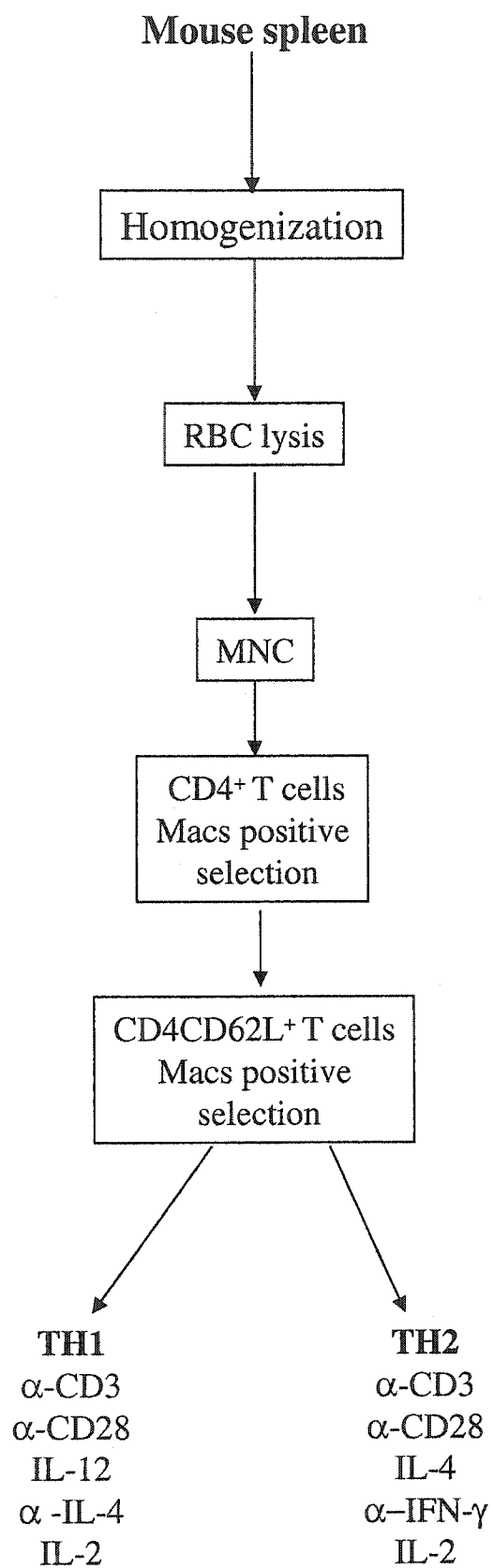
V.4.9. Development of T_H1 and T_H2 cells from IL-12Rβ2-tg BALB/c mice *in vitro*

Splenic CD4⁺CD62L⁺T cells were isolated from positive and negative VAIL-12Rβ2-tg BALB/c mice by positive magnetic selection using MACS columns (Miltenyi). Cells (1x10⁶ cells/well in a 24-well plate) were stimulated 7d with plate-bound anti-CD3 (5 µg/ml), soluble anti-CD28 (1 µg/ml), huIL-2 (10 U/ml), mIL-12 (100 U/ml) and anti-IL-4 mAb (10 µg/ml) to generate T_H1 cells or with plate-bound anti-CD3, soluble anti-CD28, murine IL-4 (200 U/ml) and anti-IFN-γ (10 µg/ml) to polarize the cells towards T_H2 phenotype (Fig. 5.4). At d3, cells were expanded in 10 to 15 x the stimulation volume of RP-10 medium containing 20 U/ml huIL-2. At d7, cells were extensively washed and 1 x 10⁶ cells/well were re-stimulated with plate-bound anti-CD3 and soluble anti-CD28 to determine the cell phenotype. At d3 of the second stimulation, cell supernatants were harvested and analyzed for IFN-γ and IL-4 secretion by capture ELISA. IFN-γ and IL-4 are considered the major indicators of T_H1 and T_H2 phenotype development, respectively.

V.4.10. Reversibility of VAIL-12Rβ2-tg T_H2 cells to T_H1 phenotype *in vitro*

Splenic CD4⁺CD62L⁺T cells were isolated from positive and negative VAIL-12Rβ2-tg BALB/c mice by positive magnetic selection using MACS columns (Miltenyi). Cells were polarized towards the T_H2 phenotype for one week as described above. At d7, cells were extensively washed and restimulated with plate bound anti-CD3 and soluble anti-CD28 in the presence or absence of mIL-12 (100 U/ml). At d3, cell supernatants were harvested and analyzed for IFN-γ and IL-4 cytokine secretion.

Fig. 5.4. CD4⁺CD62L⁺T cell separation and T cell polarization. Splenic naïve CD4⁺CD62L⁺T cells were isolated from positive and negative VAIL-12Rβ2-tg mice by positive magnetic selection using two-step separation. 1) FITC-conjugated anti-CD4 mAb/anti-FITC microbeads to enrich CD4⁺ T cells then 2) anti-CD62L microbeads to isolate naïve CD4⁺T cells. CD4⁺CD62L⁺ cell isolation was assessed by flow cytometry using biotin-conjugated anti-CD62L mAb detected with PE-conjugated streptavidin. T_H1 cell conditions were generated by stimulating naïve T cells with plate-bound anti-CD3 (5 µg/ml), soluble anti-CD28 (1 µg/ml), huIL-2 (10 U/ml), IL-12 (100 U/ml) and anti-IL-4 mAb (10 µg/ml). T_H2 cells were developed with plate-bound anti-CD3 (5 µg/ml), soluble anti-CD28 (1 µg/ml), mIL-4 (200 U/ml) and anti-IFN-γ mAb (10 µg/ml).



V.4.11. Immune response of VAIL-12R β 2-tg BALB/c mice to *L. major* infection

V.4.11.1- *L. major* promastigote parasite cell culture and mouse infection

L. major promastigotes (MHOM/IL/81/Friedlin from ATCC, Manassas, VA) were grown in 10 ml M199 complete medium (M199 medium (Life Technologies) supplemented with 10 % FBS, 1 % penicillin/streptomycin, 40 mM HEPES, 1 x L-glutamine, 0.1mM adenine and 0.25% hemin prepared in 50 % triethylamine (Sigma)) in 25 cm² cell culture flasks. At the stationary growth phase (2×10^7 parasites/ml), the parasites were pelleted 20 min at 2400 x g, washed twice with 10 ml M199 complete medium and resuspended in sterile PBS buffer (Life Technologies) at 4×10^7 parasites per ml. Five animals per group of positive and negative VAIL-12R β 2-tg BALB/c mice were infected in the right footpads with 1×10^6 promastigote parasites in a total volume of 25 μ l. Before infection, footpad thickness was measured using a metric Caliper (T=0 of infection). The disease was monitored weekly by measuring the footpad swelling. Footpad thickness was determined by subtracting the footpad thickness at T=0 of infection from the thickness reached every week.

V.4.11.2. Preparation of *L. major* total cell lysate (LmAg)

L. major promastigote parasites expanding in M 199 complete medium at 8×10^6 parasites per ml were harvested in 50 ml tubes and centrifuged at 2400 x g for 20 min at 4°C. 3×10^9 pelleted cells were resuspended in 10 ml sterile calcium chloride and magnesium chloride free PBS (Life Technologies) containing one protease inhibitor cocktail tablet (Roche). The mixture was frozen at -80°C then thawed at RT. The freeze/thaw cycles were repeated 3 times. Thawed cells were then sonicated 3 x 1 min on ice. The mixture was re-centrifuged at 2400 x g for 20 min at 4°C and supernatant, termed LmAg, was quantified in terms of total proteins using Coomassie Reagent kit (Pierce) following the manufacturer's protocol. LmAg was aliquoted (0.5 ml / tube) in 1.5 ml Eppendorf tubes and stored at -80°C.

V.4.11.3. Preparation of lymph node (LN) cells

At the end of the *L. major*-infection course, positive and negative VAIL-12R β 2-tg BALB/c mice were euthanized and draining popliteal LN were removed aseptically. LN cells were isolated by homogenization of popliteal LN in RP-10 medium using the

frosted end of two sterile glass micro-slides. Cells were then washed twice with 10 ml medium before cell culture.

V.4.12. Cytokine profile of VAIL-12R β 2-tg BALB/c mice in response to *L. major* infection

Popliteal LN cells isolated from *L. major*-infected positive and negative VAIL-12R β 2-tg mice were seeded in triplicate at (2×10^5 cells/well in a round bottom 96-well plate) in the presence or absence of 10 μ g/ml of freeze/thaw LmAg and 5 μ g/ml of anti-mouse IL-4R (M1 mAb), generously provided by Dr. Charlie Maliszewski (Immunex, Seattle, WA). M1 mAb is used to block the IL-4 consumption by the LN cells. At d3, cell supernatants were harvested and analyzed for IFN- γ and IL-4 production by capture ELISA.

V.4.13. Cytokine gene expression in *L. major*-infected VAIL-12R β 2-tg BALB/c mice

LN cells from *L. major*-infected positive and negative VAIL-12R β 2-tg mice (2×10^6 cells/well in a 24 well-plate) were stimulated in the presence of 10 μ g/ml freeze/thaw LmAg. At d2, cells were harvested for total RNA preparation. RNA was analyzed for cytokine gene expression by RNase Protection Assay (RPA) using mouse cytokine set 1 multi-probe template (mCK1) (Pharmingen) as reported above. Radiolabeled RNase protected probes were resolved on 6% polyacrylamide gels and were exposed to film for 24h to 36h at -70°C .

V.5. Results

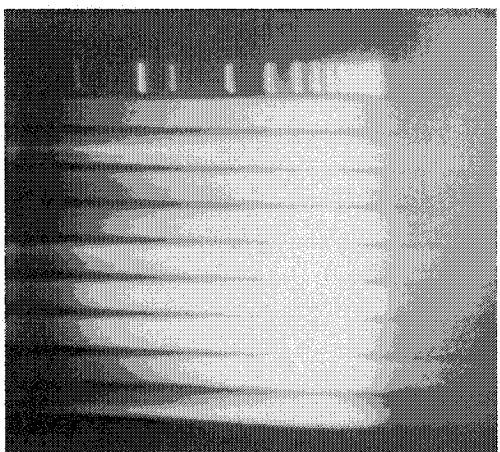
V.5.1. Integration of VAIL-12R β 2 DNA and generation of transgenic mice

At three weeks old, gDNA from fourteen (BALB/c x FVB/N) F1 mice (potential VAIL-12R β 2-tg mice) and a normal BALB/c mouse were subjected to restriction digestion with the endonuclease NcoI (Fig 5.5 A and C). The resulting Southern blot was probed with a 1600 bp radiolabeled DNA fragment revealing two DNA bands (2400 and 2000 bp) in three gDNA: #9379 (lane 5), #9380 (lane 6) and #9316 (lane 13) (Fig 5.5 B

Fig. 5.5. Southern blot analyses of potential VAIL-12R β 2-tg founders. Genomic DNA (6 μ g) from tail clip tissue of potential VAIL-12R β 2-tg founders (F1 generation) was digested with NcoI restriction enzyme (lanes 1-8 and 10-15). gDNA from a normal BALB/c mouse (lane 9) was used as a negative control. After electrophoresis on agarose gels (A and C), gDNA was transferred onto nylon membrane and hybridized with the radiolabelled probe designed in Fig. 5.3. Sizes are bp. Southern blot detected three potential VAIL-12R β 2-tg F1 founders (lanes 5, 6 and 13). Based on band density, lane 6 (mouse # 9380) appeared to have integrated higher copy number of VAIL-12R β 2 transgene (B and D). 1 kbp DNA ladder was used as DNA markers.

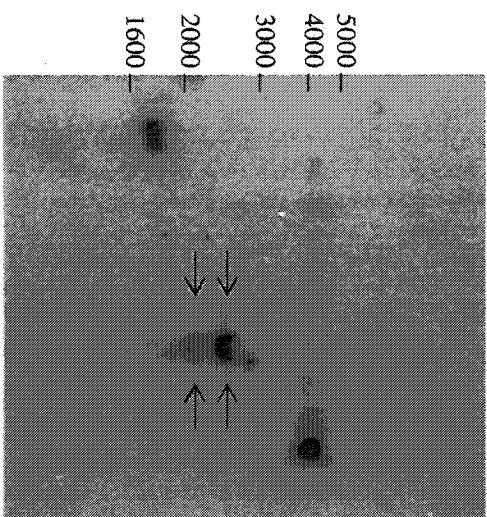
A

M 1 2 3 4 5 6 7 8 9



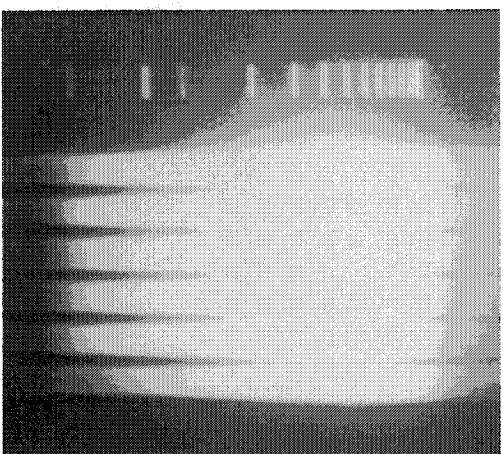
B

M 1 2 3 4 5 6 7 8 9



C

M 10 11 12 13 14 15



D

M 10 11 12 13 14 15

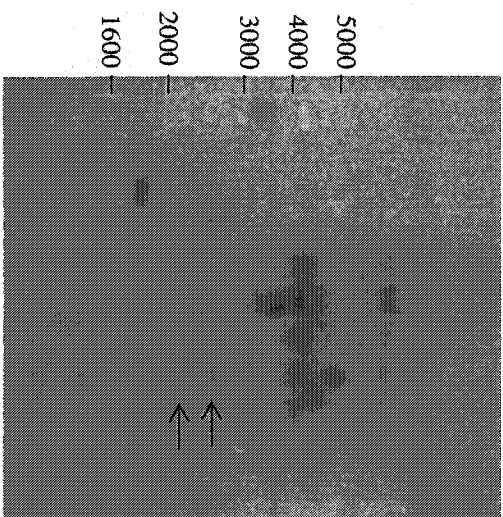
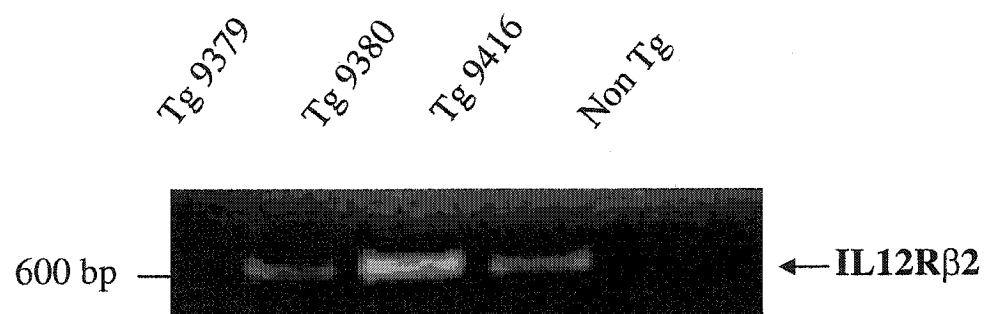


Fig. 5.6. VAIL-12R β 2 gene expression in F1 founder mice. VAIL-12R β 2 transgene expression was investigated by RT-PCR using total RNA from tail clip tissue from the three potential founders (screened by Southern Blot analysis in Fig 5.5) and compared to a non-transgenic mouse. RT-PCR was performed using 3'CD2-UTR and 5'IL-12R β 2-2000 specific primers. RT-PCR products were analyzed by electrophoresis on agarose gel. Specific bands of 600 bp confirmed the expression of the transgene at the mRNA level in all three founders. No band was detected in the non-tg control mice. The position of the VAIL-12R β 2 transgene is indicated by the arrow. 1 kbp ladder was used as DNA markers.



and D). The hybridization of the radiolabeled probe with the two gDNA fragments indicated the presence of the VAIL-12R β 2 transgene in the mouse genomes. Based on the intensity of the detected DNA fragments, it was concluded that mouse #9380 has integrated multiple copies of the transgene compared to mice #9316 and #9379.

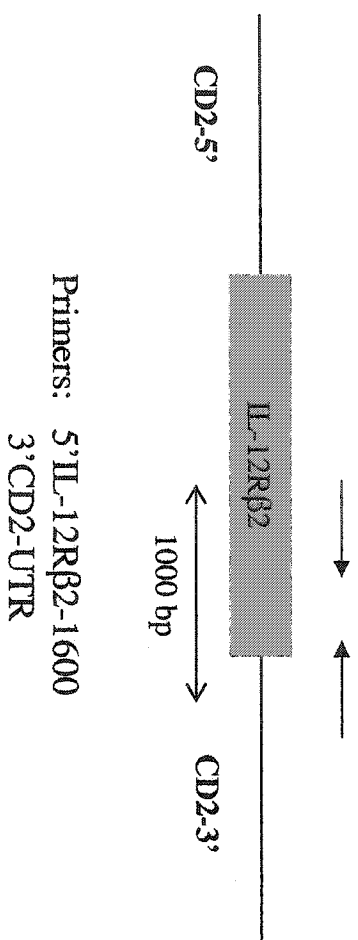
V.5.2. Expression of VAIL-12R β 2 transcripts in the transgenic mice

To ensure that VAIL-12R β 2 transgene was integrated and expressed in the three potential founders before their backcrossing to BALB/c mice, the expression of the VAIL-12R β 2 transgene was analyzed by RT-PCR using RNA isolated from tail clip tissue. Fig. 5.6 showed that all tg mice expressed the VAIL-12R β 2 transgene at the transcriptional level. No band was detected in non-tg cDNA. Thus, VAIL-12R β 2 transgene was integrated and transcribed by positive tg mice. VAIL-12R β 2-tg mouse #9380 (higher copy numbers) was then backcrossed to pure BALB/c mice for five generations. Each generation was screened for the transmission of the transgene by PCR (Fig. 5.7). A schematic representation of the VAIL-12R β 2 gene showing DNA fragments amplified from gDNA of positive tg is reported in Fig. 5.7 A. Analysis of PCR products by electrophoresis on an agarose gel demonstrated that 50% of the litter was usually receiving the transgene (Fig. 5.7 B). VAIL-12R β 2 transgene transmitted to each mouse generation was also analyzed for its transcriptional expression by RT-PCR using total RNA from splenocytes of one representative mouse. Fig. 5.8 shows the expression of the transgene in splenocytes isolated from positive VAIL-12R β 2-tg mice but not from negative tg animals.

IL-12R β 2 gene from the DNA construct used to generate VAIL-12R β 2-tg mice was also used to transfect the Jurkat cell line. The IL-12R β 2 gene was excised by digestion of VAIL-12R β 2 construct with EcoRI restriction enzyme then re-cloned in pcDNA3.1/zeo vector. The resulting plasmid (pcIL-12R β 2) was used to stably transfect Jurkat cells. This cell line was utilized because it does not express endogenous IL-12R β 2 (105). Also, to assess whether the expression and the stability of IL-12R in transfected T cells requires the participation of both IL-12R β 1 and IL-12R β 2, Jurkat cells were transfected with pcIL-12R β 2 construct in the presence or absence of pcIL-12R β 1 and

Fig. 5.7. Screening for positive VAIL-12R β 2-tg generations by PCR. Screening for VAIL-12R β 2-tg generations was routinely performed by PCR. VAIL-12R β 2 transgene was amplified from gDNA isolated from tail clip tissue using IL-12R β 2-1600-5' and CD2-3'UTR primers as reported in (A). VAIL-12R β 2 plasmid was used as positive control template and ddH₂O was used as negative control. PCR products were analyzed by electrophoresis on agarose gel (B). The position of the DNA bands was determined by using 1 kbp DNA ladder as markers. The arrow indicates the position of the amplified VAIL-12R β 2 transgene.

A



B

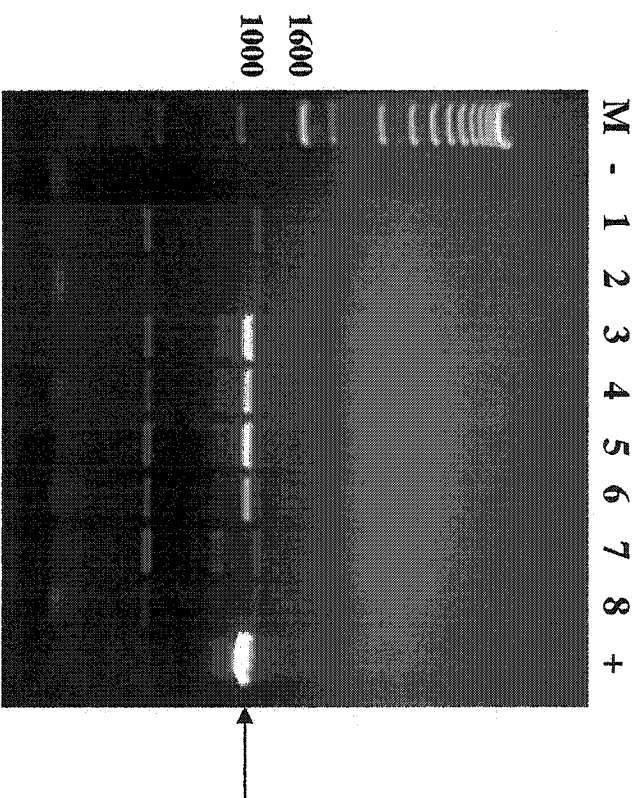
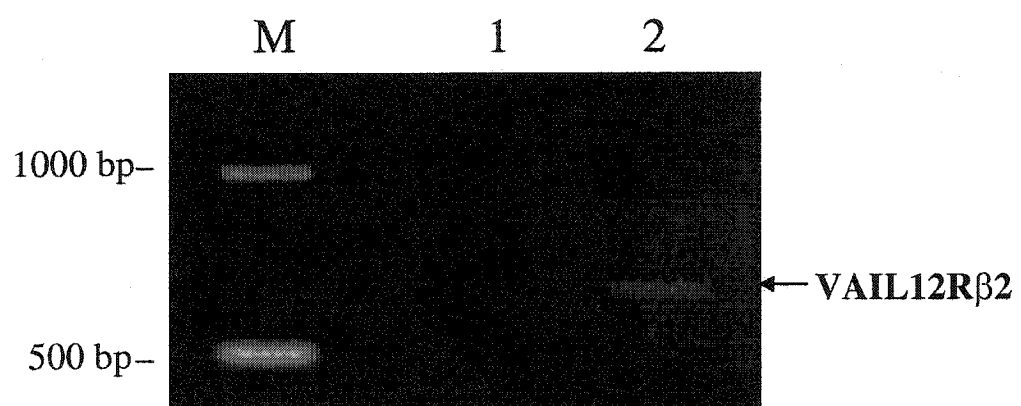


Fig. 5.8. VAIL-12R β 2 gene expression in positive VAIL-12R β 2-tg generations. Gene expression of VAIL-12R β 2 gene was analyzed in different mice generations to confirm the transmissibility and the expression of the transgene. Total RNA was prepared from splenocytes of positively VAIL-12R β 2-tg mice and investigated by RT-PCR using IL-12R β 2-2000-5' and CD2-3'UTR primers. As indicated by the arrow, a band of 600 bp corresponding to the expressed transgene was detected in positively transgenic mice but not in the negative controls. The position of the DNA bands was determined using 1 kbp DNA ladder as markers.



total RNA was analyzed for transcriptional expression of IL-12R β 2 by RT-PCR. Fig. 5.9 demonstrates that IL-12R β 2 was expressed in the presence or absence of IL-12R β 1 cDNA. No IL-12R β 2 was amplified from normal Jurkat cells confirming their deficiency in that receptor. These results again confirmed that the IL-12R β 2 complete gene used to generate VAIL-12R β 2-tg mice is “on” at the transcriptional level.

V.5.3. Functionality of the VAIL-12R β 2 transgene in IL-12R β 2KO mice

Recently, mice that are deficient in IL-12R β 2 chain (IL-12R β 2KO) have been generated offering us the opportunity to test the expression and the function of VAIL-12R β 2 plasmid *in vivo*. IL-12R β 2KO mice do not transduce IL-12 signals despite the expression of high affinity IL-12 binding sites (118) and consequently do not respond to exogenous IL-12. In order to investigate whether the VAIL-12R β 2 gene integrated in positive VAIL-12R β 2-tg BALB/c mice was translated into a functional IL-12 receptor, IL-12R β 2KO mice that express VAIL-12R β 2 transgene were generated. CD4⁺T cells from normal IL-12R β 2KO mice did not show any response to IL-12 stimulation *in vitro*. However, when these mice incorporated the VAIL-12R β 2 transgene (VAIL-12R β 2⁺/IL-12R β 2KO), CD4⁺T cells stimulated with soluble anti-CD3 and irradiated APCs became responsive to IL-12 and produced significant amounts of IFN- γ (Fig. 5.10). These data implied that the transgene was normally expressed and was able to transduce the IL-12 signals.

V.5.4. Polarization of naïve CD4⁺T cells from VAIL-12R β 2-tg mice towards T_H2 phenotype

CD4⁺CD62L⁺T cells were isolated from spleens of positive and negative VAIL-12R β 2-tg mice and were stimulated *in vitro* for 1 week under T_H1 or T_H2 polarizing conditions. Similar to non-tg mice, when CD4⁺T cells from VAIL-12R β 2-tg mice were primed in the presence of IL-12, they produced high levels of IFN- γ and very low levels of IL-4 suggesting that these cells developed normally into T_H1 cells (Fig. 5.11). However, when CD4⁺T cells were primed in the presence of IL-4, they exhibited

Fig. 5.9. VAIL-12R β 2 gene expression *in vitro*. To ensure the integrity of IL-12R β 2 complete gene used for the generation of VAIL-12R β 2-tg mice, IL-12R β 2 insert was excised from the VAIL-12R β 2 plasmid and re-cloned into pcDNA3.1 vector to transfect Jurkat cells. Total RNA from Jurkat cells transfected with pcIL-12R β 2 with or without pcIL-12R β 1 constructs was analyzed by RT-PCR. cDNA products obtained were separated by electrophoresis on agarose gel. The presence of the specific band indicated by the arrow demonstrated that the IL-12R β 2 gene is "on" at the transcriptional level. No IL-12R β 2 expression was detected in normal Jurkat cells. VAIL-12R β 2 construct was used as a positive control. The position of the DNA bands was determined using 1 kbp DNA ladder as markers.

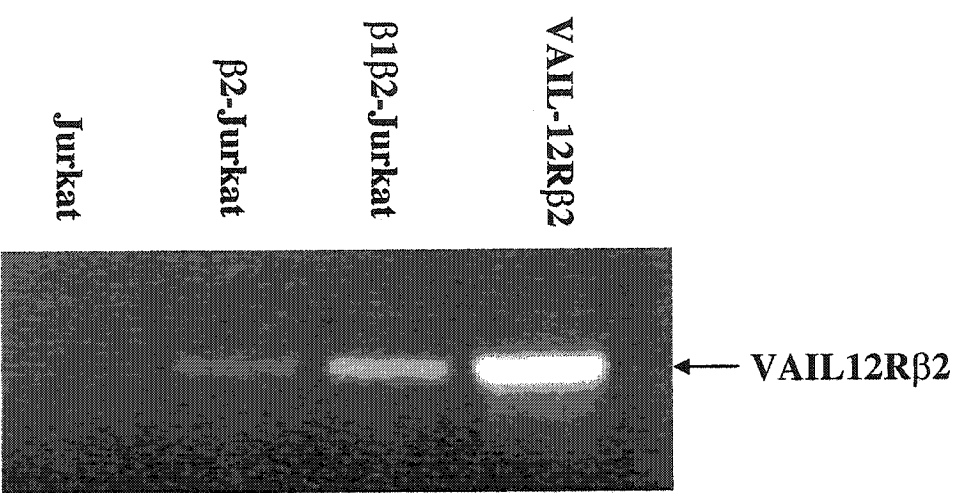
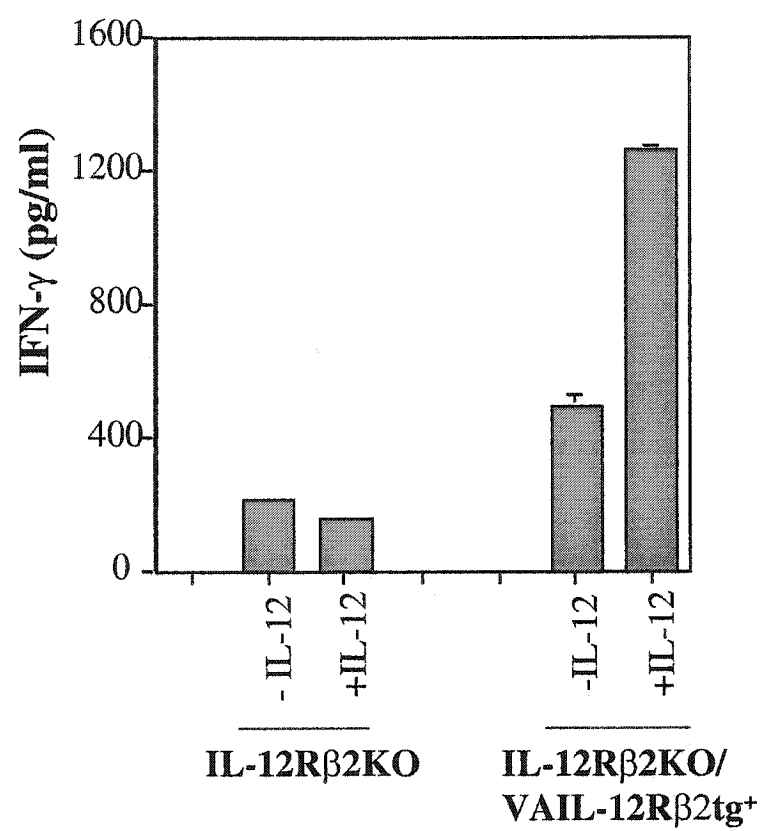


Fig. 5.10. Functionality of the VAIL-12R β 2 transgene *in vivo*. To further demonstrate that VAIL-12R β 2 transgene was expressed and functional in VAIL-12R β 2-tg mice, IL-12R β 2KO mice, reported to be defective in IL-12-induced IFN- γ production (118), were bred to VAIL-12R β 2-tg mice. Mice from the F2 generation were first screened for the presence of VAIL-12R β 2 transgene by PCR. Positive transgenic mice were then screened for the absence of endogenous IL-12R β 2. Normal IL-12R β 2KO and IL-12R β 2KO mice expressing the VAIL-12R β 2 transgene only were sacrificed for splenic T cells isolation. CD4⁺T cells (5×10^4 cells/well) were cultured in triplicate in a 96-well plate and stimulated with soluble anti-CD3 (1 μ g/ml) and 5×10^5 irradiated APCs in the absence and presence of IL-12 (100 U/ml). At d3, cell supernatants were analyzed for IFN- γ secretion by ELISA. The plotted values are the mean of replicate ELISA wells and error bars represent the SEM. The results demonstrated that in contrast to IL-12R β 2KO mice, CD4⁺T cells from VAIL-12R β 2/IL-12R β 2KO mice were able to produce IFN- γ in response exogenous IL-12 confirming that VAIL-12R β 2 transgene was functional *in vivo*.



a T_H2 phenotype characterized by elevated levels of IL-4 and very low levels of IFN- γ (Fig. 5.11). The amounts of IFN- γ and IL-4 were similar in positive and negative VAIL-12R β 2-tg cells suggesting that polarization into T_H2 cells took place despite the constitutive expression of IL-12R β 2 by positive tg CD4⁺T cells.

V.5.5. Responsiveness of T_H2 cells from VAIL-12R β 2-tg mice to exogenous IL-12

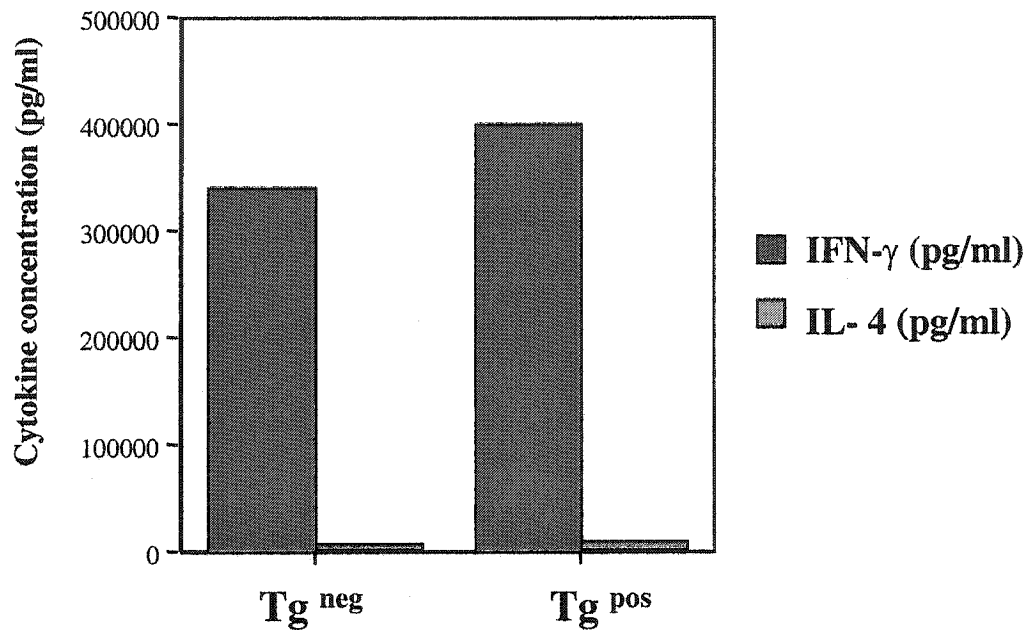
To see whether treatment of T_H2 cells constitutively expressing VAIL-12R β 2 with IL-12 would induce IFN- γ production and ultimately revert the cell phenotype from T_H2 to T_H1, T_H2 cells from tg and non-tg splenocytes, were generated. T_H2 cells were then restimulated in the presence or absence of exogenous IL-12. Fig. 5.12 A shows that, despite the constitutive expression of IL-12R β 2, T_H2 cells from VAIL-12R β 2-tg cells exhibited only a slight increase in IFN- γ production in response to high concentrations of IL-12 (100 U/ml). In addition, when CD4⁺T cells from VAIL-12R β 2-tg mice were analyzed for IL-4 secretion, they exhibited high secretion levels confirming that these cells were still of T_H2 phenotype (Fig. 5.12 B). Interestingly, when T_H2 cells were treated with IL-12, cells from positive tg mice displayed higher levels of IL-4 secretion than non-tg cells. Taken together these results imply that despite the constitutive expression of IL-12R β 2, T_H2 cells were unable to respond adequately to IL-12 as measured by IFN- γ secretion. Also, addition of high concentrations of IL-12 to these T_H2 cells did not inhibit IL-4 production and did not manifest any opposition to T_H2 cell differentiation, suggesting also that IL-12 signaling in positive tg cells (translated by the very small increase in IFN- γ observed in response to IL-12) could not induce reversion from a T_H2 to T_H1 phenotype.

V.5.6. Infection of VAIL-12R β 2-tg BALB/c mice with *L. major*

In an attempt to clarify whether persistence of IL-12R β 2 expression was relevant to the stability of T_H2 cell development *in vivo*, the immune response of VAIL-12R β 2-tg BALB/c mice to *L. major* infection was investigated. Positive and negative VAIL-12R β 2-tg BALB/c mice (5 mice per group) were infected in the rear footpad with

Fig. 5.11. T_H1/T_H2 polarization of naïve CD4⁺CD62L⁺T cells from VAIL-12Rβ2-tg BALB/c mice. CD4⁺CD62L⁺T cells were isolated from negative and positive VAIL-12Rβ2-tg splenocytes by positive magnetic selection and analyzed for T_H1 and T_H2 polarization. Cells (1x10⁶ cells/well) were primed with plate-bound anti-CD3 (5 µg/ml), anti-CD28 (1 µg/ml), IL-2 (10 U/ml), IL-12 (100 U/ml) and anti-IL-4 mAb (10 µg/ml) for T_H1 conditions or plate-bound anti-CD3 (5 µg/ml), soluble anti-CD28 (1 µg/ml), IL-2 (10 U/ml), IL-4 (200 U/ml) and anti-IFN-γ (10 µg/ml) for T_H2 conditions. At d3, cells were expanded in RP-10 medium containing 20 U/ml IL-2 for an extra 4 days. T_H cell phenotype was investigated by restimulating the cells with plate-bound anti-CD3 (5µg/ml) and soluble anti-CD28 (1µg/ml). At d3 of the second stimulation, cell supernatants were analyzed for IFN-γ and IL-4 production by capture ELISA. The plotted values are the mean of replicate ELISA wells and error bars represent the SEM.

T_H1 polarization



T_H2 polarization

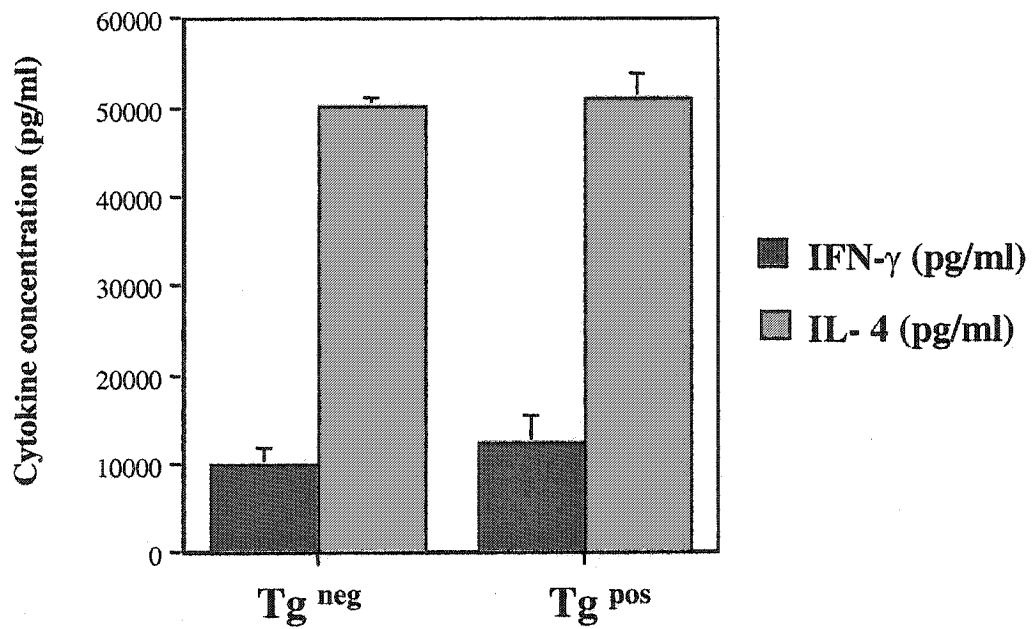
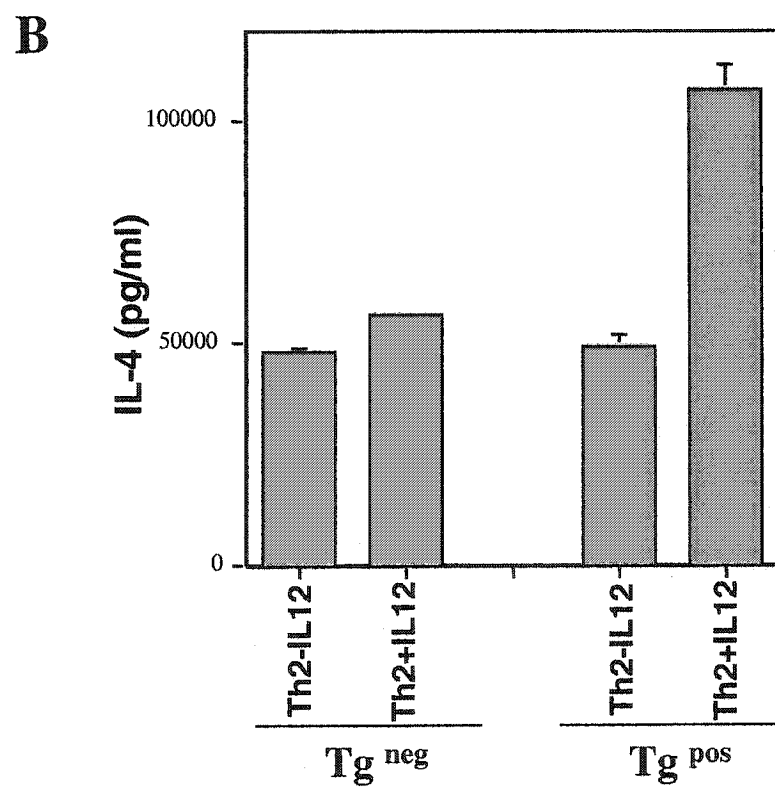
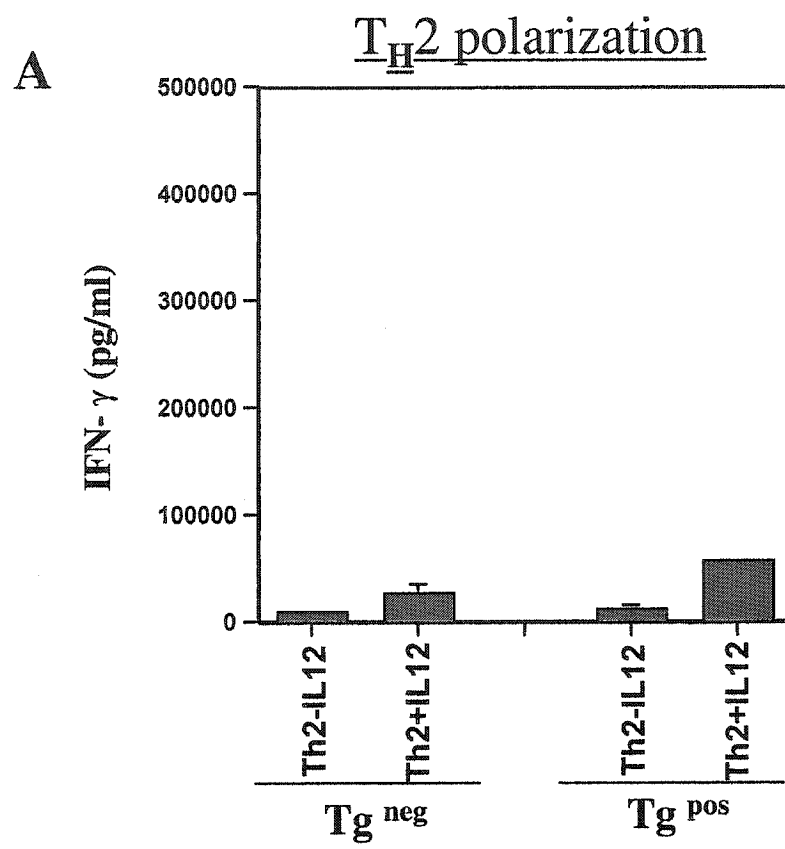


Fig. 5.12. IL-12 responsiveness of T_H2 cells from VAIL-12R β 2-tg BALB/c mice. CD4⁺CD62L⁺T cells were isolated from negative and positive VAIL-12R β 2-tg splenocytes by positive magnetic selection and analyzed for T_H2 polarization and responsiveness to IL-12. Indeed, cells (1x10⁶ cells/well) were primed with plate-bound anti-CD3 (5 μ g/ml), soluble anti-CD28 (1 μ g/ml), IL-2 (10 U/ml), IL-4 (200 U/ml) and anti-IFN- γ (10 μ g/ml) for one week. At d3 post-priming, cells were expanded in RP-10 medium containing 20 U/ml IL-2 for an additional 4 days. At d7, T_H2 cells were extensively washed and restimulated with plate-bound anti-CD3 (5 μ g/ml) and soluble anti-CD28 (1 μ g/ml) in the presence or absence of IL-12 (100 U/ml). At d3 post-stimulation, supernatants were analyzed for IFN- γ and IL-4 cytokine secretion by capture ELISA. The plotted values are the mean of replicate ELISA wells and error bars represent the SEM.



L. major promastigotes. Following infection, footpad inflammation was monitored as an indicator of disease progression. As expected, BALB/c mice that were negative for the VAIL-12R β 2 transgene displayed a significant footpad swelling starting at week 4 post-infection (Fig. 5.13). Footpads from BALB/c mice that were positive for the VAIL-12R β 2 transgene also showed a similar susceptibility pattern characterized by an increase in footpad thickness starting from week 4 post-infection. These data showed that although constitutively expressing IL-12R β 2, VAIL-12R β 2-tg BALB/c mice continued to be susceptible to *L. major* and were unable to clear the infection.

V.5.7. Cytokine production by VAIL-12R β 2-tg BALB/c mice in response to *L. major* infection

The immune response developed by VAIL-12R β 2-tg BALB/c mice to *L. major* infection was further explored by defining the phenotype of the responding immune cells. To do so, lymph node cells were isolated from draining popliteal LN or spleen of *L. major* infected positive and negative VAIL-12R β 2-tg BALB/c mice. Cells were re-stimulated *in vitro* with *Leishmania* antigens (LmAg). At d3, cell supernatants were analyzed for the T_H1-specific IFN- γ and for the T_H2-specific IL-4 cytokines by capture ELISA. As shown in Fig. 5.14, both positive and negative VAIL-12R β 2-tg BALB/c mice exhibited similar levels of IFN- γ despite the constitutive expression of IL-12R β 2 by positive tg BALB/c mice. The levels of IFN- γ produced in response to *L. major* infection seemed not sufficient to induce an adequate T_H1 immune response to allow clearance of infection. Similarly, the levels of IL-4 produced by both tg and non-tg LN cells were indistinguishable. These results demonstrated that despite the constitutive expression of IL-12R β 2, any endogenous IL-12 that might be released during infection (59) did not overcome the T_H2 development by BALB/c mice infected by *L. major* parasites.

V.5.8. Cytokine gene expression profile in *L. major*-infected VAIL-12R β 2-tg BALB/c mice

To further confirm the T_H2 phenotype developed by VAIL-12R β 2-tg mice in response to *L. major* infection, the parasite-specific cytokine gene expression was investigated by RNase protection assay (RPA) using the mCK1 mouse cytokine multi-

Fig. 5.13. Course of *L. major* infection of VAIL-12R β 2-tg BALB/c mice. Positive and negative VAIL-12R β 2-tg BALB/c mice (5 mice per group) were infected with 1×10^6 logarithmic-phase *L. major* promastigotes in the right hind footpad. Footpad swelling was monitored weekly using a metric caliper and footpad thickness was determined by subtracting the footpad thickness at T=0 of infection from the thickness reached every week. The mean and SDs are representative of two separate experiments. Both mouse groups exhibited susceptibility to *L. major* infection and the constitutive expression of IL-12R β 2 transgene *in vivo* did not confer resistance to susceptible BALB/c mice.

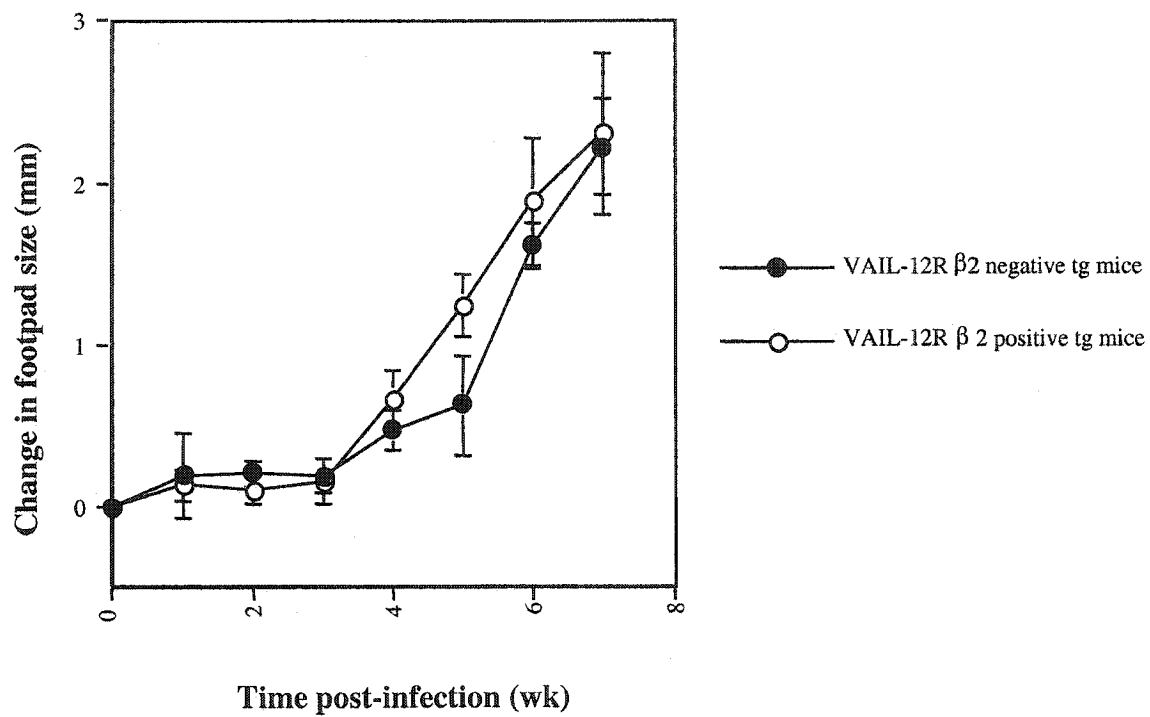
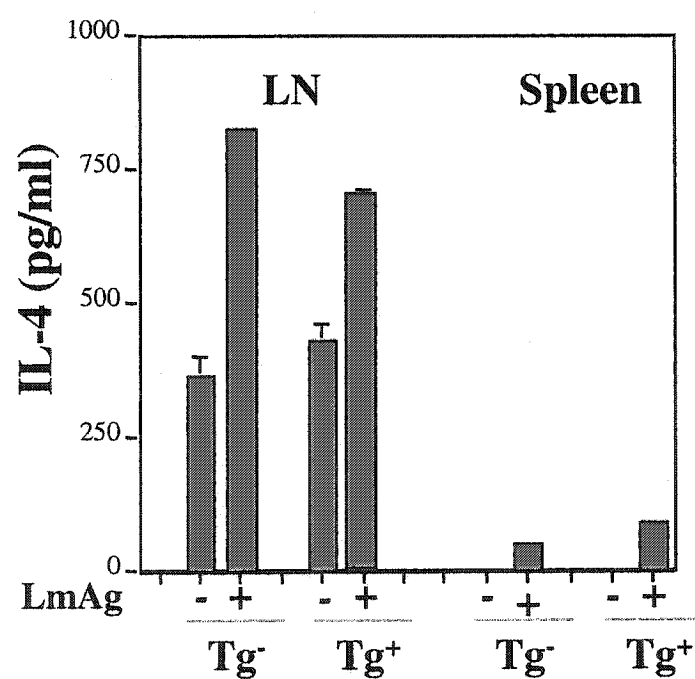
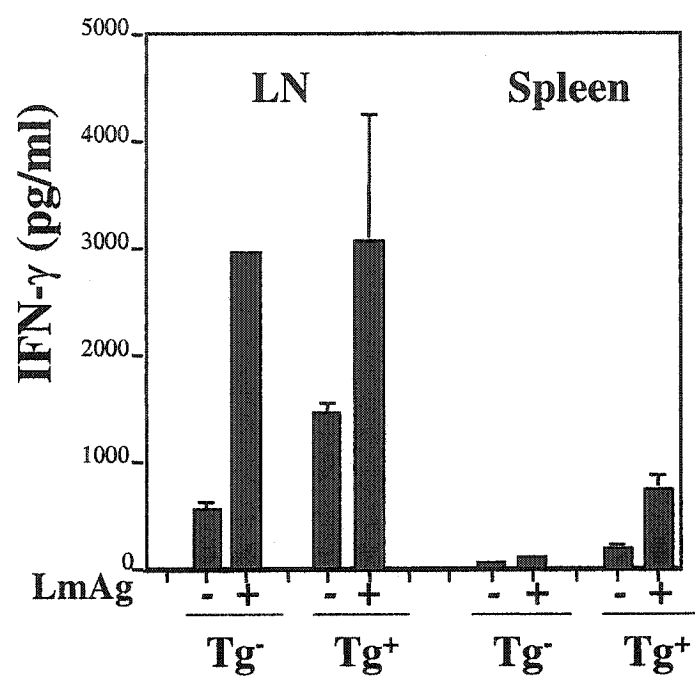


Fig. 5.14. *L. major*-infected VAIL-12R β 2-tg BALB/c mice develop a specific T_H2 immune response characteristic of susceptible mice. LN and spleen cells (5×10^5 cells/well) from *L. major*-infected positive and negative VAIL-12R β 2-tg BALB/c mice were incubated *in vitro* in the presence (+) or absence (-) of 10 μ g/ml LmAg. For IL-4 analysis, 5 μ g/ml anti-IL-4R M1 mAb was added to the culture to block IL-4 consumption. At d3, cell supernatants were investigated for IFN- γ and IL-4 secretion by capture ELISA. The results demonstrated that negative and positive VAIL-12R β 2-tg BALB/c mice displayed similar levels of IFN- γ and IL-4 characteristic of *L. major* susceptible animals. Constitutive expression of IL-12R β 2 did not induce T_H1 immune response to *L. major*.

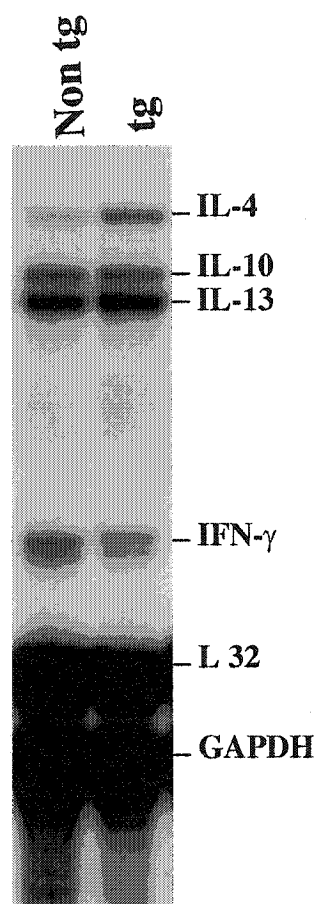


probe template set (Pharmingen). Total LN cells, isolated from draining popliteal LNs of infected positive and negative VAIL-12R β 2-tg BALB/c mice, were stimulated *in vitro* with LmAg for 2d and total RNA was analyzed for the cytokine gene expression profile. Again, positive and negative VAIL-12R β 2-tg BALB/c mice displayed a similar phenotype characterized by the expression of T_H2 cytokine genes IL-4, IL-10 and IL-13 (Fig. 5.15) consistent with the susceptibility observed by tg mice when infected with *L. major*. In accordance with previous studies (57) and with Fig. 5.14, BALB/c mice, although developing a T_H2 phenotype, still express some IFN- γ . However, the levels are insufficient to inhibit T_H2 differentiation and to induce a strong T_H1 immune response against *L. major*.

V.6. Discussion

CD4⁺T cells differentiate towards a T_H1 phenotype when naïve T cells are stimulated in the presence of IL-12 cytokine. Different studies agreed that IL-12 and its signaling via IL-12R β 2 chain is the master switch igniting T_H1 cell differentiation [reviewed elsewhere (12)] and the resulting IL-12-responsiveness (manifested by IFN- γ production) is necessary to induce a strong T_H1 immune response and clear intracellular parasitic infections. Indeed, *in vitro* data reported earlier that IL-12R β 2 and its signaling via STAT4 are critical for T_H1 cell development (274) and that in T_H2 cells, IL-12R β 2 was rapidly downregulated by IL-4 resulting in a complete extinction of IL-12 signaling (23). In the same way, *in vivo* studies reported compatible results and implied that BALB/c mice exhibit susceptibility to the intracellular parasite *L. major* by developing a non-healing T_H2 immune response (30,32,71,275). Differentiation and expansion of reactive T_H2 cells in leishmaniasis was speculated to be due to an early extinction of IL-12 signaling as a result of a downregulation of the expression of IL-12R β 2 chain by the burst of IL-4 produced by these mice early during the infection (30-32,32,57). These different data imply that downregulation of the IL-12R β 2 might be a fundamental step for T_H2 cell development and maintenance. However, in agreement with recent studies (276,277), the data presented in this chapter indicated that, despite the constitutive

Fig. 5.15. Cytokine gene expression in LN cells from *L. major*-infected VAIL-12R β 2-tg mice. Lymph node cells were isolated from positive and negative VAIL-12R β 2-tg mice infected with *L. major*. Cells (2×10^6 /well) were then stimulated *in vitro* with LmAg (10 μ g/ml) for two days and were harvested for total RNA preparation. Parasite-specific cytokine gene expression was analyzed by RNase Protection Assay (RPA) using the mCK1 mouse cytokine multi-probe template set (Pharmingen). Undigested radiolabelled probe was used as markers. LN cells from *L. major*-infected positive and negative VAIL-12R β 2-tg BALB/c mice displayed significant upregulation of IL-4, IL-10 and IL-13 type-2-specific cytokine transcripts as well as a small amount of IFN- γ mRNA. Cytokine gene expression confirmed the T_H2 profile expressed by VAIL-12R β 2-tg mice in response to *L. major* infection.



expression of IL-12R β 2, T_H2 cells generated *in vitro* from VAIL-12R β 2-tg cells exhibited only a slight increase in IFN- γ production in response to exogenous IL-12. More interestingly, in the presence of IL-12, VAIL-12R β 2-tg T_H2 cells increased the levels of IL-4 production compared to non-tg T_H2 cells. A similar observation was recently reported in developing and committed T_H2 cells transfected *in vitro* for an ectopic expression of IL-12R β 2 chain (276). Thus, the results presented herein demonstrated that downregulation of IL-12R β 2 expression was not required for T_H2 cell development and stability. Indeed, GATA-3 and c-Maf transcription factors specific for T_H2 cell differentiation were found to be induced by IL-4 and their expression was maintained despite the activation of the IL-12 signaling (277). Furthermore, ectopic expression of IL-12R β 2 chain performed *in vitro*, induced STAT4 activation in T_H2 cells but was unable to induce IFN- γ production by these cells (276). Together, these data demonstrate that loss of IL-12 signaling was not the only factor controlling T_H2 differentiation and that the lack of IL-12R β 2 expression was not critical for the stability of T_H2 phenotype. The data also showed that IL-12R β 2 expression was not sufficient to reverse the T_H2 phenotype. This also implied that IL-4 might inhibit T_H1 cell development via other mechanisms independent of IL-12 signaling.

Furthermore, the present data showed that CD4⁺CD62L⁺T cells from VAIL-12R β 2-tg mice developed normally towards T_H1 when primed in the presence of IL-12 and that overexpression of IL-12R β 2 did not affect this polarization. T_H1 cells polarized *in vitro* dramatically upregulated IFN- γ with inhibition of IL-4 secretion. Similarly, in the presence of IL-4 priming conditions, cells showed normal progression to T_H2 cell differentiation characterized by a very high production of IL-4 accompanied with a very small amount of IFN- γ .

Although constitutively expressing the IL-12R β 2 gene, polarized T_H2 cells from VAIL-12R β 2-tg mice did not produce high levels of IFN- γ and did not revert to the T_H1 phenotype in response to stimulation with a high dose IL-12 (38 ng/ml). However, these T_H2 cells produced more IL-4 in response to exogenous IL-12 compared to non-tg BALB/c mice. Since IL-12 as well as IL-4 were reported to induce proliferation of T cells (12,278), increased IL-4 production by VAIL-12R β 2 transgenic T_H2 cells in response to

exogenous IL-12 might be due to the additive proliferative effect induced in response to both IL-12 and IL-4 cytokines in the particular case of VAIL-12R β 2-tg T_H2 cells.

To further demonstrate that T_H2 cell differentiation can occur despite the constitutive expression of IL-12R β 2 chain, the *in vivo* immune response of VAIL-12R β 2-tg BALB/c mice to the infection with the intracellular parasite *L. major* was analyzed. The results showed that, similar to non-tg BALB/c mice, VAIL-12R β 2-tg BALB/c mice infected with *L. major* exhibited a progressive footpad swelling. A persistent increase in footpad thickness was the first indicator of susceptibility developed by these mice. Thus, despite the constitutive expression of IL-12R β 2, VAIL-12R β 2-tg mice were unable to reduce the inflammation at the site of infection.

As mentioned above in the discussion, BALB/c mice develop a T_H2 cell phenotype in response to *L. major* infection characterized by the production of a burst of IL-4 and that this IL-4 was responsible for the susceptible phenotype developed by these mice. However, conflicting data were published regarding the role played by IL-4 in T_H2 cell development in murine experimental leishmaniasis (65,67,279). To identify the phenotype developed by the reactive T cells, LN cells from draining popliteal LNs of VAIL-12R β 2-tg and non-tg BALB/c mice were isolated and were restimulated *in vitro* with LmAg. The results indicate that both positive and negative VAIL-12R β 2-tg BALB/c mice exhibited a similar pattern of IL-4 and IFN- γ cytokines secreted in response to parasite antigens. The parasite-specific cytokine gene profile expressed by LN cells was also investigated by RNase Protection Assay. The data demonstrated a significant expression of strong T_H2-specific cytokine transcripts IL-4, IL-13 and IL-10 and only some IFN- γ . The role of IL-13 in *L. major* infection was recently demonstrated in mouse and human leishmaniasis (280-282). Indeed, overexpression of IL-13 gene in C57BL/6 mice reverted their resistant phenotype to a susceptible one. Conversely, deletion of the IL-13 gene from susceptible BALB/c mice conferred resistance to these animals and showed healing of the infection (281). In human cutaneous leishmaniasis, IL-13 was found to be the major T_H2 cytokine produced by PBMC (282). These data suggested that IL-13 plays a key role in leishmaniasis. Indeed, IL-13 was designated as a susceptibility factor for *L. major* infection (281).

The results presented here also showed a significant upregulation of IL-10 gene expression in response to *L. major* antigens. IL-10 is a T_H2-associated cytokine that exerts a negative feedback control on IL-12 production (1,283). IL-10 has been shown to prevent parasite killing by activated macrophages by rendering them refractory to the activation effects of IFN- γ (284). While, most of the studies agree on the fact that IL-10 is acting as a T_H2 cytokine, data on the overall role of IL-10 in leishmaniasis seem to be controversial. Some authors reported that removal of endogenous IL-10 by anti-IL-10 treatment did not alter the T_H2 cytokine pattern in susceptible mice and did not affect the levels of IgE production (285). However, other reports demonstrated that non-healer BALB/c mice deficient in IL-10 gene expression controlled the infection (284) and treatment of human PBMC with neutralizing anti-IL-10 antibodies, enhanced IL-12 production in response to *Leishmania* proteins (286).

In response to *L. major* infection, BALB/c mice (susceptible strain) develop a non-healing T_H2 immune response. Whether these animals produce IL-12 early during the infection is still an enigma. Different authors reported that the most striking dysfunction observed in *Leishmania*-infected macrophages is their inability to produce IL-12, which is the main inducer of IFN- γ and T_H1 differentiation, and consequently resistance to *L. major* infection (287,288). However, others demonstrated that although these animals produce IL-12 early during the infection (59), they completely fail to heal the infection. This was attributable to the early extinguishment of IL-12R β 2 signaling by the burst of IL-4 produced by these mice (30,32,57). However, the *in vitro* studies reported herein brought into question the statement that BALB/c mice develop a susceptible T_H2 immune response because of the downregulation of IL-12 signaling and demonstrated that IL-12R β 2 downregulation was not critical and that T_H2 cells differentiated despite the constitutive expression of the IL-12 receptor. One may speculate that T_H2 differentiation was triggered because of the absence of IL-12-induced T_H1-development and that IL-12R β 2 downregulation was a **consequence** of T_H2 cell expansion in response to the early burst of IL-4 and **not a cause** as previously understood.

In conclusion, the results presented here demonstrated that constitutive expression of IL-12R β 2 was unable to shut down the T_H2 cell differentiation *in vitro* and *in vivo*.

Also, the concept stating that BALB/c mice develop susceptible phenotype due to the extinction of IL-12 signaling is put into question since the results showed that T_H2 cell differentiation occurred despite the persistence of IL-12R β 2 activity *in vitro* ((276,277) and the present findings). Furthermore, the *in vivo* data demonstrated that despite the constitutive expression of IL-12R β 2, VAIL-12R β 2-tg BALB/c mice still exhibit a parasite specific T_H2 cytokine gene profile characterized by an upregulation of the T_H2 specific cytokines IL-4, IL-13 and IL-10.

CHAPTER VI

Genetically resistant mice lacking the IL-12R β 2 gene are susceptible to *L. major* infection and develop a specific T_H2 immune response

VI.1. Summary

IL-12 and its signaling via IL-12R β 2 chain have been shown to play a critical role in T_H1 cell development and consequently in mounting an efficient *L. major*-specific T_H1 immune response. *L. major*-susceptible BALB/c mice develop a T_H2-immune response characterized by large non-healing lesions and production of T_H2-associated cytokines such as IL-4, IL-5, IL-10 and IL-13. Indeed, upon *L. major* infection, BALB/c mice display low levels of IFN- γ due probably to the early burst of IL-4 observed in the draining lymph node (30,32,57). As result, IL-12R β 2 chain is downregulated and specific CD4⁺T cells develop into IL-4 secreting cells (31,32,69). The disease-exacerbating role of IL-4 has been shown to be due to its ability to inhibit macrophage activity (289). In contrast, *L. major*-resistant C57BL/6 mice develop a T_H1 immune response characterized by expression of the key cytokine IFN- γ necessary to trigger the parasite killing process by activated macrophages (50,91). However, the data presented in the previous chapter demonstrated that extinction of IL-12R β 2 expression did not play a critical role in T_H2 cell differentiation and that persistent expression of IL-12R β 2 did not rescue BALB/c mice infected with *L. major*. These results prompted us to investigate the role of IL-12 signaling in the T_H1 cell development. As already mentioned, IL-12 and its signaling via the IL-12R β 2 chain is a mandatory event to induce and maintain T_H1 cell development and consequently to confer resistance in the murine experimental leishmaniasis model (51,59,70,71,74,290,291). Therefore, to better understand the *in vivo* role played by IL-12R β 2 activity and consequently IL-12 signaling in the development of T_H1 cells, resistant C57BL/6 mice deficient in the IL-12R β 2 gene (IL-12R β 2KO) were infected with the intracellular parasite *L. major* and the outcome of the infection was investigated. IL-12R β 2KO mice were shown to have a defect in IL-12 signaling resulting in an abrogation of IFN- γ production and STAT4 activation (118).

The findings reported in the present chapter demonstrate that, in contrast to wild-type mice, genetically resistant mice deficient for the IL-12R β 2 gene were not able to mount a curative T_H1 immune response but rather, developed a lethal T_H2 immunity. The emerging T_H2 phenotype was characterized by an increase in T_H2 cytokine and cytokine transcript expression (IL-4, IL-5, IL-10 and IL-13) and by a decrease in T_H1 cytokines

(IFN- γ and TNF- α). Furthermore, IL-12R β 2KO mice exhibited a complete loss of parasite-specific IgG2a isotypes and an upregulation of parasite-specific IgE antibodies.

VI.2.Hypothesis

IL-12 signaling is required for T_H1 cell development. CD4⁺T cells from resistant C57BL/6 mice lacking IL-12R β 2 activity (IL-12R β 2KO) are characterized by a defect in IL-12-induced IFN- γ production *in vitro* and thus these mice will develop susceptibility to *L. major* infection.

VI.3. Objective

Explore the *in vivo* role of IL-12 signaling in T_H1 cell differentiation in the murine experimental leishmaniasis model using genetically resistant mice lacking IL-12R β 2 chain in comparison with normal mice of resistant and susceptible backgrounds.

VI.4. Materials and Methods

VI.4.1. Cell preparation from *L. major*-infected mice

L. major-infected IL-12R β 2KO, resistant C57BL/6 and susceptible BALB/c, mice were euthanized when cutaneous ulcerations appeared on the infected footpads. Draining popliteal lymph nodes (LN) and spleens were removed aseptically and single-cell suspensions were prepared. Spleen suspensions were cleared of red blood cells using ammonium chloride lysis at 4°C. After three washes with RP-10 medium, cells were resuspended in the same medium and cultured in the presence and absence of 10 μ g/ml freeze/thaw LmAg. At the same time, infected and non-infected footpads were recovered for histological analyses.

VI.4.2. Histology of mice footpads infected with *L. major* promastigotes

The histological analyses reported in this chapter were performed by Louise Pelletier at the department of Pathology (Faculty of Medicine, University of Ottawa). Histology of footpads from *L. major*-infected mice was performed to investigate tissue destruction and immune cell infiltration at the site of infection. At the end of the infection time course, the infected right footpads from *L. major*-infected mice (C57BL/6, IL-

12R β 2KO and BALB/c) were excised and fixed for 24 h in 15 ml buffered formalin 10% (BDH) then processed for histology following the protocol previously reported (292). Paraffin sections were processed for routine hematoxylin and eosin (H&E) staining to analyze the tissue morphology.

VI.4.3. Proliferation of spleen cells from *L. major*-infected IL-12R β 2KO mice to LmAg

Spleen cells isolated from *L. major*-infected IL-12R β 2KO, BALB/c and C57BL/6 mice were plated in triplicates in 96-well round-bottom plates (2×10^5 /well) in a total volume of 200 μ l RP-10. Cells were then stimulated in the presence or absence of 10 μ g/ml freeze/thawed LmAg. At d5, cells were pulsed with [3 H]-thymidine (Amersham Pharmacia Biotech) at 1 μ Ci per well for an additional 24h. Cells were harvested onto filter papers and incorporated radioactivity was measured in a Matrix Direct Beta Counter (Packard).

VI.4.4. Cytokines profile induced by *L. major* infection of IL-12R β 2KO mice

In parallel to the proliferation assay, LN cells (2×10^5 /well) were plated in 96-well round-bottom plates under the same conditions for cytokine detection with addition of 5 μ g/ml of anti-mouse IL-4R (M1 mAb) to the culture medium to block IL-4 consumption by the reacting cells. At d5, the supernatants were harvested and 100 μ l were analyzed for IL-4 and IL-5 levels (as T_H2-associated cytokines) and for TNF- α and IFN- γ concentrations (as T_H1-associated cytokines) by capture ELISA.

VI.4.5. Determination of serum antibody titers against *L. major* antigens

At the end of the infection time course, serum was isolated from *L. major*-infected animals and assayed for parasite-specific antibody isotypes. Parasite-specific IgG1 and IgG2a antibody analyses were performed by our collaborator Antonio Campos-Neto at the Infectious Disease Research Institute (Seattle, WA). Parasite-specific IgE antibodies were measured in our lab following the same methodology. To determine the serum levels of parasite-specific Abs produced in response to *L. major* infection (IgG1, IgE and IgG2a), 96 well flat bottom plates (Nunc) were coated with 50 μ l/well of LmAg at 2

µg/ml in ELISA coating buffer for 4h at RT or overnight at 4°C. The plates were rinsed with ELISA wash buffer (3 x washes, soaked 10 min then 3 x washes) and blocked overnight at 4°C with 200 µl/well of ELISA blocking buffer. 100 µl/well of the serum samples, at different serial dilutions in ELISA dilution buffer, were added to the plates for 2h at RT. The plates were then washed as mentioned above and 100 µl/well of goat anti-mouse IgG1-HRP (Southern Biotechnology), goat anti-mouse IgG2a-HRP (Southern Biotechnology) or goat anti-IgE (Cedarlane) at 1:2000 (v/v) in ELISA dilution buffer were added to the plates for 1h at RT. The plates were washed and anti-IgE plates were incubated 1h at RT with 100 µl/well of anti-goat IgG peroxidase conjugate (Sigma) at 1:8000 in ELISA dilution buffer. After washing the plates, 100 µl/well of TMB peroxidase substrate (MANDEL) were used for detection. When reaching the desired color intensity, the reactions were stopped using 1N HCl in ddH₂O (BDH). O.Ds were read at 450 nm using WinSelect microplate reader (Tecan).

VI.4.6. RPA analysis of cytokine gene expression induced in response to LmAg

1 x 10⁶ LN cells from popliteal LN of IL-12Rβ2KO, susceptible BALB/c and resistant C57BL/6 mice were seeded in a 24-well plate and stimulated in the presence of 10 µg/ml freeze/thawed LmAg. At d2, cells were harvested and total RNA was prepared using Trizol reagent. Cytokine transcripts were detected by RPA using the mouse cytokine set 1 multi-probe template (mCK1) (Pharmingen) according to the manufacturer's protocol. Radiolabeled RNase protected probes were resolved on polyacrylamide gels and were exposed to film (XAR, Eastman Kodak, Rochester, NY) for 16h at -70°C.

VI.5. Results

VI.5.1. Monitoring of *L. major* infection in resistant mice defective in the IL-12Rβ2 gene

IL-12Rβ2KO mice have been recently generated and have been shown to be defective in their ability to mount T_H1-mediated immune responses *in vitro* (118). To

Fig. 6.1. Course of *L. major* infection in genetically resistant mice defective in the IL-12R β 2 gene compared to resistant C57BL/6 and susceptible BALB/c mice. Mice (5 per group) were infected with 1×10^6 logarithmic-phase *L. major* promastigotes in the right hind footpad. Footpad swelling was monitored weekly using a metric caliper and footpad thickness was determined by subtracting the footpad thickness at T=0 of infection from the thickness reached every week. The mean and SDs are representative of three separate experiments.

*IL-12R β 2KO mice infected with *L. major* that developed footpad ulceration had to be euthanized in accordance with University of Ottawa Animals Care Committee guidelines.

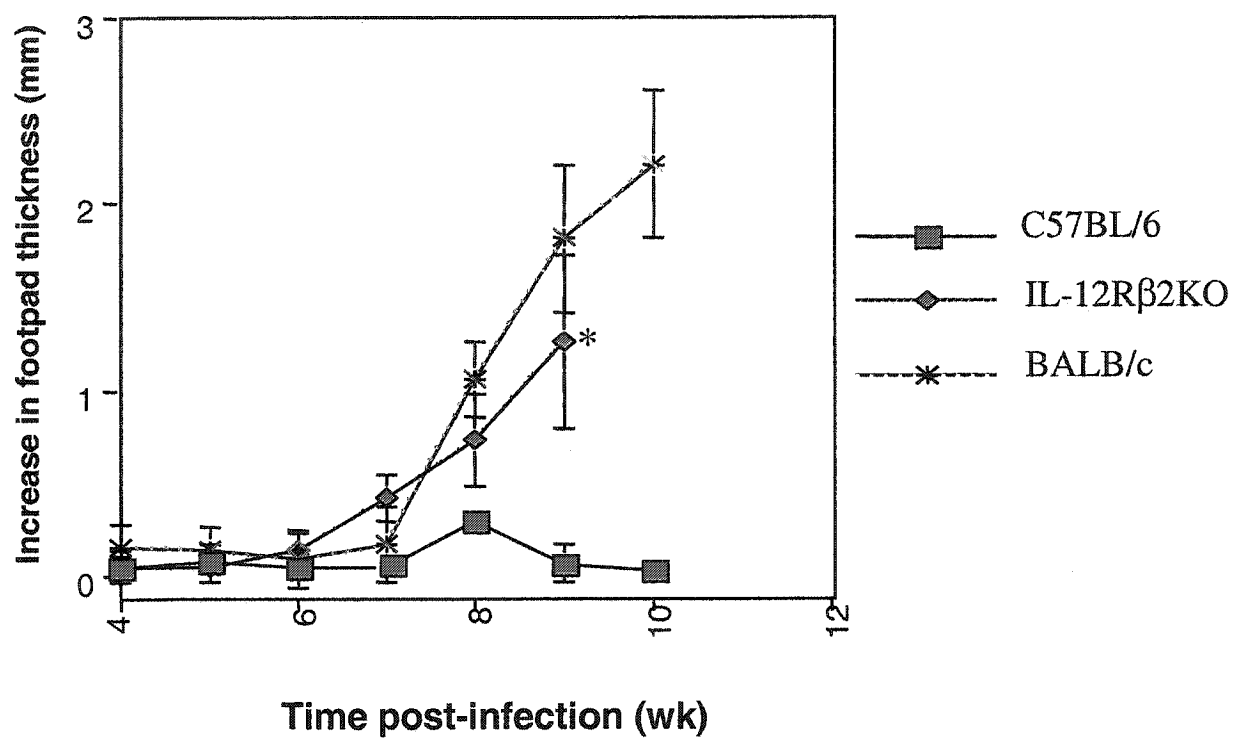
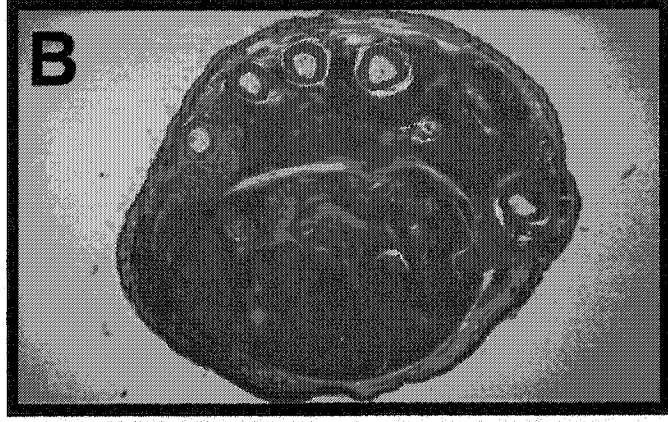


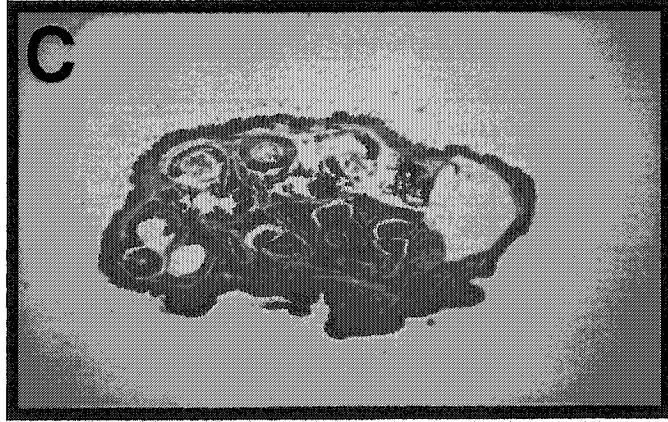
Fig. 6.2. Genetically resistant IL-12R β 2KO mice developed footpad swelling to *L. major* infection. Footpad sections from *L. major*-infected resistant mice lacking the IL-12R β 2 activity (IL-12R β 2KO) (A), susceptible BALB/c (B) and resistant C57BL/6 mice (C) were stained with hematoxylin-eosin and analyzed for tissue destruction. *L. major*-infection induced swelling and skin structure destruction in IL-12R β 2KO and BALB/c mice, whereas resistant C57BL/6 mice present less swelling and more preserved footpad size. Magnification x 2.



IL-12R β 2KO



BALB/c



C57BL/6

determine the effect of IL-12R β 2 gene deletion on the T_H1 immune responses during *L. major* infection, the course of infection was compared in resistant (C57BL/6), susceptible (BALB/c) and resistant C57BL/6 mice deficient in IL-12R β 2 gene (IL-12R β 2KO). Kinetics of footpad swelling was obtained by performing weekly measurements of the footpad thickness during the course of infection (Fig. 6.1). Resistant C57BL/6 mice developed a small footpad swelling, but were able to heal it and control the disease by 10 wk post-infection. In sharp contrast, IL-12R β 2KO mice showed an increase in footpad thickness and by wk 9, they developed ulcerated lesions that did not heal. In accordance with the Ottawa University animal care guidelines, the animals had to be euthanized at this time point. Similarly, susceptible BALB/c mice developed increasing footpad swelling accompanied with large non-healing ulceration by wk 10. Increase in footpad thickness was the first sign of susceptible phenotype developed by IL-12R β 2KO mice in response to *L. major*. These data suggested the important role played by IL-12 signaling in resistance to leishmaniasis.

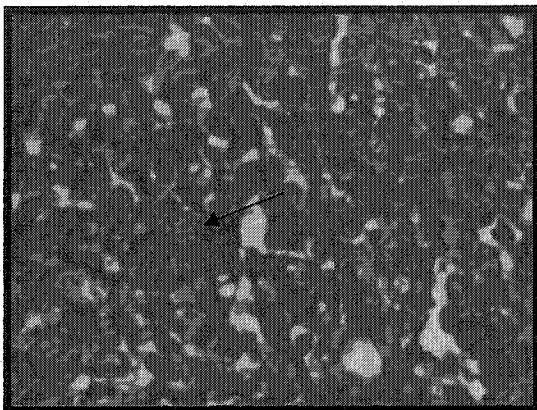
Histopathology was examined at the site of infection at the end of the infection time course using routine H&E staining. As shown in Fig. 6.2, injection of *L. major* promastigotes in the right footpads resulted in a significant footpad swelling in IL-12R β 2KO (A) and susceptible BALB/c (B) resulting in a destruction of the skin structure. However, footpad sections from *L. major*-infected resistant C57BL/6 mice revealed almost no lesions. Also, a massive inflammatory infiltrate that consists in *L. major*-infected macrophages, lymphocytes, neutrophils and eosinophils was observed in IL-12R β 2KO and BALB/c footpad sections (Fig. 6.3 A and B). This inflammatory reaction was characteristic of susceptibility to intracellular parasitic infection. However, footpad sections from C57BL/6 mice displayed more conserved structure with few inflammatory cells (Fig. 6.3 C).

VI.5.2. Proliferative response of IL-12R β 2KO splenocytes to *Leishmania* antigens

To examine the effect of IL-12R β 2 gene deletion on the capacity of spleen cells to proliferate in response to *in vitro* stimulation, splenocytes from *L. major*-infected IL-12R β 2KO, susceptible BALB/c and resistant C57BL/6 mice were cultured for 5d in the

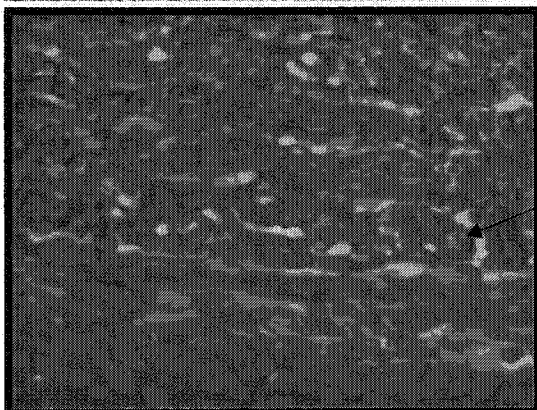
Fig. 6.3. Histological analysis of the footpad from *L. major*-infected IL-12R β 2KO mice. Footpad sections from resistant mice lacking IL-12R β 2 (IL-12R β 2KO), susceptible BALB/c and resistant C57BL/6 mice infected with *L. major* were stained with hematoxylin-eosin and analyzed for inflammatory cell infiltration. Similar to susceptible BALB/c mice, *L. major*-infected IL-12R β 2KO footpads displayed extensive inflammatory infiltration by parasite-loaded macrophages, neutrophils, eosinophils and lymphocytes. However, resistant C57BL/6 mice exhibited few inflammatory cells. *L. major* parasites residing inside macrophages are indicated by arrows. Magnification x 100.

A



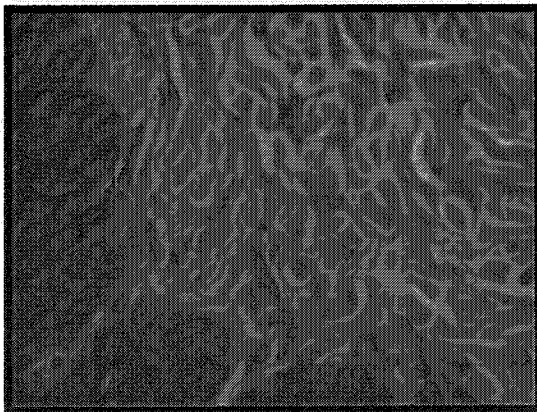
IL-12R β 2KO

B



BALB/c

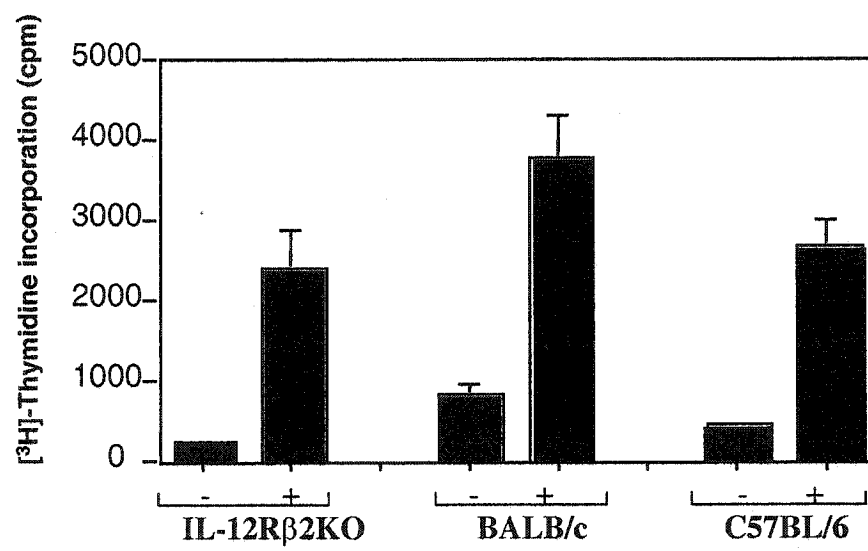
C



C57BL/6

X 100

Fig. 6.4. Proliferative response of splenocytes from *L. major*-infected IL-12R β 2KO mice to LmAg. Spleen cells from *L. major*-infected IL-12R β 2KO, BALB/c and C57BL/6 mice (5×10^5 cells/well) were incubated in the absence (-) or presence (+) of 10 μ g/ml LmAg for 72h. Cells were then pulsed for an additional 24h with tritiated thymidine and assayed for the proliferative response. The plotted values are the mean of triplicate ELISA wells and error bars represent the SEM. Similar to BALB/c and C57BL/6 mice, IL-12R β 2KO cells showed normal proliferative response to *Leishmania* antigens.



absence (-) or presence (+) of LmAg and assayed for their proliferation. As shown in Fig. 6.4, splenocytes from parasite-infected BALB/c, C57BL/6 and IL-12R β 2KO mice exhibited similar levels of proliferation in response to LmAg challenge, indicating that parasite-reactive cells from IL-12R β 2KO mice had no defect in their proliferative capacity.

VI.5.3. Analysis of parasite-specific antibodies in *L. major*-infected IL-12R β 2KO mice

Next, the antibody response to *L. major* antigens was analyzed. At the end of the infection course, parasite-specific serum IgG1, IgE and IgG2a levels were determined in *L. major*-infected IL-12R β 2KO mice and compared to susceptible BALB/c mice (Fig. 6.5). IL-12R β 2KO mice and BALB/c mice showed similar levels of parasite-specific T_H2-associated antibodies (IgG1 and IgE) (Fig. 6.5 A and B). More interestingly, in the absence of IL-12 signaling, no parasite-specific T_H1-associated antibody (IgG2a) was detected in IL-12R β 2KO mice whereas BALB/c mice contained substantial amounts of parasite-specific IgG2a isotypes (Fig. 6.5 C).

VI.5.4. Cytokine profile developed by IL-12R β 2KO mice in response to murine leishmaniasis

To determine whether the non-healing state of *L. major*-infected IL-12R β 2KO mice correlated with the development of T_H1 or T_H2 immune responses, an assessment of IFN- γ and TNF- α (as T_H1 specific cytokines) and the T_H2-associated IL-4 and IL-5 cytokine levels were determined (Fig. 6.6). Total LN cells isolated from *L. major*-infected IL-12R β 2KO and control mice (BALB/c and C57BL/6) were stimulated *in vitro* with LmAg and analyzed for cytokine secretion. LN cells from *L. major*-infected IL-12R β 2KO and susceptible BALB/c mice produced lower levels of IFN- γ in response to LmAg than did C57BL/6 mice. The decrease in IFN- γ was accentuated in IL-12R β 2KO mice (Fig. 6.6 A). The residual IFN- γ produced by IL-12R β 2KO cells was IL-12-independent since previous data have shown that activated splenocytes from these mice failed to produce IFN- γ in response to IL-12 stimulation (118). IFN- γ was also assessed in cell culture supernatants from cells plated in the absence of the anti-IL-4R M1 mAb

Fig. 6.5. In response to *L. major* infection, resistant C57BL/6 mice lacking IL-12R β 2 activity exhibited a parasite-specific T_H2-associated antibody profile. At the end of *L. major* infection course, parasite-specific T_H2-associated IgG1 (A) and IgE (B) antibodies as well as parasite-specific T_H1-associated IgG2a (C) antibodies were determined in blood sera from IL-12R β 2KO and BALB/c mice using capture ELISA. Blood sera were subjected to serial dilutions and parasite-specific isotype antibody levels were measured as the OD at 405 nm.

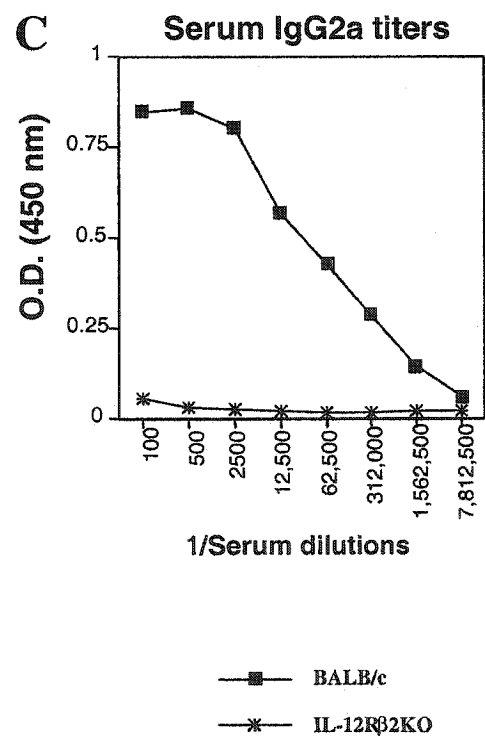
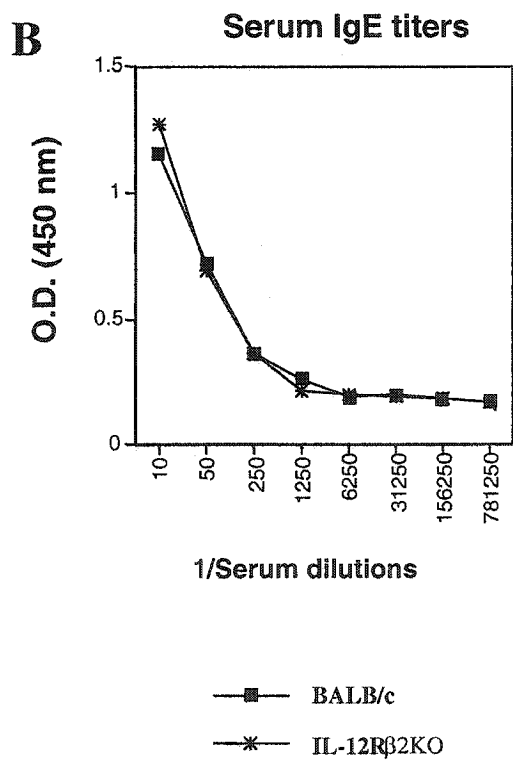
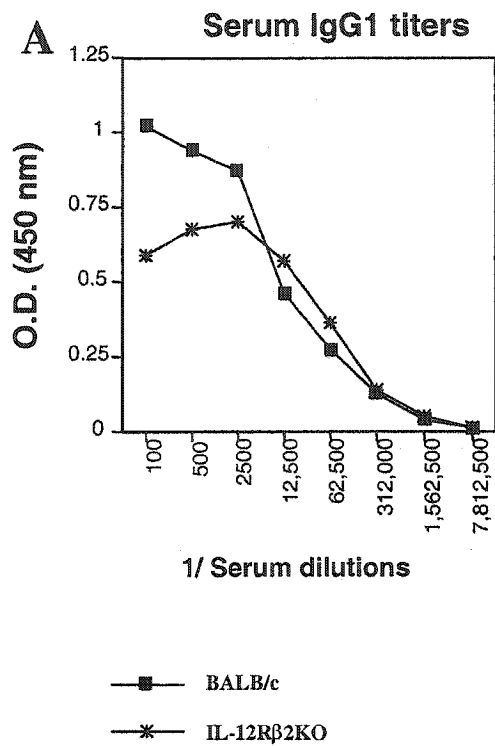
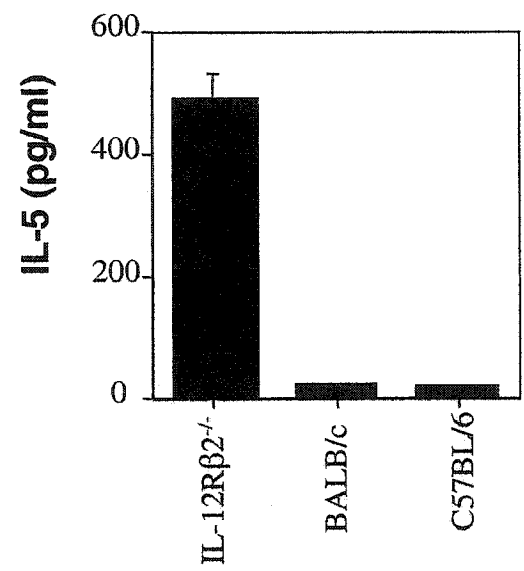
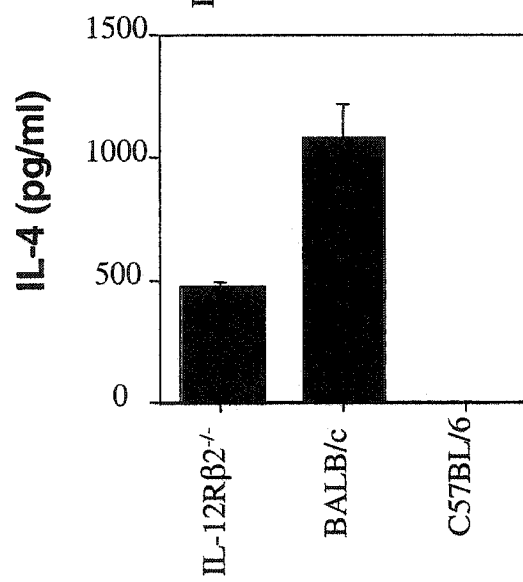
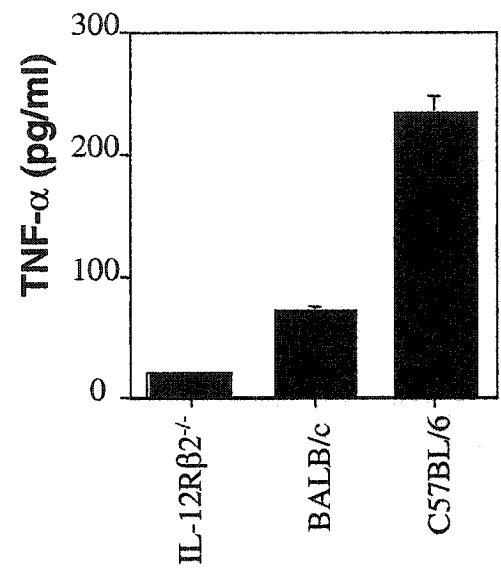
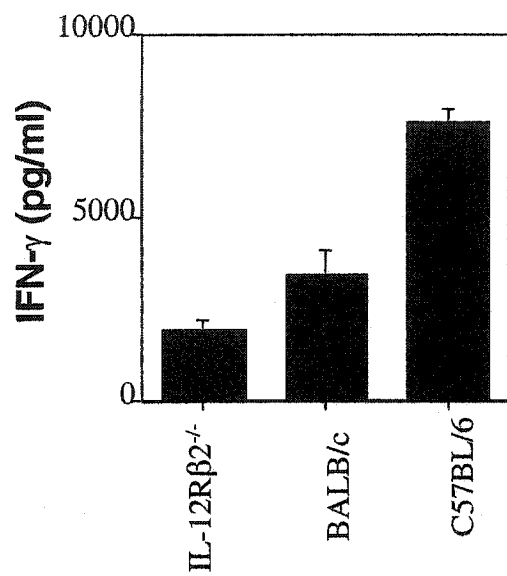


Fig. 6.6. The absence of IL-12R β 2 activity was associated with a decrease in parasite specific T_H1 and an increase in T_H2 cytokine secretion. Total LN cells from *L. major*-infected IL-12R β 2KO, susceptible BALB/c and resistant C57BL/6 mice were cultured at 5×10^5 cells/well in the presence of 10 μ g/ml of LmAg. At d3, cell culture supernatants were harvested and assayed by capture ELISA for IFN- γ , TNF- α (as T_H1 cytokines) and IL-4 and IL-5 production (as T_H2 cytokines). The plotted values are the mean of replicate ELISA wells and error bars represent the SEM.



and they exhibited a similar level of IFN- γ production (data not shown). Similarly, infected IL-12R β 2KO mice failed to upregulate TNF- α at levels similar to resistant C57BL/6 wild-type mice (Fig. 6.6 B). In contrast, IL-12R β 2KO and BALB/c mice produced much higher levels of IL-4 in response to LmAg than did C57BL/6 mice (Fig. 6.6 C). Interestingly, the amount of the T_H2-associated IL-5 cytokine produced by IL-12R β 2KO mice was significantly higher than BALB/c and C57BL/6 mice (Fig. 6.6 D). Taken together these results showed that *L. major*-infected resistant mice lacking the IL-12R β 2 chain developed a non-healing T_H2 phenotype characterized by an increase in IL-4 and IL-5 T_H2-associated cytokines and a decrease in IFN- γ and TNF- α T_H1-specific ones.

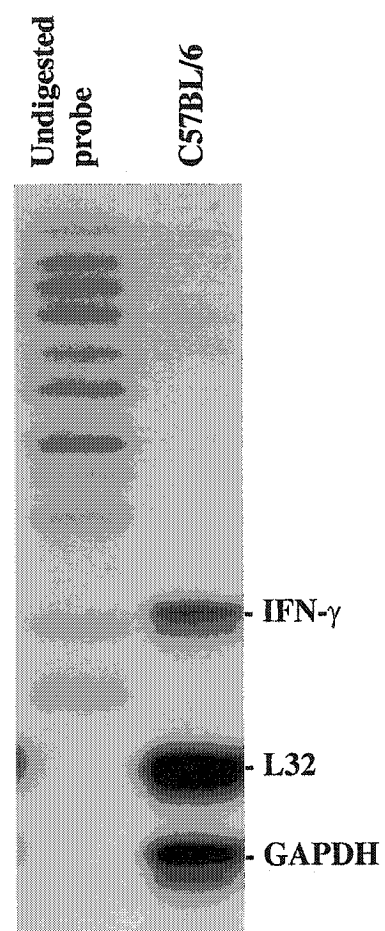
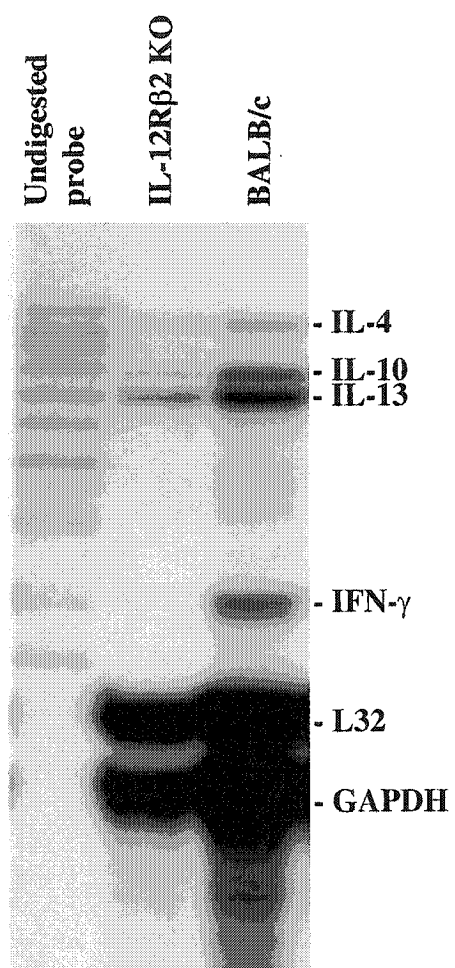
VI.5.5. Cytokine gene expression in IL-12R β 2KO mice in response to *L. major* infection

To further characterize the immune response in *L. major*-infected IL-12R β 2KO mice, C57BL/6 and BALB/c mice, the cytokine gene expression of LN cells from these different groups of mice was analyzed using RNase Protection Assay (Fig. 6.7). In response to LmAg stimulation, susceptible BALB/c mice expressed a mixed cytokine gene expression profile comprised of low levels of T_H1 (IFN- γ) and significant levels of T_H2 transcripts (IL-4, IL-10 and IL-13). IL-12R β 2KO LN cells produced only T_H2-associated IL-4, IL-10 and IL-13 mRNAs with no expression of IFN- γ mRNA. This may correspond to an accentuated T_H2 phenotype. In contrast, resistant C57BL/6 mice exhibited significant expression of IFN- γ mRNA but no T_H2 cytokine transcripts. This confirmed again the non-healing T_H2 pattern induced in resistant mice defective in IL-12 signaling in response to the murine leishmaniasis.

VI.6. Discussion

Resolution of lesions induced by the intracellular parasite *L. major* in genetically resistant mice, such as C57BL/6, results from the development of T_H1 immune responses inducing the production of elevated levels of IFN- γ (25). The development of T_H1 effector cells was shown to be strongly dependent on the early production of IL-12 by

Fig. 6.7. Parasite-specific cytokine gene expression confirmed the T_H2 phenotype developed by IL-12R β 2KO mice in response to *L. major* infection. Total lymph node (LN) cells isolated from *L. major*-infected IL-12R β 2KO, susceptible BALB/c and resistant C57BL/6 were stimulated *in vitro* with 10 μ g/ml LmAg at 2.5×10^6 cells/well in a 12-well plate. At d2, total RNA was harvested and analyzed for parasite-specific cytokine gene expression by RNase Protection assay (RPA) using the mCK1 mouse cytokine multi-probe template set (Pharmingen). In contrast to resistant C57BL/6 wild-type, cells from *L. major*-infected IL-12R β 2KO mice exhibited a complete absence of T_H1-associated cytokine transcripts and a significant upregulation of IL-4, IL-10 and IL-13 transcripts characteristic of a strong T_H2 response. C57BL/6 mice that showed resistance to *L. major* infection, showed no detectable levels of any T_H2 mRNA.



macrophages and dendritic cells, which triggers IFN- γ production and maintains the upregulation of the IL-12R β 2 chain (12,288,290,293). The resulting IFN- γ is required for downregulation of T_H2 differentiation and maintenance of T_H1 development (294). This was well demonstrated in resistant mice lacking IL-12 (77), IFN- γ (72) or IFN- γ receptor (73), which failed to induce T_H1 but instead, developed a fatal T_H2 immunity.

In the present study, genetically resistant mice lacking the transducing subunit of IL-12 receptor (IL-12R β 2 chain) were infected with *L. major* to analyze the role of IL-12 signaling in T_H1/T_H2 differentiation and consequently in conferring protective immunity to this intracellular parasite. Similar to IL-12R β 1KO mice (117), IL-12R β 2KO mice are reported to be incapable of expressing IFN- γ in response to IL-12 *in vitro* (118). Nevertheless, IL-12R β 2KO mice continue to express normal IL-12R β 1 and bind IL-12 with low and high affinities (117,118). The results presented here strongly demonstrated that signaling via IL-12R β 2 chain is crucial for protective T_H1 immunity against the intracellular parasite *L. major*. Indeed, the data showed that in contrast to resistant wild-type mice, *L. major* infection of IL-12R β 2KO mice displayed a significant increase in footpad swelling after infection with *L. major* promastigote parasites. This was accompanied with the development of large non-healing cutaneous lesions. Infection was also associated with extensive tissue destruction due to significant numbers of *L. major*-infected-macrophages, lymphocytes, eosinophils and neutrophils infiltrated at the site of infection.

The mechanisms reported to underlie the development of non-healing lesions in susceptible BALB/c mice in response to *L. major*-infection include a lack of expansion of T_H1 effector cells (72,73). In addition, T_H2-associated cytokines exacerbate the disease's state by deactivating macrophages (1,281,284). The T_H2 immune response in susceptible BALB/c mice is thought to be generated by the burst of IL-4 that occurs early during infection (31,69). This IL-4 results in inhibition of IL-12 production (287,288) and failure to induce the IL-12-dependent appropriate T_H1 responses (103). Although there are conflicting data regarding *Leishmania* susceptibility in IL-4-deficient mice (26,65), neutralization of *L. major*-induced IL-4 promoted a protective T_H1 immune response in susceptible BALB/c mice and repressed the fatal T_H2 cell response (295). Furthermore, overexpression of IL-4 in transgenic mice of a resistant background rendered these mice

susceptible to this parasite (66). Similar to IL-4, a recent study demonstrated that overexpression of the T_H2-specific IL-13 in C57BL/6 mice rendered these animals susceptible to leishmaniasis, whereas BALB/c mice defective in IL-13 and/or IL-4 cytokine genes developed resistance to the disease (281). The role of IL-10 in promoting disease progression in murine leishmaniasis model has also been investigated (284) and susceptible BALB/c mice deficient in the IL-10 gene were able to control *L. major* infection compared to normal BALB/c mice. In agreement with the role of IL-12 in leishmaniasis (59,77), the results reported herein indicated that resistant mice lacking IL-12R β 2 gene exhibited a cytokine profile characterized by the expression of IL-4 and IL-5 cytokines at the protein levels and by the upregulation of IL-4, IL-10 and IL-13 genes at the transcriptional levels, which correlated with a strong T_H2 phenotype. In contrast, IFN- γ was lower at the protein level and non-detectable at the transcriptional levels. The residual IFN- γ production by BALB/c mice observed herein was in agreement with previous studies showing that LN cells from infected BALB/c mice are able to produce both IL-4 and a small amount of IFN- γ in response to LmAg stimulation but that the amount of IFN- γ is insufficient to confer protection in the context of a strong T_H2 response (296). *L. major*-infected IL-12R β 2KO mice also produced parasite-specific T_H2-associated IgG1 and IgE antibodies but did not produce T_H1-associated IgG2a isotype antibodies. Although protective immunity to *L. major* was shown to be independent of parasite-specific Abs (297), parasite-specific Ab production reflected the ability of the host to respond to antigen efficiently. Indeed, expression of serum isotype antibodies is reported to mirror the defaults towards T_H1 or T_H2 (50). In accordance with previous reports, the data reported herein demonstrated that switching to IgG2a isotype is critically dependent upon T_H1-like immune responses and consequently upon intact IL-12 signaling, whereas switching to IgG1 and IgE correlates with T_H2 responses (298). However, the outcome observed in the present study is in contrast with a study showing that resistant mice deficient in STAT4 signaling molecules exhibited susceptibility to *L. major* infection but did not default towards the T_H2 phenotype (78). As explained in the general introduction (Chapter I), IL-12 is reported to signal via multiple STAT4-independent pathways such as the STAT1 and STAT3 pathway (111,113), p56^{lck} (125), NF- κ B (63) and different MAP kinase pathways (126-128). The study using STAT4^{-/-}

mice implies the presence of a STAT4-independent pathway that is able to downregulate T_H2 cell development. However, this “alternate” pathway was not sufficient to generate an appropriate T_H1 immune response.

The data presented herein demonstrated that *L. major*-infected IL-12R β 2KO mice produced lower levels of TNF- α compared to susceptible BALB/c and resistant C57BL/6 wild-type mice. This is consistent with different reports showing that in murine leishmaniasis, TNF- α was associated with protective immunity (299,300). Indeed, in contrast to susceptible BALB/c mice, resistant mice upregulate TNF- α expression in draining LN in response to *L. major* infection (301). Moreover, different studies have suggested a role for TNF- α in reducing the size of *Leishmania*-induced lesions and containing parasite dissemination (302-304). Indeed, infection of resistant C57BL/6 mice lacking the TNF- α gene with *L. major* parasites led to a dramatic development of a visceral form of the disease and resulted in a rapid death of the animals (305) suggesting that TNF- α is required for the control of *Leishmania* parasites *in vivo*.

IL-18 is another pro-inflammatory cytokine capable of inducing high levels of IFN- γ by immune cells. The results reported in chapter III and IV demonstrated that IL-18 was able to induce IFN- γ secretion from T and NK cells independently of IL-12 signaling. However, after infection of IL-12R β 2KO mice with *L. major* parasites, only a negligible amount of IFN- γ protein was detected in draining LN. This inability of IL-18 to compensate for the IL-12 signaling loss might be due to two phenomena: 1) Despite its capacity to increase IFN- γ production, IL-18 stimulation also results in T_H2 -associated cytokine expression known as strong antagonists of IFN- γ (149,158). In addition, IL-18 was reported to be unable to induce T_H1 cell differentiation but instead, synergizes with IL-12 to develop a type-1 immune response (163). 2) In reference to a recent study (306), IL-18 was shown to not be expressed during canine leishmaniasis.

Recently, a novel cytokine named IL-23 has been reported to have the capacity to bind the IL-12R β 1, but not the IL-12R β 2 chain (129). IL-23 is composed of a p19 protein that combines with the p40 subunit of the IL-12 cytokine to form the biologically active cytokine (115). IL-23 acts via a heterodimeric receptor comprised of the β 1 subunit of IL-12R and a recently reported subunit termed IL-23R (307). IL-23 shares many

activities with IL-12. Indeed, similar to IL-12, IL-23 activates STAT4 signaling proteins and induces proliferation and IFN- γ production in human PHA-blasts and murine memory T cells (129). However, IL-23 is unable to induce proliferation and IFN- γ production from murine naïve T cells (115). Over-expression of IL-23 in transgenic mice induced high levels of circulating TNF- α and resulted in multi-organ inflammation and premature death (308). Although sharing homology and some functions with IL-12 (115,129,308), *in vivo* induction of IL-23 expression is still unknown. The study on the immune response of resistant mice lacking IL-12R β 2 to *L. major* infection indicates that in *L. major*-infected IL-12R β 2KO mice, IL-23 (if induced at appropriate levels) was not able to compensate for the loss of IL-12 signaling through the IL-12R β 2 chain. In addition, even though the cytoplasmic domain of murine IL-12R β 1 chain binds Tyk2 kinase during JAK/STAT pathway activation (12,90), this chain alone was not able to overcome the loss of IL-12R β 2 chain to induce T_H1 cell development.

In conclusion, C57BL/6 mice that are deficient for IL-12R β 2 became susceptible to *L. major* infection and unlike STAT4-deficient C57BL/6 mice, defaulted to a T_H2-specific cytokine pattern. Furthermore, IL-12R β 2KO mice completely downregulated parasite-specific T_H1-associated isotype antibodies (IgG2a) and expressed high levels of parasite-specific T_H2-associated ones (IgG1 and IgE). Despite the increasing interest expressed towards the new IL-12-related IL-23 cytokine, the results presented in this chapter confirmed that signaling via IL-12R β 2 chain is still required for appropriate T_H1 cell polarization and for conferring resistance following infection with the intracellular parasite *L. major*.

Concluding Remarks

Through its ability to induce IFN- γ from NK and T cells, IL-12 plays a critical role in both innate and adaptive immunity of the host defense against intracellular pathogens. The results reported in the present thesis are summarized in Fig. 1. DC cells constitute a major source of IL-12 and IL-18 production after infection with pathogens (Fig. 1-A). IL-12 or IL-18 act on the cells of the innate immune system such as NK cells and also on T cells, part of the adaptive immune system. NK cell responsiveness to IL-12 requires co-activation of these cells with IL-2 (Fig. 1-B). Two novel observations emerged from the NK cell study reported in the present work: 1) Upon activation with IL-2, NK cells upregulate IL-12R β 2 chain and differentiate *in vitro* towards two cell subsets, IL-12R β 2^{lo} and IL-12R β 2^{hi}, expressing different levels of IL-12R β 2 subunit (Fig. 1-C). Both NK cell subsets respond similarly to IL-12 by increasing the production of IFN- γ , the proliferation to low dose IL-12 and the cytolytic activity against susceptible target cells. However, IL-12R β 2^{hi} cells produce higher levels of IFN- γ than IL-12R β 2^{lo} cells in response to the combination of IL-2+IL-12+IL-18. 2) The second interesting observation was revealed by phenotypic analyses of these two NK cell subsets that demonstrated that only IL-12R β 2^{hi} cell subset expresses the NK inhibitory receptor Ly49G2. Although these NK cell subsets differ in the expression of the NK inhibitory receptor Ly-49G2 and respond differently to the IL-2+IL-12+IL-18 cytokine combination by producing different amounts of IFN- γ *in vitro*, the actual development of these two NK subsets *in vivo* and their roles in disease and immunity is still to be determined.

IL-15, produced by DC and endothelial cells, also upregulates IL-12R β 2 expression in NK cells allowing their responsiveness to IL-12 stimulation. This suggests the use of IL-15 as an alternate stimulator to sensitize NK cells to the presence of IL-12 in the milieu (Fig. 1-B). At this stage of NK cell stimulation, responsiveness to IL-12 is manifested by an increase in IFN- γ and IL-10 production (Fig.1-D). The ability of IL-12 to increase IFN- γ secretion and to activate the cytolytic activity of NK cells is at the basis of its potential role in cancer therapy. Indeed, anti-tumor activity of IL-12 has been proven in several mouse models and in clinical trials and understanding the regulation of IL-12R may contribute to the development of efficient strategies in tumor therapy.

Activated NK cells are also responsive to IL-18. Similar to IL-12, IL-18 induces IFN- γ production by NK cells, however, in contrast to IL-12 stimulation, IFN- γ production in response to IL-18 is accompanied with the expression of an increased level of the T_H2-cytokine IL-13 (Fig.1-D).

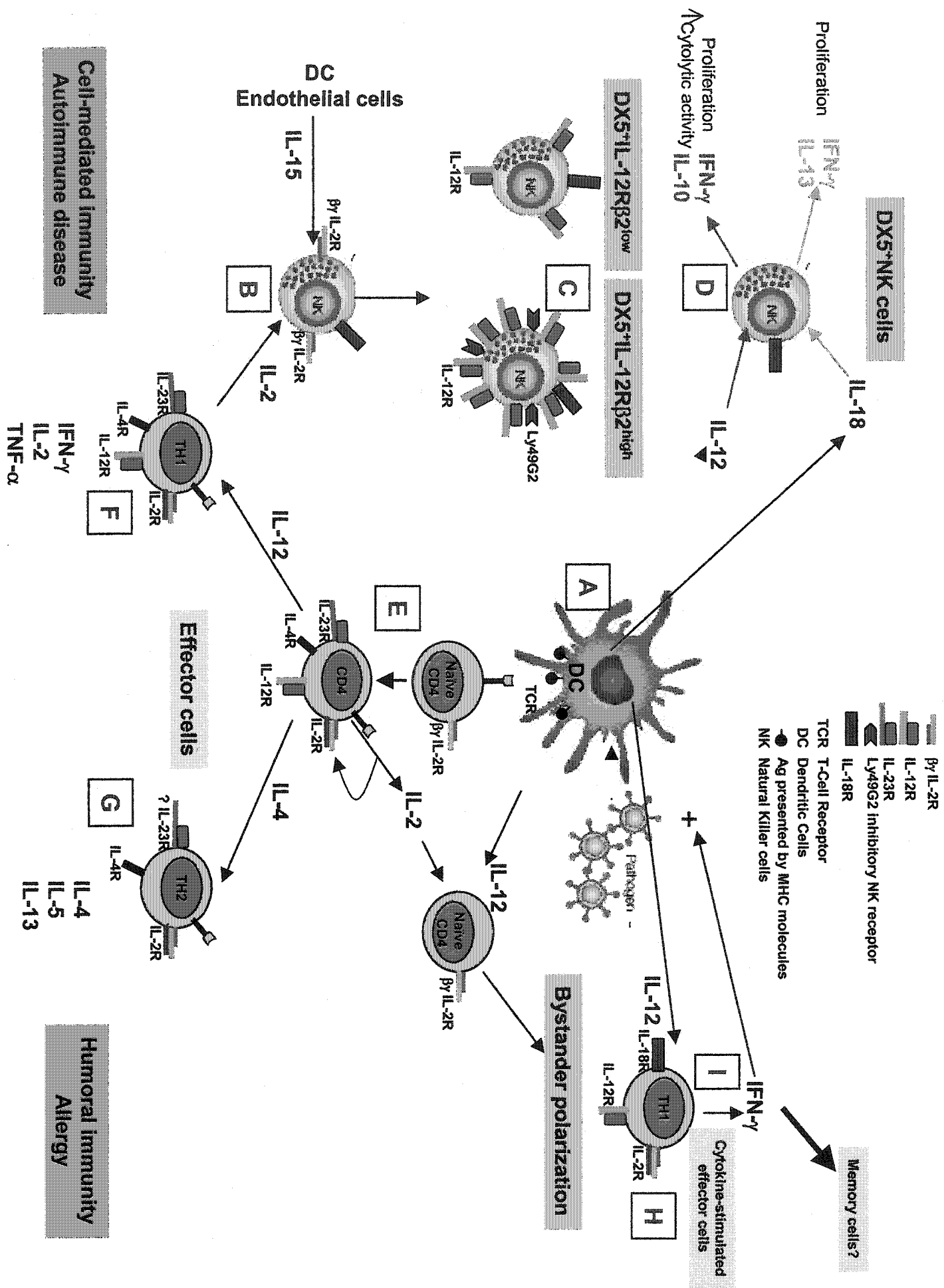
The data presented in the present thesis also provide evidence that IL-12 and IL-18 act on NK cells independently of each other and that IL-18 signaling is able to induce the production of high levels of IFN- γ in NK cells independently of IL-12 signaling, a point which has been controversial in the literature until now.

DC cells are professional APC able to present specific peptides to T cells via their MHC molecules. Upon antigen encounter, naive CD4⁺T cells are activated. They upregulate their high affinity IL-2 receptor and produce IL-2 cytokine that act on T cells in an autocrine manner (Fig. 1-E). Activated CD4⁺T cells also express both β 1 and β 2 chains of IL-12R as well as IL-23R and are able to respond to IL-12. The presence of IL-12 drives the differentiation of CD4⁺T cells towards T_H1 phenotype responsible for cell-mediated immunity (Fig. 1-F). However, if IL-4 is present in the environment at the time of stimulation, CD4⁺T cells develop towards a T_H2 phenotype and promote humoral immunity (Fig. 1-G). Developed T_H2 cells do not express IL-12R β 2 chain and do not respond to IL-12. The importance and the novelty of the work presented herein also resides in the discovery that murine naïve CD4⁺T cells are capable of responding to high doses of IL-2 *in vitro* in the absence of any TCR-ligation. Indeed, naive CD4⁺T cells respond to IL-2 via the β and γ chains of IL-2R (intermediate affinity receptor). Activation with IL-2 upregulated the expression of IL-12R β 2 and rendered naïve T cells responsive to IL-12 and/or IL-18 (Fig. 1-H). More interestingly, cytokine-activated murine naïve CD4⁺T cells were able to show signs of polarization towards a T_H1 phenotype when stimulated in the presence of IL-2+IL-12 (Fig. 1-I). This phenomenon, termed "Bystander polarization", may occur *in vivo* in response to a strong immune response to an unrelated antigen that is capable of generating sufficient IL-2 cytokine in the milieu to activate naïve T lymphocytes. Bystander polarized T cells might then help to enrich the pool of T_H1 memory cells present in the organism.

IL-12 also plays a central role in the regulation of the response to infection and T_H1/T_H2-related diseases. Understanding the mechanisms governing IL-12R β 2 regulation

might contribute to our knowledge of T_H1/T_H2 regulation that remains a priority to prevent or cure T_H1 -associated autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and insulin-dependent diabetes mellitus as well as T_H2 -associated diseases such as allergy and asthma. Analysis of the immune response of susceptible transgenic BALB/c mice constitutively expressing IL-12R β 2 gene to the intracellular parasite *L. major* indicated that these transgenic BALB/c mice developed a T_H2 -immune response characteristic of susceptible animals and that they could not heal the parasitic disease. This clarifies that loss of IL-12R β 2 activity is not required for the differentiation and the stability of T_H2 effector cells *in vivo* and consequently focusing on IL-12 might not be a suitable therapeutic target for T_H2 -dependent diseases. However, the study of the immune response of resistant mice deficient in the IL-12R β 2 gene to *L. major* infection showed that these mice were unable to develop any T_H1 immune response and become susceptible to the infection by defaulting towards a fatal T_H2 phenotype. These data suggest that the loss of IL-12R β 2 activity was not compensated by either the newly described IL-23 cytokine or by IL-18. This finding indicates that intact IL-12 signaling via IL-12R β 2 is critical for the development of T_H1 effector cells. Consequently targeting the IL-12 cytokine may help to develop immune therapies to cure intracellular parasitic infections and T_H1 -associated autoimmune diseases.

Fig. 1: Proposed model to summarize the main data reported in the present thesis. Upon activation with pathogens, DC produce IL-12 and IL-18 (A). IL-12 acts on NK cells, however, a co-stimulation of these cells with IL-2 is required to induce IFN- γ production by these cells. Activation of NK cells with IL-2 results in the development of two NK cell subsets *in vitro*, characterized by the expression of different levels of IL-12R β 2 chain. These subsets were named IL-12R β 2^{lo} and IL-12R β 2^{hi} (C). NK cells also upregulate IL-12R β 2 expression in response to IL-15 released by DC and endothelial cells *in vivo*. IL-15-stimulated NK cells respond to IL-12 or IL-18 by increasing IFN- γ production suggesting that IL-15 may be used as a substitute for IL-2 activation of NK cells (B). IL-2-activated NK cells respond to IL-12 by upregulating IFN- γ secretion and IL-10 production (D). Activated NK cells also respond to IL-18 by increasing IFN- γ production, however, in contrast to IL-12, IL-18 responsiveness is characterized by the upregulation of the T_H2-cytokine IL-13 (D). DC are professional APC capable of activating T cells by presenting specific antigenic peptides via their MHC molecules. Activation of CD4⁺T cells via TCR results in upregulation of high affinity IL-2R, IL-12R, IL-23R and secretion of IL-2 that acts on T cells in an autocrine manner (E). When IL-12 is present in the environment during T cell activation, naive CD4⁺T cells develop towards T_H1 phenotype responsible for cell-mediated immunity (F). However when IL-4 is present in the milieu, naive CD4⁺T cells differentiate towards a T_H2 phenotype characterized by the absence of IL-12R β 2 expression (G). CD4⁺ T cells are also able to respond to high dose of IL-2 *in vitro* by upregulating the IL-2R α chain, IL-12R and IL-18R (H). Interestingly, IL-2-activated CD4⁺T cells responded to IL-2+IL-12 and showed signs of polarization towards T_H1 phenotype in the absence of any TCR ligation. This phenomenon was termed “bystander polarization” (I).



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Appendix A

A.1. Mice

For all the different experiments reported in the present dissertation, mice were used at 10 to 24 weeks of age.

- Normal C57BL/6 and IL-12R β 2-deficient C57BL/6 female mice were obtained from Jackson Laboratory (Bar Harbor, ME).
- Rag2^{-/-}/TCR-transgenic-DO11.10 female mice were from Taconic (Germantown, NY)
- BALB/c mice were bred in the animal facility at Ottawa University (Ottawa, ON) and held under specific pathogen-free conditions

A.2. ELISA antibodies

- For IL-4 ELISA, anti-mouse IL-4 (11B11) capture mAb was supplied by Dr.Craig Reynolds from NCI (Bethesda, MD) and biotinylated rat anti-mouse IL-4 detection mAb (BVD6-24G2) from Pharmingen. Recombinant murine IL-4 standards were from Life Technologies.
- For IL-5 ELISA, the capture Ab, purified rat anti-mouse/human IL-5 mAb (TRFK5), and the detection mAb, biotinylated rat anti-mouse IL-5 mAb (TRFK4), were purchased from Pharmingen. Recombinant murine IL-5 standards were from R&D systems.
- For IFN- γ ELISA, IFN- γ capture Ab (R4-6A2), biotinylated rat anti-mouse IFN- γ (XMG1.2) and recombinant murine IFN- γ (utilized as standards) were obtained from Pharmingen.
- For TNF- α ELISA experiments, OptEIA set: Mouse TNF- α (Mono/Poly) from Pharmingen was used.

Table 2. List of the antibodies used for flow cytometry and cell stimulations

Antibodies against mouse	Clones	Sources
B220 (CD45R)	RA3-6B2	Pharmingen
CD3	145-2C11	Cedarlane
CD3-biotin	145-2C11	Cedarlane
CD3-FITC	145-2C11	Cedarlane
CD4-FITC	L3T4	Pharmingen
CD8-FITC	53-6.7	Sigma
CD19-PE	1D3	Pharmingen
CD25-PE	PC61.5.3	Cedarlane
CD28	37.51	Pharmingen
CD44-PE	1M7	Pharmingen
CD45RB-biotin	16A	Pharmingen
CD62L-PE	MEL-14	Cedarlane
IFN- γ	R4-6A2	Pharmingen
IFN- γ -PE	XMG1.2	Pharmingen
IFN- γ -PE matching isotype control (Rat IgG1)	R3-34	Pharmingen
IL-4 mAb	11B11	Dr. Craig Reynolds (NCI, Bethesda, MD)
IL-4R mAb	muIL4RM1	Dr. Charlie Maliszewski (Immunex, Seattle, WA)
IL-12p40 hybridomas	C17.8	Dr. Giorgio Trinchieri (The Wistar Institute, Philadelphia, PA)
Ly49A	YE 1/32	Dr Suzanne Lemieux (Institut Armand Frappier, Laval)
Ly49C-biotin	4LO3311	Dr Suzanne Lemieux (Institut Armand Frappier, Laval)
Ly49G2-biotin	4LO439	Dr Suzanne Lemieux (Institut Armand Frappier, Laval)
Ly49G2-FITC	4D11	Pharmingen
NK1.1-PE	PK136	Cedarlane
Pan-NK/biotin mAb	DX5	Pharmingen
Pan-NK/ PE	DX5	Pharmingen
Rat Ig-FITC (polyclonal Ab)	-----	Pharmingen

Table 4. Controls used in flow cytometry for extracellular cell surface markers and for intracellular cytokine staining.

Cell staining	Abs used	Controls
IL-12R β 2	- Rat anti-mouse IL-12R β 2 antiserum/ FITC conjugated anti-rat Igs	Non-stained cells (autofluorescence) Non-immune rat serum FITC conjugated anti rat Igs only
CD3	- Anti-CD3-FITC - Biotin-anti-CD3/STV-PE	- Non-stained cells (autofluorescence)
CD4	Anti-CD4-FITC	- Non-stained cells (autofluorescence)
DX5	Anti-DX5-PE	- Non-stained cells (autofluorescence)
NK 1.1	Anti-NK 1.1-PE	- Non-stained cells (autofluorescence)
B 220	Anti-B220-PE	- Non-stained cells (autofluorescence)
CD 19	Anti-CD19-PE	- Non-stained cells (autofluorescence)
CD 25	Anti-CD25-PE	- Non-stained cells (autofluorescence)
CD 44	Anti-CD44-PE	- Non-stained cells (autofluorescence)
CD 45	Biotine anti-CD45RB STV-PE	- Non-stained cells (autofluorescence)
CD 62L	Anti-CD62L-PE	- Non-stained cells (autofluorescence)
Ly49G2	Anti-LY49G2-FITC	- Non-stained cells (autofluorescence)
Ly49A	Anti-Ly49A-FITC	- Non-stained cells (autofluorescence)
Ly49C	Anti-Ly49C	- Non-stained cells (autofluorescence)
Intracellular IFN- γ	Anti-IFN- γ -PE	Non-stained cells (autofluorescence) Isotype matching Ig-PE IFN- γ -PE in cells not producing IFN- γ

A.3. Cytokines and RPA probes

- Human IL-2 (huIL-2), provided by Dr. Craig Reynolds (NCI, Bethesda, MD).
- Murine IL-12 (muIL-12), provided by Genetics Institute (Cambridge, MA).
- Murine IL-4, IL-15 and IL-18 were purchased from PeproTech.
- Recombinant murine IFN- γ were from Pharmingen.
- Mouse cytokine multi-probe mCK1 template sets (Pharmingen) contain IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, IFN- γ , L32 and GAPDH templates.
- Mouse cytokine multi-probe mCR3 template sets (Pharmingen) contain IL-10R, IL-11R, IL-12R β 1, IL-12R β 2, IL-6R α , gp130, IFN- γ R α , IFN- γ R β , L32 and GAPDH.

A.4. Buffers and solutions

A.4.1. Bacterial media, solutions and buffers

A.4.1.1. LB medium

10 g tryptone peptone (Difco, Becton Dickinson), 5g yeast extract (Difco, Becton Dickinson), 5g NaCl (Sigma) and 1 ml 1N NaOH (VWR). Complete to 1L with ddH₂O.

A.4.1.2. LB plates

15 g of agar (bacto Agar, Difco, Becton Dickinson) is added per liter of LB medium. The mixture is autoclaved at 121°C for 45 min. Upon cooling, LB agar was supplemented with 100 μ g/ml ampicilline or 35 μ g/ml chloramphenicol.

A.4.1.3. Calcium Chloride solution

60 mM CaCl₂ (Sigma), 15 % glycerol and 10 mM PIPES (Sigma) pH 7 in ddH₂O. The solution was autoclaved and kept cold at 4°C.

A.4.1.4. Bacterial lysis buffer

50 mM NaH₂PO₄ (pH8) containing 300 mM NaCl, 1 mM PMSF (Sigma), 1 mg/ml lysozyme (Sigma), 10 mM β 2-ME and one protease inhibitor cocktail tablet (Roche).

A.4.1.5. Bacterial freezing solution

65% glycerol (BDH), 0.1 M MgSO₄ (BDH), 0.25 M Tris buffer (VWR) pH8 in ddH₂O and filtered using syringe driven-filter units (Millipore).

A.4.2. 100 x Denhardt's solution

2 % (w/v) BSA, 2 % (v/v) Ficoll (Sigma) and 2 % (w/v) polyvinylpyrrolidone (Sigma) in ddH₂O.

A.4.3. Table 3. SDS polyacrylamide gel composition

Components	Stacking Gel	Resolving Gels	
		10 %	12 %
40 % Acryl. / Bis (29:1)	1 mL	2.5 mL	3 mL
0.5 M Tris HCl, pH 6.8	2.5 mL	-----	-----
1.5 M Tris - HCl, pH 8.8	-----	2.5 mL	2.5 mL
10% SDS	100 μ L	100 μ L	100 μ L
dd H ₂ O	6.4 mL	4.8 mL	4.35 mL
TEMED	10 μ L	5 μ L	5 μ L
10% APS	50 μ L	50 μ L	50 μ L
Total Volume	10 mL	10 mL	10 mL

A.4.4. Oligo-labeling buffer (OLB)

OLB is composed of solution A (10 μ l), solution B (25 μ l) and solution C (15 μ l) and stored at -20°C . The different solutions were prepared as follows:

Solution A (Stored at -20°C): 1 ml of solution O [1.25 M Tris-HCl, 0.125 M MgCl_2 pH 8.0]; 18 μ l β 2-ME and 5 μ l each of dATP, dTTP and dGTP (dNTP solutions were prepared at 0.1 M in TE buffer and stored at -20°C).

Solution B (Stored at 4°C): 2 M free-acid HEPES, pH 6.6.

Solution C (Stored at -20°C): Hexadeoxynucleotides (Amersham Pharmacia Biotech # 2166)

A.4.5. Starving medium for Jurkat cell transfection

RPMI 1640 containing 10% FCS, 25 mM HEPES, penicillin/streptomycin and L. glutamine. The medium was filtered in 500 ml Vacuum driven filtration system Stericup (Millipore, Bedford, MA). This medium is depleted from β 2-ME.

A.4.6. TE (Tris-EDTA) buffer

For 1 X stock solution: 10 mM Tris-HCl, pH 8 (Sigma); 1 mM EDTA pH 8 (Sigma) in ddH₂O.

A.4.7. Western-blotting buffers.

A.4.7.1. 5 x SDS electrode running buffer (pH 8.3)

To make up 1L with ddH₂O, 15 g Tris base (VWR Canlab); 72 g glycine and 5 g SDS. SDS electrode running buffer was used at 1 x dilution.

A.4.7.2. BSN (Bierum & Shafer-Nielson) transfer buffer (pH 9.2)

To make up 1L with ddH₂O: 5.82 g Tris base (VWR); 2.93 g glycine (Sigma); 200 ml Methanol (BDH); 3.75 ml 10% SDS (Sigma).

A.5. Probe and primer sequences (5'...3')

A.5.1. IL-12R β 1 primer sequences for PCR amplification of murine IL-12R β 1 complete cDNA

Sense: ACGTGAATTCATGGACATGATGGGGCTGCCA

Anti-sense: CATGGAATTCTCAGGCCTCTTGCCCCAAAGT

A.5.2. IL-12R β 2 primer sequences for PCR amplification of murine IL-12R β 2 complete cDNA

Sense: ACGTGAATTCATGGCACAGACTGTTAGAGAA
(IL-12R β 2-5'-EcoRI)

Anti-sense: CATGGAATTCTCAAGCCTCATTACTCATGAG
(IL-12R β 2-3'-EcoRI)

A.5.3. Primer sequences for PCR amplification of murine IL-12R β 2 cDNA (for anti-mouse IL-12R β 2 antisera production)

Sense: ATGGCTAGCCATCACCATCACCATCACAA
TATAGATGTGTGCAAGCTT
Anti-sense: CATGGAATTCTCAAGCCTCATTACTCATGAG

A.5.4. Primer sequences for VAIL-12R β 2 transgene amplification

A.5.4.1. For VAIL-12R β 2 amplification from gDNA (screening for positive VAIL-12R β 2-tg mice)

Sense: AGCACCAGTGAGTGGCCCTC (IL-12R β 2-1600)
Anti-sense: GGCCCGGGTACGTAGAATTC (CD2-UTR)

A.5.4.2. For VAIL-12R β 2 amplification by RT-PCR

Sense: CTCCTGTCTACTCTCAAACC (IL-12R β 2-2000)
Anti-sense: GGCCCGGGTACGTAGAATTC (CD2-UTR)

A.5.4.3. For endogenous IL-12R β 2 amplification from IL-12R β 2KO tail gDNA

Sense: ATGGCACAGACTGTTAGAGA (IL-12R β 2-5')
Anti-sense: TTGCTTTAATCAGCAGCCAC (IL-12R β 2-3'-70)

A.5.4.4. For IL-12R β 2 amplification by RT-PCR from the Jurkat cells

Sense: CTCCTGTCTACTCTCAAACC (IL-12R β 2-2000-5')
Anti-sense: GGA GTA GGA ACT GGG CAG AT (IL-12R β 2-3'UTR)

All the primers cited above were prepared by the Biotechnology Research Institute at the faculty of Medicine (University of Ottawa, Ottawa, ON).

A.5.5. Probes and primers used in real-time PCR analyses

A.5.5.1. IL-12R β 2

Sense: CCT GCC CCT GTG ATT CCT C
Anti-sense: AGC CCT GCT TGG GAT TTA AAG

Probe: TGG GTC AGC TGC CAA TAT

A.5.5.2. *IL-18R α*

Sense: AGC CGT CAA GCC ATG GAG

Anti-sense: GTC TGG AGC TCA AGG TGC TGA

Probe: AGG AGT CGG AGG CGC GGT CTG

A.5.5.3. *IL-18R β*

Sense: AAA AAG GCT CTG CGG GTT CT

Anti-sense: GGA ACT GGC GTG GAC CG

Probe: CCC ACA GTC ACA TGG AAA GGC TTG AAG

A.5.5.4. *IFN- γ*

Sense: AAG TGG CAT AGA TGT GGA AGA AAA G

Anti-sense: TGC AGG ATT TTC ATG TCA CCA T

Probe: CTT TTG CCA GTT CCT CCA GAT ATC CAA

A.5.5.5. *IL-13*

Sense: AGG ATA TTG CAT GGC CTC TGT AA

Anti-sense: AGT TGC TTT GTG TAG CTG AGC AGT T

Probe: CAC TAC GGT CTC CAG CCT CCC CGA T

A.5.5.6. *rl19*

Sense: CAA GCG GAT TCT CAT GGA ACA

Anti-sense: TGG TCA GCC AGG AGC TTC TT

Probe: TCC ACA AGC TGA AGG CAG ACA AGG C

A.6. Protocols

A.6.1. Immunization protocol for antibody production

To generate the anti-mouse IL-12R β 2 anti-sera, 100 μ g or 500 μ g of rIL-12R β 2 protein resuspended in PBS was mixed to equal volume of CFA and the emulsion was

injected subcutaneously in the area of the ventral abdomen of female Lewis rat and New Zealand white rabbit respectively. The animals were boosted three times at 28d intervals with 100 µg and 500 µg of rIL-12Rβ2 in IFA respectively. At 7d after each boost, a 1 ml blood sample was recovered to evaluate the immune response. 7d after the last boost, total blood was harvested by cardiac puncture in non-heparinized tubes. Before immunization, 1 ml blood samples were taken from the animals and non-immune serum was used as negative control in flow cytometry and western blot experiments.

A.6.2. Phenol/chloroform DNA precipitation

This method was used to extract high concentrations of DNA. In 1.5 ml Eppendorf tube containing the DNA, one volume of buffer-saturated phenol (Life Technologies) and one volume of chloroform (BDH)/Isoamyl alcohol (BDH) mixture at a ratio of 24:1 (v/v) were added. The solution was mixed by rotating the tube 5 min at RT. The mixture was centrifuged for 5 min at RT, 10,000 x g. The DNA-containing upper aqueous phase was transferred into new 1.5 ml Eppendorf tube. DNA was precipitated by adding 1/10 volume of 3M sodium acetate pH 5.5 (Sigma) and 2 x volume of 100% ice-cold ethanol (BDH). The mixtures were vortexed and kept at least 30 min at -20°C. The tube was centrifuged 30 min at 10,000 x g at RT and the supernatant was discarded. The pellet was washed twice with 1 ml of 70 % ice-cold ethanol followed by a centrifugation of 5 min at 10,000 x g. The pellet was dried at RT and resuspended in TE buffer (Appendix A.4.6). DNA-containing tube was finally incubated at 55°C for a complete dissolution of DNA in TE buffer. The purity of DNA was analyzed by UV spectrophotometry at 260 and 280 nm using Beckman DU640B photometer (Fullerton). The RNA integrity was analyzed by electrophoresis on 0.8% agarose gel containing ethidium bromide (Bio-Rad).

A.6.3. Total RNA preparation

For RT-PCR and RPA experiments, total RNA was prepared from the different cell types using Trizol reagent. Resting or stimulated murine cells were harvested in 1.5 ml Eppendorf tube by centrifugation at 10,000 x g for 15 sec at RT. The pellets were then resuspended in 1 ml Trizol reagent per $5-10 \times 10^6$ cells. The cells were resuspended well

and incubated for 5 min at RT for complete cell lysis and nucleoprotein dissociation. The cell lysates were stored at -20°C for less than two weeks until use. Frozen lysates were thawed at RT and mixed with 200 μl chloroform (BDH) per ml Trizol for 2-3 min for nucleic acid separation. After 15 min. centrifugation at $12,000 \times g$ at 4°C , the upper aqueous phases were transferred to new eppendorf tubes. RNA was precipitated for 10 min at RT using 0.5 ml isopropanol alcohol (BDH) per ml Trizol followed by a 10 min centrifugation at $10,000 \times g$ at 4°C . The RNA pellets were washed twice with centrifugations at $7500 \times g$ at 4°C with 1ml ice-cold 75% ethanol (BDH) per ml Trizol used. The pellets were air dried at RT, and resuspended in Depc-treated H_2O with an incubation of 10 min at 55°C for complete RNA dissolution. The purity of RNA was analyzed by UV spectrophotometry at 260 and 280 nm. The RNA integrity was analyzed by electrophoresis on 1% agarose gel containing ethidium bromide (Bio-Rad).

A.6.4. Serum preparation

After collection in non-heparinized tubes, blood was allowed to clot at RT. The clot was stored overnight at 4°C or centrifuged at $10,000 \times g$ at 4°C for 15 min to isolate the serum. 0.5 ml aliquots of the separated serum were mixed to 0.5 ml glycerol in 1.5 ml Eppendorf tubes and stored at -20°C .

A.6.5. T helper cell polarization

Murine $\text{CD4}^+\text{T}$ cells were polarized towards $\text{T}_\text{H}1$ or $\text{T}_\text{H}2$ subsets as previously described (7). Briefly, 0.5×10^6 cells/ml of splenic $\text{CD4}^+\text{T}$ cells were cultured in a total volume of 2 ml RP-10 medium using a 12 well-plate. The cells were stimulated in the presence of plate-bound anti-CD3* (5 $\mu\text{g/ml}$) plus soluble anti-CD28 (1 $\mu\text{g/ml}$) plus 10 U/ml IL-2 or in the presence of 5 to 10×10^6 of irradiated APCs. $\text{T}_\text{H}1$ cells were generated by adding 100 U/ml mIL-12 and 10 $\mu\text{g/ml}$ of anti-IL-4 mAb (11B11). $\text{T}_\text{H}2$ cells were differentiated in the presence of 200 U/ml mIL-4 and 10 $\mu\text{g/ml}$ anti-IFN- γ mAb in the medium. Cells incubated in the absence of cytokines represent the undifferentiated $\text{T}_\text{H}0$ cells. For primary stimulation, the cells were cultured in the indicated conditions for 7 days at 37°C . At d3, the cells were transferred into 25 ml flasks and expanded in 10 to 15 ml RP-10 containing 20 U/ml huIL-2. At day 7, the cells were

harvested, extensively washed and counted. Aliquots of 0.5×10^6 cells/ml were re-cultured in the same conditions cited above for a second round of 7d stimulation in the presence of irradiated APCs or in the presence of plate-bound anti-CD3 plus anti-CD28. After the third round of stimulation, T_H2 cells were completely polarized.

*Soluble anti-CD3 was diluted in sterile PBS (Life Technologies) at 5 $\mu\text{g/ml}$ and 100 $\mu\text{l/well}$ was dispensed in a 12 well-plate. The plate was incubated 90 min at 37°C. The excess anti-CD3 mAb was removed from the wells before cell seeding.

A.6.6. Transformation of competent DH5 α *E. coli*

Max Efficiency DH5 α competent cells (Life Technologies) were used in all the bacterial transformation for plasmid amplification experiments used in the present dissertation. Competent cells stored as 100 μl aliquots were removed from -80°C freezer and thawed in ice. Cells were gently transferred into 5 ml chilled propylene tubes (Falcon). 50 pg of pUC19 DNA (control DNA plasmid) or 1-10 ng of the transformant DNA was added to the tubes containing DH5 α competent cells and gently mixed with moving the pipette through the cells while dispensing. Cells/DNA mixtures were incubated on ice for 30 min, then heat-shocked 45 sec at 42°C in the water bath (Isotemp 210, Fisher Scientific). The tubes were then placed on ice for 2 min, 0.9 ml of LB medium (Appendix A.4.1.1) was added and the cells were placed on a Orbital Shaker (Forma Scientific) at 225 rpm at 37°C for 1h. Reactions containing control DNA or experimental DNA plasmids were diluted 1:100 in LB medium and 100 μl of these dilutions were spread on LB plates containing 100 $\mu\text{g/ml}$ ampicilline. Plates were incubated overnight at 37°C . Next day, the DH5 α colonies were counted in both control and experimental plates to evaluate the transformation efficiency. Colonies from experimental plates were grown separately overnight in 14 ml propylene tubes (Falcon) containing 10 ml LB broth and the plasmids were purified from the transformed DH5 α cells using Qiafilter Maxi kit following the manufacturer's protocol (Qiagen). Purified plasmids were subjected to digestion with specific restriction enzymes followed by electrophoresis on 1% agarose gels to verify if the transformation yielded the expected

product. 0.5 ml from the colonies cultures containing the desired plasmid were mixed to 0.5 ml of *E. coli* freezing solution (Appendix A.4.1.5) and stored at -80°C .

A.6.7. Transformation of competent BL21 *E. coli* for protein expression

To generate the anti-murine IL-12R β 2 antiserum, a recombinant IL-12R β 2 protein of 32 kDa has been expressed in competent BL21 (DE3) pLysS cells (Novagen).

A.6.7.1. Preparation of BL21(D3) competent cells

BL21 (DE3) pLysS strain (Novagen) was streak out on LB plate with no antibiotics to isolate colonies and incubated overnight at 37°C . The next day, cells were collected from single colony to inoculate 50 ml sterile LB growth media containing 35 $\mu\text{g/ml}$ chloramphenicol in 250 ml flask at 37°C on a shaker overnight (225 rpm). The following day, 4 ml from overnight BL21 cultures was diluted in 400 ml LB broth 35 $\mu\text{g/ml}$ chloramphenicol in a sterile 2.5 L Erlenmeyer flask and incubated at 37°C with shaking until a cell density of 0.375 OD_{600nm} is reached. The 400 ml BL21 culture was dispensed in 8 autoclaved pre-chilled 250 ml sterile centrifuge bottles and left on ice for 5 to 10 min. The cells were then pelleted 10 min at 1600 x g. All the subsequent steps were performed on ice. Cells were gently resuspended in 10 ml ice-cold calcium chloride solution (Appendix A.4.1.3) and held on ice for 30 min. The cells were centrifuged at 1100 x g and the pellets were resuspended in 2 ml ice-cold CaCl₂ solution. 0.5 ml cell aliquots were transferred to sterile pre-chilled cryogenic tubes (Falcon) and immediately stored at -80°C until use. Competent cells were checked for transformation efficiency using 0.1 ng and 1 ng of any ampicilline resistant plasmid such as pUC 19 on LB plates containing 100 $\mu\text{g/ml}$ ampicilline. The yield, reported as number of cells transformed per μg of plasmid DNA, was approximately of 1 to 5 10×10^7 .

A.6.7.2. Transformation of Competent BL21 *E. coli*

pET17/IL-12R β 2/EcoRV plasmid construct encoding for the N-terminal ECD of the murine IL-12R β 2 chain was used to transform BL21 (D3) competent *E. coli* to express a 32 kDa recombinant IL-12R β 2 protein (aa 24-275) bearing a 6 X His tag at the N-terminus. Cell transformation has been performed following the protocol described above.

A.6.8. Viability staining of cells

Murine cell viability is determined using trypan blue exclusion test. The dye is excluded by live but not by dead cells. Aliquots (20 μ l) from murine cells resuspended in RP-10 were transferred to 1.5 ml Eppendorf tubes and mixed with same volume of 0.4% trypan blue stain (Life Technologies). A drop of trypan blue/cell mixture was applied to the hemacytometer (Hausser Scientific, Horsham, PA). Cells were counted on a binocular microscope Olympus BX40 (Carsen).

Appendix B. Curriculum Vitae

Habiba Chakir

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PERSONAL DATA

Citizenship: Canadian, Moroccan
Languages: French, English and Arabic
Married, two children

EDUCATION

- Jan. 1999- Present: PhD (Microbiology and Immunology)
Department of Biochemistry, Microbiology and Immunology
University of Ottawa, Ottawa, Ontario
Permission for thesis writing: Jan 30, 2002.
Thesis title: **"Role of Interleukin-12 and Interleukin-18 in murine immune cell regulation"**
Supervisor: Dr. John R. Webb
- 1998-1999 Volunteer student at SIMS institute (NRC, Ottawa)
Supervisor: Dr. Jenny Phipps
- 1986-1989 M.Sc. (Biochemistry)
Department of Biology, Faculty of Sciences
University Hassan II, Rabat, Morocco in collaboration with
"Centre National de la Recherche Scientifique" Marseille, France.
Thesis title: **"Adaptation of the morphology and the enzymatic apparatus of the small intestine to cold, fasting and hibernation in Gerbil, *Jaculus orientalis*"**
Supervisors: Dr. Abdelwahab Benajiba AND Dr. Suzanne Maroux.

1985-1986	CEA (Certificat des Etudes Approfondies in Biochemistry-Microbiology) Department of Biology, Faculty of Sciences University Mohamed V, Rabat, Morocco.
1981-1985	B.Sc. (Biology) Department of Biology & Geology Faculty of Sciences University Hassan II, Casablanca, Morocco
1981	High School Diploma Lycée AL-Khansa Casablanca, Morocco

SCHOLARSHIPS AND ACADEMIC AWARDS

I was awarded the following:

- 1- The Ontario Graduate Scholarship by the Ontario ministry of Education and training (2002-2003) (declined).
- 2- The scholarship for the 3rd and 4th year of doctoral Research by the Faculty of Graduate and Postdoctoral Studies (FGPS), University of Ottawa (2002-2003).
- 3- The scholarship for the 3rd and 4th year of doctoral Research by the Faculty of Graduate and Postdoctoral Studies (FGPS), University of Ottawa (2001-2002).
- 4- The Ontario Graduate Scholarship in Science and Technology (OGSST) by FGPS, University of Ottawa (2001-2002).
- 5- The BMI travel award of Biochemistry, Microbiology and Immunology department, Faculty of Medicine, University of Ottawa (2002).
- 6- The FGPS Travel award of University of Ottawa (2001).
- 7- The BMI travel award of Biochemistry, Microbiology and Immunology department, Faculty of Medicine, University of Ottawa (2001).
- 8- The BMI second prize for Ph.D. Poster Presentations, Faculty of Medicine, University of Ottawa (2001).

- 9- The Ottawa University Excellence Scholarship (2000-2001).
- 10- The Ontario Graduate Scholarship in Science and Technology (OGSST) by FGPS of University of Ottawa (2000-2001).
- 11- The Strategic Areas of Development (SAD) scholarship by University of Ottawa (1999-2000).
- 12- The Ottawa University Excellence Scholarship (1999-2000).
- 13- The Special Opportunity Grants (SOG) program for Female Doctoral Students by the Ontario ministry of Education and Training (1999).
- 14- The Ministry of Education Excellence scholarship (Morocco) for M.Sc studies (1986-1989).
- 15- The French-Moroccan Cooperation scholarship from the Embassy of France in Morocco for the M.Sc collaboration program (1987-1988).
- 16- The Ministry of Education Excellence scholarship (Morocco) for "Diplome des Etudes Approfondies: DEA" (1986).
- 17- The "Royal award for best national Students" by His Majesty Hassan II, King of Morocco (1985).
- 18- The Ministry of Education Excellence scholarship (Morocco) for B.Sc studies (1981-1985).
- 19- First prize for M.Sc's poster at the National Day of Mohamed V University, Rabat, Morocco (November 1988). "Study of the Adaptation of Intestinal Enzymatic Machinery to Cold, Fasting and Hibernation in Gerbil".
H. Chakir, S. Maroux and A. Benajiba.
- 20- Second prize for Poster presentation at the National Day of University Mohamed V, faculty of Sciences, Rabat, Morocco (November 1987). "Contribution to the Analysis of Intestinal Aminopeptidase Expression during Fasting and Induced Experimental Hibernation".
H. Chakir and A. Benajiba.
- 21- Highest grades in B.Sc at the faculty of Sciences, University Hassan II, Casablanca, Morocco.

ACADEMIC ACCOMPLISHMENTS.

Publications

- 1- "Bystander Polarization" of CD4⁺T cells. Activation with high-dose IL-2 renders naïve T cells responsive to IL-12 and/or IL-18 in the absence of TCR ligation.
H. Chakir, AM. Lemay and J.R. Webb (2003).
Accepted for publication in "European Journal of Immunology" (in press).
- 2- Genetically Resistant Mice Lacking IL-12R β 2 gene are Susceptible to *Leishmania major* Infection and Develop a Specific Th2 Immune Response.
H. Chakir and J.R. Webb (2003).
Microbes and Infection 5(4): 241-9.
- 3- T-bet/GATA3 ratio as a measure of T_H1/T_H2 cytokine profile in mixed cell populations in rat.
H. Chakir, H. Wang, D. Lefebvre, J. R. Webb and F. W. Scott (2002).
Accepted for publication in "The Journal of Immunological Methods" (in press).
- 4- Cytokine Expression by Murine DX5⁺ cells in Response to IL-12, IL-18 or the Combination of IL-12 and IL-18.
H. Chakir, AM. Lemay and J.R. Webb.
Cellular Immunology (2001) 212, 71-81
- 5- Differentiation of murine NK cells into distinct subsets based on variable expression of the IL-12R beta-2 subunit. **H. Chakir**, A.A. Camilucci, L.G. Filion and J.R. Webb. *J. Immunol.* 2000 Nov 1; 165(9). 4985-93.

POSTER PRESENTATIONS AND LECTURES.

- 1- Poster presentation at the Experimental Biology annual meeting (FASEB), New Orleans, Louisiana, (USA) April 2002. "Genetically resistant mice lacking the IL-12R β 2 gene are susceptible to *Leishmania major* infection and develop a specific T_H2 immune response"
H. Chakir, A. Campos-Neto and J.R. Webb
- 2- Poster presentation at the Experimental Biology annual meeting (FASEB), New Orleans, Louisiana, (USA) April 2002. "Bystander Polarization" of CD4⁺T cells. Activation with high-dose IL-2 renders naïve T cells responsive to IL-12 and/or IL-18 in the absence of TCR ligation.
H. Chakir, AM. Lemay and J.R. Webb

- 3- Poster presentation at the Experimental Biology annual meeting (FASEB), Orlando, Florida, (USA) April 2001. "Cytokine Expression by Murine NK cells in Response to IL-12, IL-18 and IL-12+IL-18"
H. Chakir, AM. Lemay and J.R. Webb
- 4- Poster presentation at the Third Annual Celebration of Research Excellence at the University of Ottawa, April 2001. "Cytokine Expression by Murine NK cells in Response to IL-12, IL-18 and IL-12+IL-18"
H. Chakir, AM. Lemay and J.R. Webb
- 5- Poster presentation at the Experimental Biology annual meeting (FASEB), Seattle, Washington, May 2000. "Differentiation of Murine LAK cells into Distinct Subsets Based on Variable Expression of the IL-12R β 2 Subunit"
H. Chakir, A.A. Camillucci, L.G. Fillion and J.R. Webb
- 6- Poster presentation at the Fourth Year Poster Day, University of Ottawa, April 2000. "Analysis of Expression of Murine Interleukin-12 receptor beta 1 chain"
A.A. Camilucci, **H.Chakir** and J.R. Webb.
- 7- Lecture at the "National Day on Olympic Competitions and Doping" organized by the Ministry of Sports and Youth, Casablanca, Morocco. June, 1990.
- 8- Oral presentation at the Fifth Congress of Environmental Sciences, Rabat, Morocco. October 1988. "Gerbil and Hibernation Phenomena". Master's work.
- 9- Poster presentation at the National Day of Mohamed V University, Rabat, Morocco. November 1988. "Study of the Adaptation of Intestinal Enzymatic Machinery to Cold, Fasting and Hibernation in Gerbil". **H. Chakir**, S. Maroux and A. Benajiba (Master's work).
- 10- Oral presentation at the 9th National Symposium of Molecular Biology, CNRS, Marseille, France (Master's thesis). April 1988.
- 11- Poster presentation at the National Day of University Mohamed V, faculty of Sciences, Rabat, Morocco, November 1987.
- 12- Poster presentation at the National Day of University Hassan II, faculty of Sciences, Casablanca, Morocco, November 1985.

PROFESSIONAL ASSOCIATIONS.

The Kuwait Group of Clinical Biochemists (KGCB)

TEACHING EXPERIENCE.

- 2002 Teaching assistant: BCH 2336 (Biochemistry Lab), department of Biochemistry, Ottawa University.
- Teaching assistant: BCH 3356 (Molecular Biology Lab), department of Biochemistry, Ottawa University.
- 2001 Teaching assistant: BCH 3356 (Molecular Biology Lab), department of Biochemistry, Ottawa University.
- Teaching assistant: BCH 2336 (Biochemistry Lab), department of Biochemistry, Ottawa University
- 2000 Teaching assistant: BCH 3756 (Molecular Biology Lab), department of Biochemistry, Ottawa University.
- 1998 Part-time research technician. University of Ottawa, faculty of Medicine, Department of Biochemistry, Microbiology and Immunology. Dr. John R. Webb's laboratory.
- 1993-1997 Medical technologist at AL-Sabah Hospital, Biochemistry laboratory, Kuwait.
- 1995-1996 Part-time gymnastics coach at "Lycée Français du Kuwait", Kuwait.
- 1990-1993 Full-time teaching assistant of biology/biochemistry at the "Institut National de la Formation des Cadres". Ministry of Youth and Sports, Rabat, Morocco.

OTHER ACTIVITIES

- 2001-2005 International judge (Xth cycle) in Women Artistic Gymnastics (WAG)(Toronto, Ontario), International Gymnastics Federation (FIG).
- 2002-present WAG national judge in Canada Gymnastics Canada (CGC, Canadian Federation of Gymnastics)
- 2001-present WAG brevet judge in Ontario Gymnastics Federation (OGF, Ontario, Canada)
- 1988-1992 Intercontinental judge (VIIth cycle) in Women Artistic Gymnastics (Thonon-les-Bains, France), International Gymnastics Federation (FIG)

- 1984-1988 International judge (VIth cycle) in Women Artistic Gymnastics (Lausanne, Switzerland), International Gymnastics Federation (FIG).
- 1980-1984 Intercontinental judge (Vth cycle) in Women Artistic Gymnastics (Thonon-les-Bains, France), International Gymnastics Federation (FIG).

I judged many competitions in WAG at the international level around the world and at the national level in Canada and Morocco. I was responsible of the WAG national judging program (Royal Federation of Gymnastics, Morocco). I occupied many key positions in the Royal Federation of Gymnastics, in the Moroccan Olympic Committee, in the Women Technical Committee of the Royal Federation of Track and Field (Morocco) and in the Kuwait National Club of women sports (Kuwait).

Appendix C. Contributions of collaborators

The data reported in the present thesis represents original work performed by Habiba Chakir under the unique supervision of Dr. John R. Webb at the University of Ottawa, faculty of Medicine, Department of Biochemistry, Microbiology and Immunology (Ottawa, Ontario, Canada).

Dr. Ruth Tal-Singer from GlaxoSmithKline (Collegeville, PA, USA) performed the real-time PCR experiments reported herein and Dr. Antonio Campos-Neto from the Infectious Disease Research Institute (Seattle, WA, USA) determined the parasite specific IgG1 and IgG2a antibodies in sera from *Leishmania*-infected mice.