UNIVERSITE DE LAUSANNE - FACULTE DE BIOLOGIE ET DE MEDECINE
CENTRE OMS DE RECHERCHE ET DE FORMATION EN IMMUNOLOGIE
(EPALINGES)

The early IL-4 response to *Leishmania major* responsible for progressive disease in BALB/c mice is subject to the control of autocrine IL-2 production and regulatory $CD4^+CD25^+$ T cells

THESE

préparée sous la direction du Professeur associé ad personam Jacques A. Louis, et présentée à la Faculté de biologie et de médecine de l'Université de Lausanne pour l'obtention du grade de

DOCTEUR EN MEDECINE

par

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Lausanne
2009

Résumé

La majorité des souches de souris de laboratoire sont résistantes à l'infection par le parasite *Leishmania major* (*L. major*). A l'opposé, les souris de la souche BALB développent une maladie évolutive. La résistance et la sensibilité sont corrélées avec l'apparition de lymphocytes T $CD4^+$ spécifiques du parasite, Th1 (de l'anglais T helper) ou Th2 respectivement. La réponse aberrante Th2 chez les souris de la souche BALB/c dépend, au moins en partie, de façon critique de la production rapide d'IL-4 suite à l'infection. Ce pic précoce d'IL-4 est produit par une population de lymphocytes T $CD4^+$ restreinte aux molécules du MHC de classe II, exprimant les chaînes du récepteur des cellules T Vβ4-Vα8. Ces lymphocytes sont spécifiques d'un épitope de l'homologue *Leishmania* de la molécule RACK1 des mammifères, appelée LACK. Il a été clairement démontré que l'IL-4 rapidement produite par ces cellules T $CD4^+$ Vβ4-Vα8 induit la maturation Th2 responsable de la sensibilité vis-à-vis de *L. major*.

Des expériences ont été entreprises pour étudier la régulation de cette réponse précoce d'IL-4. Dans ce travail, nous avons documenté, dans les cellules provenant des ganglions de souris sensibles infectées par *L. major*, une augmentation de la transcription de l'ARNm de l'IL-2 qui précède la réponse précoce d'IL-4. La neutralisation de l'IL-2 durant les premiers jours d'infection induit la maturation des cellules Th1 et la résistance vis-à-vis de *L. major*. Ces effets de l'anticorps anti-IL-2 neutralisant sont liés à sa capacité d'interférer avec la transcription rapide d'IL-4 des cellules $CD4^+$ réactives à l'antigène LACK. Une augmentation similaire d'IL-2 survient chez les souris résistantes C57BL/6 qui sont incapables de générer la réponse précoce d'IL-4. Cependant, la protéine LACK induit une transcription précoce d'IL-2 uniquement chez les souris sensibles. Des expériences de reconstitution utilisant des souris C.B.-17 SCID et des cellules T $CD4^+$ réactives à LACK provenant de souris BALB/c IL-$2^{-/-}$ démontrent un mode d'action autocrine de l'IL-2 sur la régulation de la réponse précoce d'IL-4. Par conséquent, chez les souris C57BL/6, l'absence du pic précoce d'ARNm de l'IL-4 important pour la progression de la maladie paraît liée à l'incapacité des cellules T $CD4^+$ réactives à LACK de produire de l'IL-2.

Un rôle dans le contrôle de la production précoce d'IL-4 par les cellules T régulatrices $CD4^+CD25^+$ a été investigué en déplétant *in vivo* cette population de cellules. La déplétion induit une élévation du pic précoce de l'ARNm de l'IL-4 dans les ganglions drainant de souris BALB/c, ainsi qu'une exacerbation du cours de la maladie avec des taux augmentés d'IL-4 dans les ganglions. La réponse rapide d'IL-2 vis-à-vis de *L. major* est aussi significativement augmentée chez les souris BALB/c déplétées en cellules $CD4^+CD25^+$. De plus, nous avons démontré que le transfert de 10^7 cellules provenant de la rate de souris BALB/c déplétées en cellules T régulatrices $CD4^+CD25^+$ rend les souris SCID sensibles à l'infection et permet la différentiation Th2. Au contraire, les souris SCID reconstituées avec 10^7 cellules de la rate de souris BALB/c contrôle sont résistantes à infection par *L. major* et développent une réponse Th1. Chez les souris SCID reconstituées avec des cellules de rate déplétées en cellules exprimant le marqueur CD25, le traitement avec un anticorps neutralisant l'IL-4 au moment de l'infection par *L. major* prévient le développement de la réponse Th2 et rend ces souris résistantes à l'infection. Ces résultats démontrent que les cellules T régulatrices $CD4^+CD25^+$ jouent un rôle dans la régulation du pic précoce d'IL-4 responsable du développement cellulaire Th2 dans ce modèle d'infection.

Summary

Mice from most strains are resistant to infection with *Leishmania major* (*L. major*). In contrast, BALB mice develop progressive disease. Resistance and susceptibility result from parasite-specific $CD4^+$ Th1 or Th2 cells, respectively. The aberrant Th2 response in BALB/c mice depends, at least in part, upon the production of IL-4 early after infection. The $CD4^+$ T cells responsible for this early IL-4 response to *L. major* express a restricted TCR repertoire (Vβ4-Vα8) and respond to an $I\text{-}A^d$-restricted epitope of the *Leishmania* homologue of mammalian RACK1, designated LACK. The role of these cells and the IL-4 they produce for subsequent Th2 cell development and disease progression in BALB/c mice was demonstrated.

Experiments have been undertaken to study the regulation of the rapid IL-4 production to *L. major*. In this report, we document an IL-2 mRNA burst, preceding the reported early IL-4 response, in draining lymph nodes of susceptible mice infected with *L. major*. Neutralization of IL-2 during the first days of infection redirected Th1 cell maturation and resistance to *L. major*, through interference with the rapid IL-4 transcription in LACK-reactive $CD4^+$ cells. A burst of IL-2 transcripts also occurred in infected C57BL/6 mice that do not mount an early IL-4 response. However, although the LACK protein induced IL-2 transcripts in susceptible mice, it failed to trigger this response in resistant C57BL/6 mice. Reconstitution experiments using C.B.-17 SCID mice and LACK-reactive $CD4^+$ T cells from $IL\text{-}2^{-/-}$ BALB/c mice showed that triggering of the early IL-4 response required autocrine IL-2. Thus, in C57BL/6 mice, the inability of LACK-reactive $CD4^+$ T cells to express early IL-4 mRNA transcription, important for disease progression, appears due to an incapacity of these cells to produce IL-2.

A role for $CD4^+CD25^+$ regulatory T cells in the control of this early IL-4 production was investigated by depleting *in vivo* this regulatory T cell population. Depletion induced an increase in the early burst of IL-4 mRNA in the draining lymph nodes of BALB/c mice, and exacerbated the course of disease with higher levels of IL-4 mRNA and protein in their lymph nodes. The rapid IL-2 response to *L. major* is also significantly enhanced in BALB/c mice depleted of $CD4^+CD25^+$ cells. We further showed that transfer of 10^7 BALB/c spleen cells that were depleted of $CD4^+CD25^+$ regulatory T cells rendered SCID mice susceptible to infection and allowed Th2 differentiation while SCID mice reconstituted with 10^7 control BALB/c spleen cells were resistant to infection with *L. major* and developed a Th1 response. Treatment with a mAb against IL-4 upon infection with *L. major* in SCID mice reconstituted with CD25-depleted spleen cells prevented the development of Th2 polarization and rendered them resistant to infection. These results demonstrate that $CD4^+CD25^+$ regulatory T cells play a role in regulating the early IL-4 mRNA and the subsequent development of a Th2 response in this model of infection.

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Abbreviations

ABLE	LACK T cell receptor-specific transgenic
Ag	Antigen
AICD	Activation Induced Cell Death
APC	Antigen Presenting Cell
c-	cellular
CD	Clusters of Differentiation
cDNA	complementary Deoxyribonucleic Acid
CR3	Complement Receptor 3
Cy	Cy-Chrome
DC	Dendritic Cell
DD	Death Domain
DMEM	Dulbecco Modified Eagle's Medium
DTHR	Delayed Type Hypersensitivity Response
ES	Embryonic Stem
FACS	Fluorescence Activated Cell Sorter
FADD	Fas-associated DD
FAP	Fas-associated phosphatase
FasL	Fas Ligand
FITC	Fluorescine isothyocyanate
FLICE	Fas-associated death-domain-like IL-1β-converting enzyme
FLIP	FLICE-inhibitory protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HKLM	Heat-Killed *Listeria monocytogenes*
HPRT	Hypoxantine Guanine Phosphoribosyl Transferase
^{3}H-TdR	^{3}H-methylthymidine
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin-
iNOS	inducible Nitric Oxide Synthetase
i.p.	intraperitoneal
i.v.	intravenous

JAKs	Janus Kinases
KLH	Keyhole Limpet Hemocyanin
LACK	*Leishmania* homolog of receptors for activated C kinase
L. major	*Leishmania major*
LPG	Lipophosphoglycan
LT	Lymphotoxin
mAbs	monoclonal Antibodies
MAP kinase	Mitogen-activated protein kinase
MHC	Major Histocompatibility Complex
MMTV	Mouse Mammary Tumor Virus
NF-AT	Nuclear Factor of Activated T cells
NO	Nitric Oxide
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Salt Buffer
PCR	Polymerase Chain Reaction
PE	Phycoerythrine
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
r	recombinant
R	Receptor
RBC	Red Blood Cells
RE	Responsive Element
RT	Reverse Transcription
sAg	superantigen
s.c.	subcutaneously
STAT	Signal Transducer and Activator of Transcription
TCR	T Cell Receptor
tg	transgenic
TGF	Tumor Growth Factor
TNF	Tumor Necrosis Factor
Th	T helper
UV	Ultraviolet
v-	viral

Introduction

The murine model of infection with *Leishmania major* and maturation of T helper subsets.

1 The murine model of infection with *Leishmania major.*

Upon experimental infection with *Leishmania major* (*L. major*), distinct features of the spectrum of clinical manifestations seen in patients with cutaneous leishmaniasis can be reproduced in inbred mice of different genetic backgrounds.[1] [2] Mice from the majority of inbred strains (C3H/He, CBA, C57BL/6, 129Sv/Ev) develop locally cutaneous lesions which spontaneously resolve after four to eight weeks. These mice do not develop lesions after a second inoculation of *L. major* and belong to the resistant phenotype. Mice from a few strains (BALB/c, DBA/2) develop severe and uncontrolled lesions without becoming immune to reinfection and are representative of the susceptible phenotype.[3] This murine model of infection has been used to characterize the immune responses developing in both resistant and susceptible mice.

1.1 The parasite life cycle.

L. major exist in the sand fly vector as extracellular, flagellated, spindle-shaped forms termed promastigotes that develop in close approximation to epithelial cells in the insect midgut (Figure A (1)). The major surface glycoconjugate lipophosphoglycan (LPG) constitutes a dense glycocalyx that covers the entire surface of the parasite including the flagellum.[4] Immature organisms, called procyclics, express shorter LPG molecules.[5] During development in the insect, LPG undergoes developmental modification, named metacyclogenesis, that involves capping of terminal residues that obscures the epithelial-binding domain of LPG, allowing the mature forms, called metacyclics, to be released from the midgut (Figure A (2)) and to migrate to the salivary glands of the fly (Figure A (3)) from where they are inoculated during the blood meal of the sand fly, into the dermis of its mammalian host (Figure A (4)).[6] The parasite invades preferentially mononuclear cells, either in a direct way or by receptor-mediated uptake through the complement receptor 3 (CR3) (Figure A (5)). Once inside the phagolysosomes of the macrophage, the main host cell of the parasite, the promastigotes transform themselves into a non-flagellated intracellular form called amastigotes, a developmental form in which they remain in the vertebrate host (Figure

A (6)). Amastigotes replicate by binary fission (Figure A (7)), eventually rupturing the macrophage (Figure A (8)) and spreading to uninfected cells (Figure A (9)). A new sand fly will take its blood meal from an infected mammalian host and the cycle is closed (Figure A (10)). In the fly, the macrophages taken up by the blood meal are digested and the amastigotes transform into promastigotes in the stomach of the insect (Figure A (11)).[6]

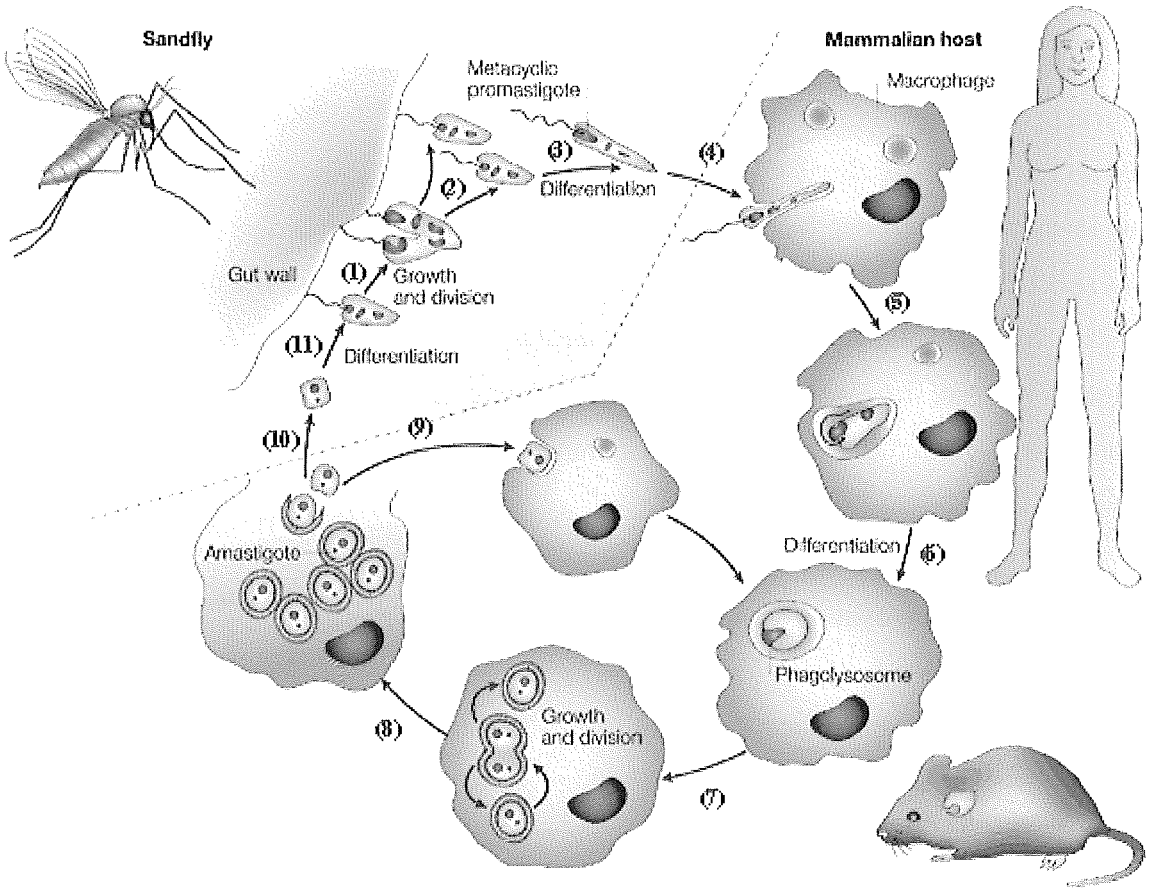

Figure A. The parasite life cycle. Modified from Nature Reviews Immunology *2*, 845-858 (2002).

1.2 Expression of resistant or susceptible phenotypes during infection with *L. major* is the result of a $CD4^+$ T helper 1 (Th1) or T helper 2 (Th2) response.

As described in chapter 1.1, *L. major* is an intracellular parasite located within the phagolysomal compartment of macrophages where major histocompatibility complex (MHC) class II processing for antigen presentation also takes place. Infection with *L. major* induces the expansion of parasite-specific $CD4^+$ T cells that recognize *Leishmania* antigens presented in association with MHC class II molecules on the surface of antigen presenting cells (APCs).

The role of T cells in the resistance of mice to infection with *L. major* has been demonstrated by results showing that adoptive transfer of T lymphocytes into susceptible nu/nu mice allowed them to control the disease.[3] Using monoclonal antibodies directed against $CD4^+$ T cells, it has been further demonstrated that parasite-specific T cells from the $CD4^+$ subpopulation were responsible for the spontaneous resolution of primary lesions in mice of resistant strains. Further adoptive transfer studies have also demonstrated that $CD4^+$ T cells were also responsible for susceptibility to infection.[7-11]

Following the description of two functionally distinct $CD4^+$ T cell subsets, Th1 and Th2, the characterization of $CD4^+$ subpopulations playing a role in resistance or susceptibility to infection with *L. major* was possible.

1.2.1 Defining Th1 and Th2 phenotypes.

The characterization of cytokine production from long term murine $CD4^+$ T cell clones was first published more than 10 years ago.[12] In these studies, it was shown that distinct $CD4^+$ T cells produced two distinct patterns of cytokines, which were designated Th1 and Th2 (Figure B). Th1 cells were found to exclusively produce interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxin (LT), while Th2 clones exclusively produced IL-4, IL-5, IL-6 and IL-13.[13] Somewhat later, additional cytokines, IL-9 and IL-10, were isolated from Th2 clones.[14 15] Finally, other cytokines, such as IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) were found to be secreted by both Th1 and Th2 cells.

The observation that $CD4^+$ T cells could be segregated into distinct subsets based on the types of cytokines they produced was of great interest, because Th1 cytokines (i.e., IL-2, IFN-γ) were associated with cellular immune functions such as delayed type hypersensitivity response (DTHR),[16-19] while Th2 cytokines (i.e., IL-4, IL-5, IL-6, IL-10, IL-13) enhance antibody production from B cells and induce several aspects of the allergic response.[17] In addition, it was soon appreciated that these specific cytokines produced from Th1 and Th2 cells (IFN-γ and IL-4, respectively) were also potent cross-inhibitors of the two cell types.[20] Thus, these differences in cytokine profiles allowed for different effector functions, as well as the ability to cross-regulate each other's function.

Recently, $CD4^+CD25^+$ regulatory T cells (T_{reg} cells) and Th17 cells have been described as two subsets distinct from Th1 and Th2 cells. T_{reg} cells expressing the forkhead/winged helix

transcription factor (Foxp3) have an anti-inflammatory role and maintain tolerance to self components by contact-dependent suppression or releasing anti-inflammatory cytokines [IL-10 and transforming growth factor-β1 (TGF-β1)] (discussed in chapter 1.5),[263] while Th17 cells expressing retinoic acid-related orphan receptor γt (RORγt) play critical roles in the development of autoimmunity and allergic reactions by producing IL-17 and, to a lesser extent, TNF-α and IL-6.[264] So the balance between Th17 and T_{reg} cells may be important in the development/prevention of inflammatory and autoimmune diseases (exhaustively reviewed in Refs. 263 to 268).

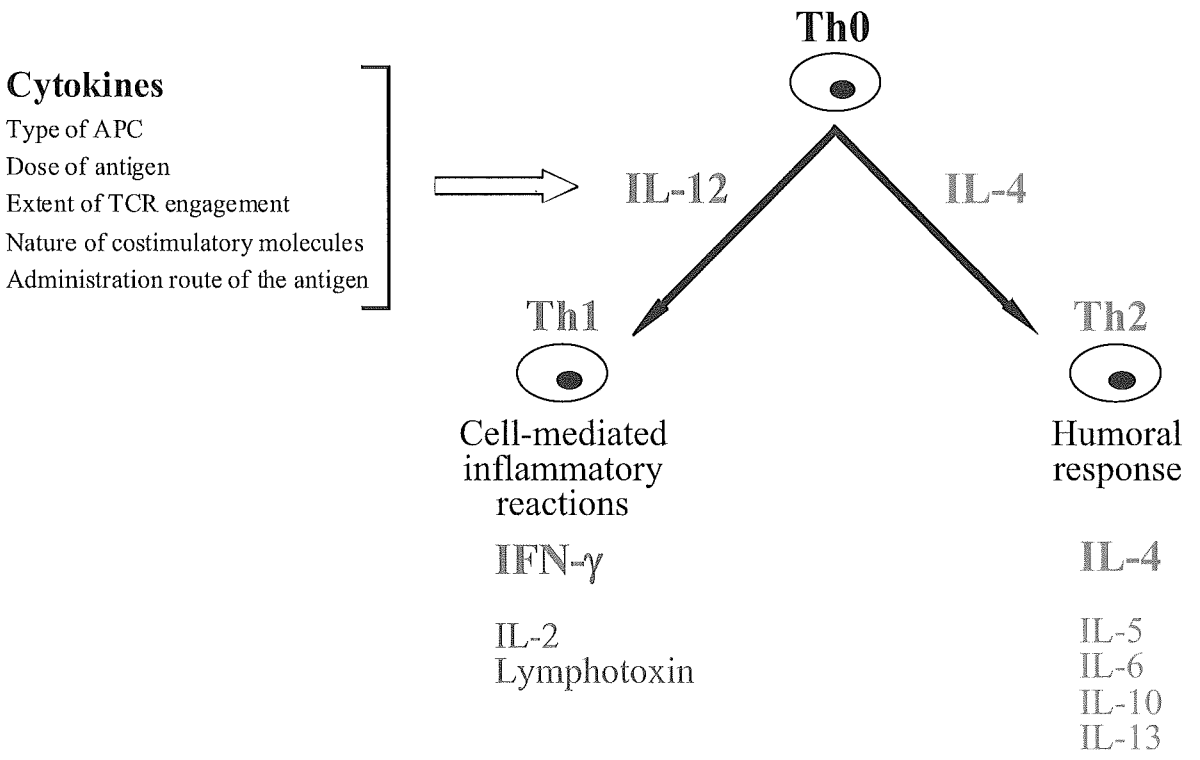

Figure B. Naive $CD4^+$ T helper cells can develop into two different effector subsets: Th1 and Th2.

The murine model of infection with *L. major* provided the first correlation *in vivo* between 1) the development of protective immunity and an expansion of Th1 $CD4^+$ T cells in resistant mice and 2) the expression of progressive disease and the development of a $CD4^+$ Th2 cell response in susceptible mice (Figure C).[21 22] If similar levels of IFN-γ have been later described in either resistant or susceptible mice, the inverse relationship between IL-4 production and resistance to infection is a paradigm of the *L. major* infection.[23] Indeed, the expression of IL-4 in susceptible BALB/c mice is persistent during the course of infection and this IL-4 production correlates with the number of parasites in the lesions and the

development of lesions.[24] Furthermore, the correlation between resistance to infection and the absence of a Th2 response was confirmed by results showing that treatment of susceptible mice with anti-IL-4 monoclonal antibodies during the first days of infection with *L. major* inhibited the maturation of a Th2 response rendering these mice resistant to infection.[25] Differentiated *L. major*-specific Th1 and Th2 cells can by themselves mediate respectively resistance and susceptibility to infection with *L. major*. This contention is supported by studies, which have shown that adoptive transfer of Th1 cells derived from infected BALB/c rendered resistant to infection as a result of treatment with anti-CD4 antibodies[9] to immuno-deficient SCID mice allowed them to resolve *L. major*-induced lesions whereas transfer of Th2 cells derived from infected BALB/c mice led to the development of larger lesions.[26]

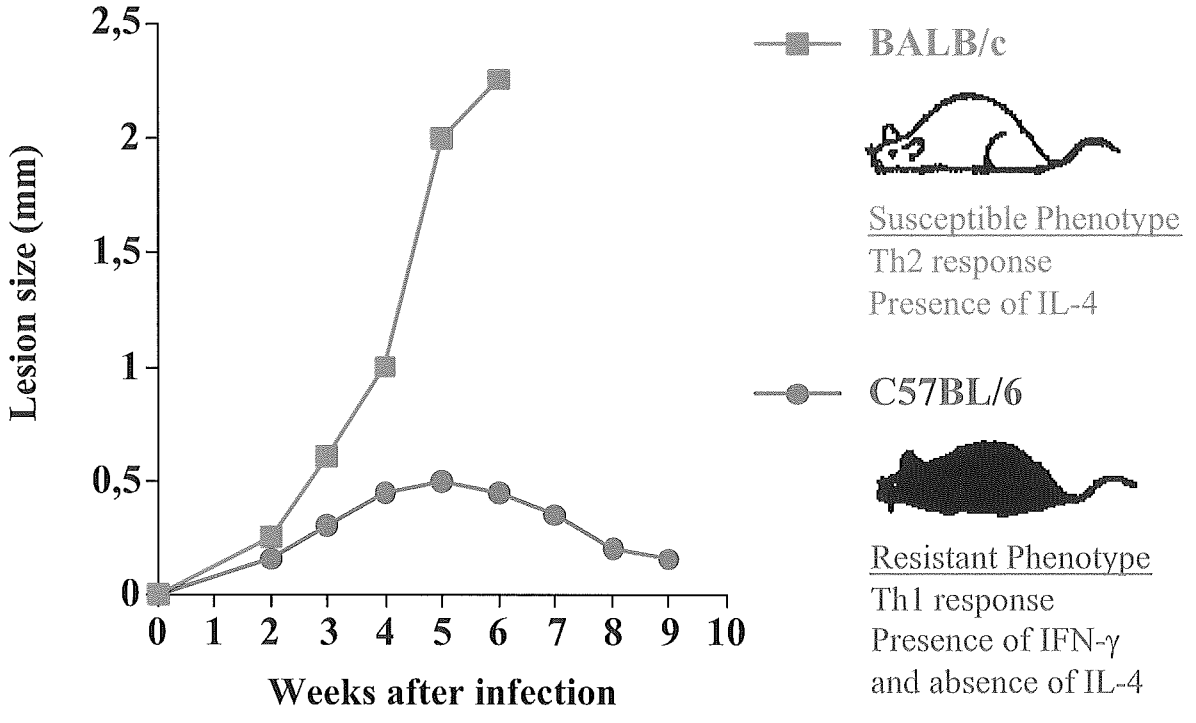

Figure C. Expression of a resistant or susceptible phenotype during infection with *L. major* is the result of a Th1 or Th2 response.

1.3 Acquired immune effector mechanisms mediating protection or susceptibility to infection with *L. major*.

Activation of macrophages to a parasiticidal state is the main anti-*Leishmania* effector mechanism. IFN-γ produced by Th1 cells in synergy with TNF-α has been demonstrated to induce the synthesis of nitric oxide (NO) synthase that leads to the L-arginine dependent

production of reactive nitrogen radicals toxic for the parasite.[27-30] Thus, genetically resistant mice lacking either IFN-γ or the IFN-γ receptor gene are unable to control parasite growth.[31 32] Neutralization of nitric oxide radicals by inhibitors of the iNOS pathway (N-iminoethyl L-ornithine, L-N-iminoethyl-Lysine or L-Nω monoethyl-Arginine) renders the macrophages unable to destroy the parasite.[27 30] Furthermore, administration of these inhibitors to resistant mice renders the mice susceptible to infection[28 33 34] and resistant mice, in which the gene for the iNOS has been inactivated, are susceptible to infection with *L. major*.[35]

It had been shown *in vitro* that Th1 $CD4^+$ T cell-mediated cytotoxicity requires a functional Fas (CD95)-Fas ligand (FasL) pathway[36-38] and that Th2 cells do not mediate apoptosis of target cells through Fas/FasL interactions. Unlike Th1 cells, Th2 effectors express high levels of Fas-associated phosphatase (FAP-1), a protease that is thought to be an inhibitor of Fas signaling.[39] In contrast to wild-type C57BL/6 resistant mice, C57BL/6 mice lacking either a functional FasL or Fas were unable to heal lesions induced by *L. major* in spite of the fact that they developed a $CD4^+$ Th1 response and their macrophages produced normal levels of reactive nitrogen in response to IFN-γ *in vitro*. These results suggested that the phenotype observed was due to a defective Fas/FasL pathway of cytotoxicity. This conclusion is supported by results showing that restoration of a functional Fas-FasL cytotoxicity pathway by exogenous FasL in FasL-deficient mice allowed complete resolution of the cutaneous lesions.[40] An apoptotic death of infected macrophages would result in a decrease in the ratio of infected macrophages to IFN-γ-producing Th1 cells, thus increasing the efficiency of macrophage activation to a microbicidal state. In contrast, Th2 cells produce IL-4, a cytokine which exerts a macrophage deactivating function since it inhibits the IFN-γ-triggered activation of macrophages.[41]

1.4 Factors influencing the development of Th1 or Th2 $CD4^+$ T cells.

Th1 and Th2 $CD4^+$ T cells develop from a common naïve $CD4^+$ T cell precursor.[42 43] Several parameters have been reported to influence the pathway of differentiation of $CD4^+$ T cell precursors, including the type of APCs,[44] the nature of the costimulatory signals,[45] the extent of T cell receptor (TCR) engagement,[46] the dose of antigen,[47] the route of antigen administration,[48] the number of cell cycles.[49] Among these different T cell polarizing signals, cytokines have now been recognized as crucial inducers of $CD4^+$ T cell differentiation (Figure B).

1.4.1 The role of cytokines in the development of polarized $CD4^+$ Th responses.

1.4.1.1 The role of IL-12 in Th1 cell development.

IL-12 is a cytokine produced by many cells such as dendritic cells (DCs), macrophages, polymorphonuclear cells and B cells.[50] Biologically active IL-12 was determined to be a heterodimeric protein, composed of a heavy chain (p40) and a covalently associated light chain (p35).[51] Noteworthy, IL-12 producers typically express far more IL-12p40 chain than IL-12p35 chain,[52 53] resulting in the formation and the secretion of IL-12p40 homodimers.[54 55] The IL-12p40 homodimer binds to the IL-12R, but fails to mediate a signal, thereby serving as a functional antagonist to IL-12p70 heterodimers.[56 57]

The possibility to obtain large population of naïve $CD4^+$ T cells specific for a single T cell epitope, derived from TCR-transgenic mice, allowed the study of the role of IL-12 in T cell differentiation. Addition of exogenous IL-12 during priming of $CD4^+$ T cells *in vitro* induced the development of Th1 cells, characterized by high levels of IFN-γ and virtual absence of IL-4 in the supernatants.[58] Interestingly, examining how endogenous production of IL-12 by APCs could influence the Th1 response, it was shown that macrophages exposed to heat-killed *Listeria monocytogenes* (HKLM) induced Th1 development and inhibited the generation of IL-4-producing cells.[59] Furthermore, anti-IL-12 inhibited the ability of macrophages exposed to HKLM to induce IFN-γ, suggesting that IL-12 produced by APCs in response to some pathogens plays an important role in Th1 development *in vivo*. The most convincing evidence for IL-12 having a role in regulating IFN-γ responses was shown in studies using IL-12-deficient mice.[60] Following *in vitro* and *in vivo* stimulation, there was impairment in IFN-γ production and type 1 cytokine responses. Studies in IL-12-deficient mice have demonstrated an essential role for endogenous IL-12 in resistance to different pathogens such as *L. major*,[61] *Toxoplasma gondii*[62] and *Mycobacterium tuberculosis*.[63] Resistance to other intracellular bacteria such as *Listeria monocytogenes* involved IL-12-dependent and -independent mechanisms.[64]

The role of IL-12 *in vivo* was most widely studied in the murine model of infection with *L. major*. Exogenous IL-12 injected during the first week of infection with *L. major* in susceptible BALB/c mice resulted in the development of a $CD4^+$ Th1 response associated with decreased production of IL-4 by lymph node T cells *in vitro* and allowed these otherwise

susceptible BALB/c mice to resolve their lesions.[65 66] Conversely, neutralization of IL-12 by polyclonal antisera against IL-12 in resistant mice led to an increase in the production of IL-4 (a Th2 cytokine) and to the generation of a susceptible phenotype.[66 67]

The importance of IL-12 in Th1 cell development during infection with *L. major* has been further tested using mice genetically deficient in either the p35 or p40 subunits of IL-12. These mice, generated on a resistant genetic background, developed progressive lesions. This was associated with an important increase in the levels of IL-4 mRNA in their lymph nodes reaching values comparable to those observed in similarly infected susceptible BALB/c mice. Conversely, levels of IFN-γ were markedly decreased in IL-12-deficient mice, which did not exhibit a Th1-mediated DTHR to *L. major* antigens normally observed in mice from resistant strains.[61] In spite of the evidence for the role of IL-12 in directing Th1 cell maturation in mice infected with *L. major*, the precise timing of IL-12 production *in vivo* following infection with this parasite is still a matter of debate.[68 69] Results from one study have shown that IL-12 transcripts were neither detected in susceptible nor in resistant mice before 7 days post-infection, a period corresponding to the transformation of promastigotes into amastigotes *in vivo*, suggesting a stage-specific evasion of IL-12 induction by promastigotes.[68] Other observations, however, have found elevated levels of IL-12 in the lymph nodes of mice from the C3H resistant strain, early (1 or 2 days) after infection, which remained elevated during the first week of infection. A burst of IL-12 production was also seen in susceptible BALB/c mice during the first two days of infection, but in these mice levels of IL-12 returned rapidly to baseline values. In mice from another resistant strain, i.e. C57BL/6, no increase in IL-12 production was observed during the first days of infection.[69 70]

1.4.1.2 The role of IFN-γ in Th1 cell development.

Th1 development is also dependent on IFN-γ,[59 71] although the requirement for this factor is still unclear. Results from several studies using *in vitro* stimulated naïve $CD4^+$ TCR αβ-transgenic T cells indicate that IFN-γ is required for the induction of Th1 cell development.[59 72 73] However, other studies, also using $CD4^+$ T cells from αβ TCR-transgenic mice did not identify a requirement for IFN-γ in Th1 cell maturation.[74] Nevertheless, the effects of IFN-γ on Th1 development may be mediated via action on the macrophage to up-regulate IL-12 production[50] or by direct effects on the T cell. The molecular basis of IL-12 unresponsiveness of Th2 cells (discussed in chapter 1.4.1.3.4) has been delineated in both

mouse and human systems.[75 76] This is at least in part due to down-regulation of the IL-12Rβ2 by IL-4 and furthermore the up-regulation of the IL-12Rβ2 by IFN-γ, which counteracts the inhibitory effects of IL-4.[75 76] Thus, BALB/c mice, which produce a substantial amount of IL-4,[77] may inhibit the IL-12Rβ2 expression, imposing the reported requirement for IFN-γ in Th1 development.[76] Moreover, since IFN-γ has some anti-proliferative effects on $CD4^+$ Th2 cells but not on $CD4^+$ Th1 cells,[78] it could favour Th1 differentiation simply by inhibiting Th2 development.

The data are also debated concerning the importance of IFN-γ in favouring Th1 cell response and inhibiting Th2 cell response during infection with *L. major*. Administration to resistant mice (C57BL/6 or C3H/He) of specific anti-IFN-γ antibodies within the first 2 days of infection rendered these mice unable to resolve their lesions and lead to the appearance of a Th2 response.[25 71 79 80] In agreement with these findings, IFN-γ-deficient C57BL/6 mice developed a Th2 response after *L. major* infection.[81] In contrast, resistant 129/Sv/Ev mice deficient for the binding chain of the IFN-γ receptor (IFN-γR), which are exquisitely susceptible to infection, still developed a Th1 response.[32] Interestingly, these conflicting results have been obtained using mice from different genetic backgrounds. In as much as i) IFN-γ is capable of superseding the inhibition of IL-12Rβ2-chain expression caused by IL-4[76] and ii) in contrast to anti-IFN-γ-treated C57BL/6 mice,[80] Sv129 IFN-$\gamma R^{-/-}$ mice do not produce an early burst of IL-4 mRNA expression in their draining lymph nodes in response to *L. major* and maintain IL-12Rβ2-chain mRNA expression on their $CD4^+$ T cells at least up to 6 days after infection,[82] it has been proposed that in the absence of IL-4, activated $CD4^+$ T cells do not require IFN-γ signaling for the maintenance of IL-12Rβ2-chain expression and IL-12 signaling *in vivo*.[82]

1.4.1.3 The role of IL-4 in Th2 cell development.

The role of IL-4 in directing the differentiation of IL-4-producing cells from naïve $CD4^+$ T cells was first elucidated in *in vitro* experiments in which murine cells were stimulated with mitogens for several days under various conditions and then restimulated to evaluate the types of cytokines produced. In these studies, it was clearly shown that the development of Th2 cells from naïve precursors is regulated by the presence of IL-4 in the priming cultures.[83-86] IL-4 could lead to the differentiation of IL-4-producing cells in an APC-

independent manner, providing evidence for a direct effect of IL-4 on $CD4^+$ T cells.[85] Similar studies were done using $CD4^+$ T cells from transgenic mice for a TCR specific for a particular antigen, such as cytochrome c or ovalbumin. These systems provided a more physiologic model than did the priming experiments highlighted above, in which polyclonal mitogens were used to stimulate T cells. In addition, it allowed to vary the type of APCs and the amount of antigen to see if they also had an effect on the differentiation of IL-4-producing cells. Similar to the findings discussed above, the presence of IL-4 in priming cultures directed the development of IL-4-producing cells regardless of the type of APCs used in the priming culture.[87 88] The effect of IL-4 in the development of Th2 responses appears to dominate over Th1 polarizing cytokines, since Th2 differentiation is observed even when both cytokines (i.e. IL-4 and IL-12) are present.[20] The dominant effect of IL-4 could be related to the ability of IL-4 to down-regulate the IL-12Rβ2-chain on $CD4^+$ T cells.[76] The most definitive evidence for IL-4 having a role in regulating IL-4 production was shown *in vivo* using IL-4-, STAT6-, or IL-4Rα-deficient mice.[89-95]

The critical role of IL-4 in Th2 cell development *in vivo* has been established in several experimental systems, among which murine Leishmaniasis has been studied most intensively. The genetic mapping of resistant loci suggests that the outcome to *L. major* infection is a complex polygenetic trait, controlled by at least six genes.[96] One of these loci maps to chromosome 11, which contains the IL-4/IL-13 gene cluster. However, it is important to keep in mind that the presence of all six loci was not essential for healing, and no single locus appeared to be necessary, clearly showing the genetic complexity of the healer phenotype. Studies *in vivo* using the murine model of infection with *L. major* have established that IL-4, during the early stages of infection, has an important role on the subsequent development of specific $CD4^+$ Th2 cells. The first evidence was that administration of anti-IL-4 antibodies to BALB/c mice at the onset of infection abrogated Th2 polarization, caused an expansion of Th1 cells and consequently led to resistance to infection.[25] The importance of IL-4 has been further established by results showing that all other immune manipulations, effective only at the initiation of infection, that are able to revert the Th2 phenotype of the *L. major* specific immune response in BALB/c mice towards a healing Th1 phenotype, appear to diminish the IL-4 production during the first week of infection.[9 25 65 66 97-99] Advances of genetic manipulations knocking out the gene for IL-4 have not yet helped in understanding the exact role of IL-4 in the murine model of infection with *L. major*. Genetically pure IL-4-deficient BALB/c mice were generated from a BALB/c embryonic stem (ES) cell line.

Conflicting results were obtained by two independent groups.[92 100] One group reported that these mice still express a susceptible phenotype to infection with *L. major*[100] and that IL-4-deficient mice lose their IL-12 responsiveness equally as do wild-type BALB/c mice.[101] Furthermore, susceptibility to infection with *L. major* could be prevented by administration of IL-12 to these IL-4-deficient BALB/c mice.[101] On the opposite, the other group described that these IL-4-deficient BALB/c mice express a resistant phenotype to infection with *L. major*.[92] Factors that are believed to contribute to the divergent results with the IL-4-knockout mice include differences in both i) the *L. major* strain used; differences in the virulence of these two substrains were suggested to be one possible explanation for these conflicting results[102] and ii) the dose of the parasites used in the various studies; at very low parasite doses, even wild-type BALB/c mice can control *L. major* infection.[103]

1.4.1.3.1 Susceptible BALB/c mice exhibit a burst of IL-4 in the draining lymph nodes within the first day after infection with *L. major*.

As discussed previously, studies using neutralizing anti-IL-4 antibodies or mice with disruption of the gene encoding IL-4 have defined a critical role for this cytokine in mediating the differentiation of the Th2 subset *in vivo* and the failure of BALB/c mice to control *L. major* infection.[25 92] Direct evidence has been obtained that BALB/c mice produce a burst of IL-4 extremely rapidly in response to infection with *L. major* (Figure D).[104] Indeed, 16 hours after subcutaneous infection BALB/c mice exhibited a peak in IL-4 mRNA expression in the draining lymph nodes, before returning to baseline levels by 48 hours. Importantly, this IL-4 production occurs during the period when neutralizing IL-4 antibodies are capable of redirecting protective Th1 development in BALB/c mice.[25 105] From day 5, a second wave of IL-4 mRNA was observed that remained elevated during the entire course of infection, reflecting the Th2 cell differentiation normally observed in these susceptible mice.[104 106] In contrast, no increase in IL-4 mRNA expression was observed in resistant mice (i.e. C57BL/6) during the first 2 days of infection with *L. major* (Figure D).[104]

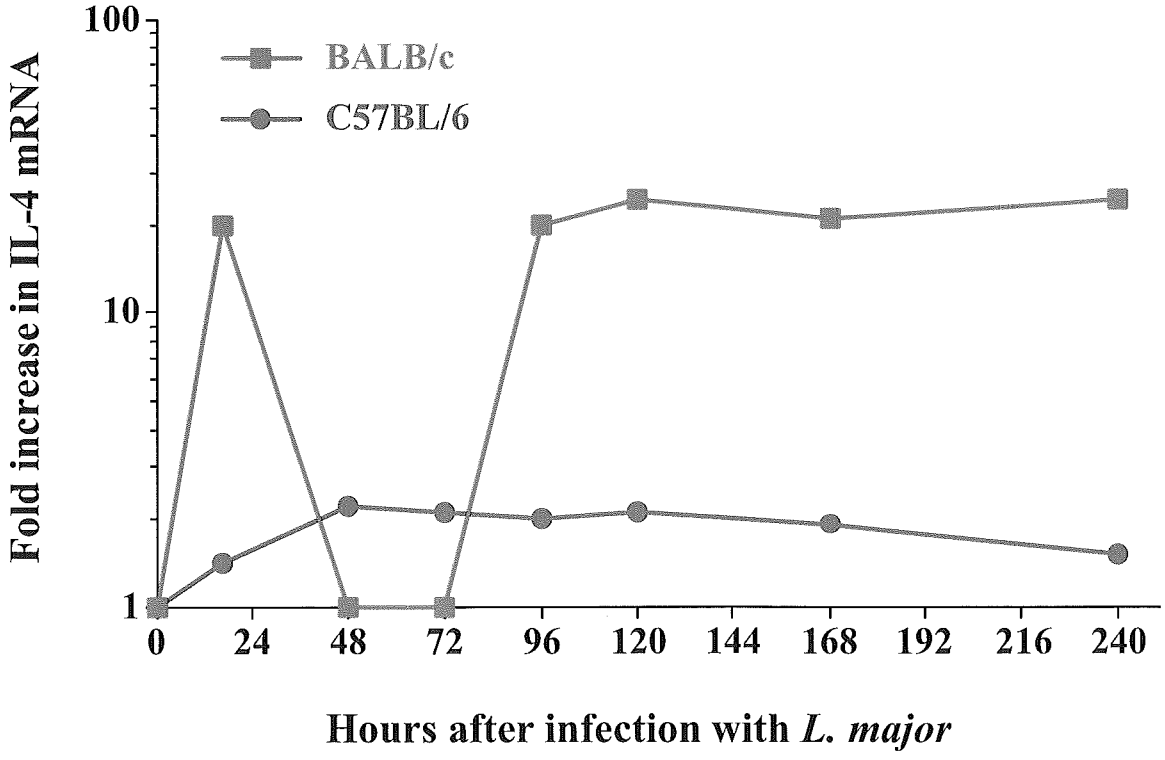

Figure D. Kinetics of IL-4 mRNA expression in draining lymph nodes of mice infected with *L. major*.

1.4.1.3.2 Cellular origin of the IL-4 produced in susceptible BALB/c mice within the first day of infection with *L. major*.

There are several candidates for IL-4 production early in an immune response, which may be responsible for Th2 differentiation; these include MHC class II-restricted $CD4^+$ T cells (memory and possibly naïve),[17 47 107 108] the $NK1.1^+$ subset of $CD4^+$ and double negative (DN) T cells,[109] and non-T cells, such as mast cells, basophils and eosinophils.[110 111]

The increased IL-4 mRNA expression during the first day of infection with *L. major* was not observed in draining lymph nodes of BALB/c mice depleted of $CD4^+$ T cells by treatment with anti-CD4 monoclonal antibodies, thus demonstrating that $CD4^+$ T cells contributed to this early IL-4 burst.[104] Quantification of IL-4 mRNA in $CD4^+$ T cell populations purified from lymph nodes of BALB/c mice 16 hours after infection directly identified $CD4^+$ T cells as the source of IL-4.[104] These $CD4^+$ T cells did not belong to the $CD4^+$ $NK1.1^+$ minor subpopulation of $CD4^+$ T cells known to produce IL-4 very rapidly in response to injections of anti-CD3 or anti-IgD antibodies,[112 113] as demonstrated by experiments characterizing the Vβ chain usage of the TCR in these $CD4^+$ T cells. The IL-4 mRNA expression seen after *L. major* infection in BALB/c mice, did not occur in $CD4^+$ T cells expressing the Vβ 8,7,2

chains which are used by $CD4^+$ $NK1.1^+$ cells.[104] Analysis of the TCR Vβ and Vα usage of $CD4^+$ T cells producing IL-4 within the first day of infection with *L. major* demonstrated that all of the IL-4 mRNA present at this time was produced by $CD4^+$ T cells that expressed the Vβ4 and the Vα8 TCR chains.[114] The contribution of these Vβ4-Vα8 $CD4^+$ T cells to the early burst of IL-4 mRNA expression in BALB/c mice in response to infection was confirmed by experiments showing that this response was absent in BALB/c mice rendered selectively deficient in the $V\beta4^+CD4^+$ T cell population.[114] These results are consistent with experiments showing an expansion of cells with the same TCR in draining lymph nodes 10 days after infection.[115] Cloned T cell lines and hybridoma derived from $CD4^+$ T cells that expressed the Vβ4-Vα8 TCR were shown to recognize the *Leishmania* homolog of mammalian RACK1 designated LACK (*Leishmania* Activated c Kinase).[116] Interestingly, the LACK antigen from *L. major* induced comparably early IL-4 mRNA expression in the same Vβ4-Vα8 population of BALB/c mice as did the intact parasites.[114] This response was driven by a single dominant $I\text{-}A^d$-restricted T cell epitope in the fourth WD domain of LACK, comprising amino acid residues 156-173.[114] In addition, there is some degree of homology between LACK and RACK1, particularly within the region of the immuno-dominant $I\text{-}A^d$ epitope of LACK eliciting the rapid IL-4 response by Vβ4-Vα8 $CD4^+$ T cells in BALB/c mice.[114 116]

In contrast to susceptible BALB/c mice, C57BL/6 and other resistant mice do not mount an early IL-4 response following infection with *L. major* or injection of LACK.[104 114] Treatment of resistant C57BL/6 mice with either anti-IFN-γ or anti-IL-12 antibodies at the onset of infection has been shown to result in the development of a Th2 response and in susceptibility to infection.[67 79] Interestingly, recent findings have demonstrated that neutralization of either IL-12 or IFN-γ in C57BL/6 mice at the initiation of infection allows the expression of this rapid IL-4 response to *L. major* or LACK (Figure E).[80] Strikingly, LACK-reactive $CD4^+$ T cells that express the Vβ4-Vα8 TCR chains were also the source of this early IL-4 response in these mice and were found to be required for reversal of the natural resistance of C57BL/6 mice following a single administration of anti-IL-12 or –IFN-γ mAbs.[80] Analysis of the epitopes recognized by these Vβ4-Vα8 $CD4^+$ T cells from C57BL/6 mice is being carried out. Preliminary results clearly show that C57BL/6 Vβ4-Vα8 $CD4^+$ T cells recognize an $I\text{-}A^b$-restricted epitope different from the $I\text{-}A^d$-restricted $LACK_{(156\text{-}173)}$ peptide seen by BALB/c Vβ4-Vα8 $CD4^+$ T cells (P. Launois, unpublished data). Noteworthy, the IL-4 produced during the early stage of infection in resistant mice treated with anti-IL-12 antibodies at the initiation of infection accounted for the increased susceptibility of these mice.[80] Together with data

showing that treatment of BALB/c mice with recombinant IFN-γ or recombinant IL-12 suppresses the early IL-4 response to *L. major*,[104] these results indicate that an impairment in mechanism(s) down-regulating the early IL-4 response by Vβ4-Vα8 $CD4^+$ T cells might underlie the susceptibility of BALB/c mice.

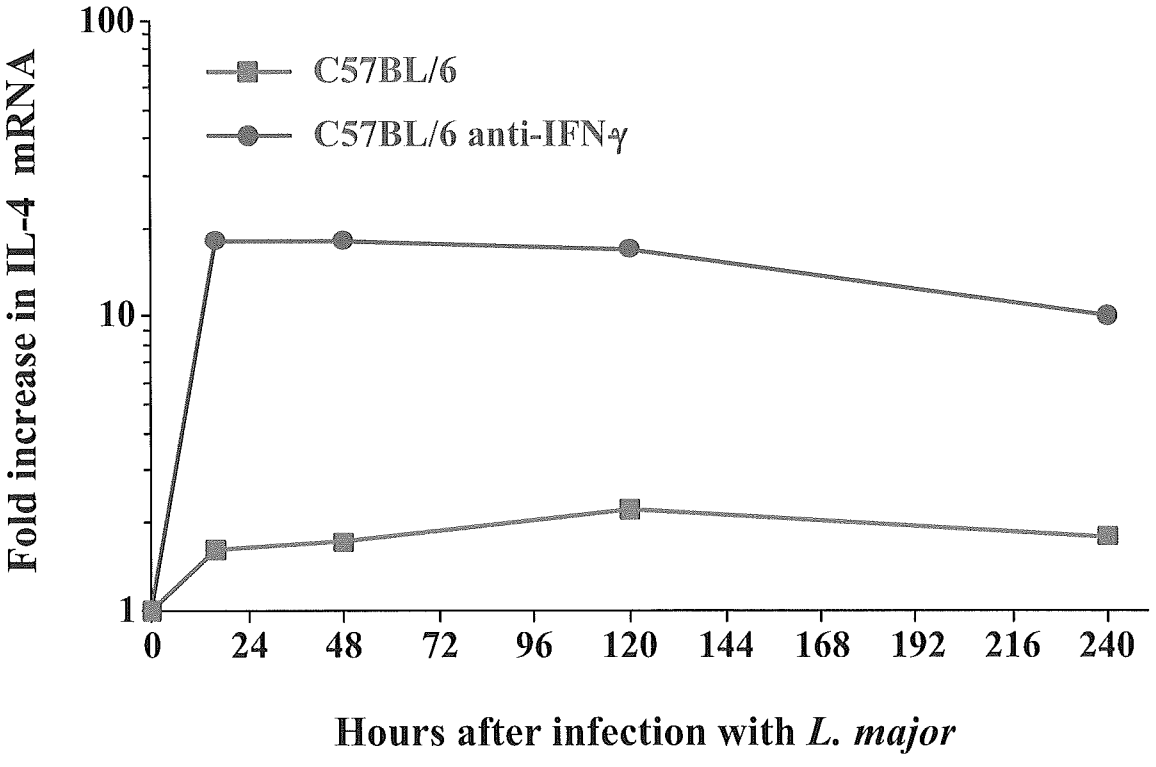

Figure E. An early burst of IL-4 instructs Th2 cell development in anti-IFN-γ-treated C57BL/6 mice infected with *L. major*.

1.4.1.3.3 The IL-4 produced by LACK-reactive Vβ4-Vα8 $CD4^+$ T cells within the first day of infection of BALB/c with *L. major* instructs subsequent Th2 cell development and susceptibility to *L. major*.

The mouse mammary tumor viruses MMTV(SIM) and MMTV(SW) encode a superantigen that ultimately leads to systemic deletion of $CD4^+$ T cells expressing the Vβ4 or Vβ6 TCR chain, respectively.[117 118] In contrast to wild-type BALB/c mice or BALB/c mice deficient in $V\beta6^+CD4^+$ T cells as a result of neonatal exposure to MMTV(SW), BALB/c mice deficient in $V\beta4^+CD4^+$ T cells by prior infection with MMTV(SIM) were not capable of generating early IL-4 transcripts in $CD4^+$ T cells following infection with *L. major*, developed a Th1 response and were resistant to infection.[114] Conversely, administration of exogenous

IL-4 to Vβ4-deficient BALB/c mice only during the first 64 hours of infection restored Th2 cell development at later stage and susceptibility to infection.[119] Furthermore, the induction of a specific unresponsive state in LACK-reactive Vβ4-Vα8 $CD4^+$ T cells following treatment of BALB/c mice with altered LACK proteins that differ by a single amino acid from the natural $I\text{-}A^d$-restricted epitope antagonizes early IL-4 response to the wild-type LACK epitope, inhibits Th2 cell development, redirects Th1 cell maturation and results in long term protection.[120] These data are also supported by results which have shown that BALB/c mice tolerant to LACK, as a result of the transgenic expression of this molecule in the thymus under MHC class II promoters, are resistant to *L. major* and develop a Th1 response.[121] Collectively, these results imply that the role of these Vβ4-Vα8 $CD4^+$ T cells is limited to providing the IL-4 necessary for Th2 maturation and suggest that in BALB/c mice, a single antigen (LACK) from this highly complex micro-organism drives the early IL-4 response that underlies subsequent Th2 cell maturation resulting in progressive disease. These data also show that Vβ4-Vα8 $CD4^+$ T cells are not essential at the effector phase of the Th2 response.

1.4.1.3.4 The IL-4 produced in BALB/c mice by Vβ4-Vα8 $CD4^+$ T cells within the first day of infection with *L. major* rapidly renders parasite-specific $CD4^+$ T cells unresponsive to IL-12.

Elegant *in vitro* studies using $CD4^+$ T cells from αβ TCR-transgenic mice of BALB/c and B10.D2 genetic background, demonstrated a T cell intrinsic bias towards a Th2 or Th1 response, respectively.[77 122] Guler *et al.*, proposed that Th2 development may be a functional consequence of a rapid loss of IL-12 responsiveness in BALB/c mice during priming *in vitro*.[77] Interestingly, it has been shown that a rapid down-regulation of the IL-12Rβ2-chain represents the molecular mechanism responsible for this loss of IL-12 responsiveness.[76] The IL-12 receptor is composed of two chains termed β1[123] and β2.[124] The β2 subunit is necessary to form a high affinity receptor[124] and to provide IL-12 signaling through the STAT/JAKS pathway.[76] Expression of the IL-12Rβ2-chain is controlled by IFN-γ and IL-4. Engagement of the TCR induces the expression of the IL-12Rβ1- and β2-chains. The IL-4 mediated down-regulation of IL-12Rβ2-chain expression in $CD4^+$ T cells from αβ TCR-transgenic mice could be overridden by addition of IFN-γ into the primary cultures.[76] Results obtained by our laboratory have documented that there was a short period of time of less than 48 hours after infection during which the IL-4, produced as a result of the 16 hours burst in IL-4

transcription, must be biologically active in order to enforce subsequent Th2 cell development. During that time IL-4 rendered *L. major*-specific $CD4^+$ T cells totally unresponsive to IL-12.[106] Neutralization of the early IL-4 production in *L. major*-infected BALB/c mice maintained IL-12 responsiveness and as a result led to Th1 development.[106] Interestingly, extinction of IL-12 signaling in BALB/c mice is due to a rapid down-regulation of IL-12Rβ2-chain mRNA expression in $CD4^+$ T cells, from 48 hours and at least up to day 8 after infection. Neutralization of the IL-4 produced in BALB/c mice by Vβ4-Vα8 $CD4^+$ T cells during the first days of infection resulted in maintenance of the IL-12Rβ2-chain mRNA expression. Resistant C57BL/6 mice, which do not mount an early IL-4 mRNA burst following infection with *L. major*,[104] maintain the expression of the IL-12Rβ2-chain on their specific $CD4^+$ T cells, which remain responsive to IL-12.[82]

1.4.1.4 The role of IL-2 in Th2 cell development.

1.4.1.4.1 The IL-2/IL-2 receptor system.

IL-2 is a 15,5 kDa glycoprotein produced principally by activated T cells (i.e. Th1 cells),[17] although activated B cells may also have the ability to produce small amounts of IL-2.[125-127] IL-2 acts on a large variety of target cells including T and B lymphocytes, NK cells, and macrophages/monocytes.[128] This cytokine plays a critical role in the regulation of immune responses. It is an important growth factor for T cells following stimulation by antigen and also functions to promote T cell survival, probably by inducing the expression of Bcl-2 and related proteins.[128-132] However, mice that lack IL-2 or a functional IL-2 receptor (IL-2R) accumulate activated T cells and develop autoimmunity,[133-135] suggesting that the dominant role of IL-2 *in vivo* is to terminate T cell responses and to maintain tolerance. A possible mechanistic explanation of this function is provided by the observation that IL-2 renders activated T cells susceptible to activation induced cell death (AICD),[131 136 137] a pathway of cell death that serves to eliminate autoreactive T cells.[138] Surprisingly, IL-2-deficient mice have little functional impairment and are able to generate *in vivo* CTL responses, produce B and T helper responses against viral challenge, and show *in vivo* T cell proliferation.[139]

The high affinity IL-2 receptor is comprised of three component chains, IL-2Rα (CD25), IL-2Rβ and IL-2Rγ.[128 140 141] IL-2Rβ and IL-2Rγ are present constitutively in resting lymphocytes.[142 143] In contrast IL-2Rα is expressed only following activation.[128 140 144] IL-2Rβ is part of the IL-15 receptor,[145] and appears to be required for peripheral immune regulation, since mice lacking this chain exhibit T and B cell activation *in vivo* as well as autoimmunity.[134] IL-2Rγ is shared by the receptors specific for IL-4, IL-7, IL-9 and IL-15.[141 146-148] Mutations in the IL-2Rγ gene result in X-linked severe combined immune deficiency in humans and a similar severe defect in lymphoid development in mice, presumably owing to defects in several of these signaling pathways.[141 149 150] Together, the IL-2Rβ and IL-2Rγ chains form a low affinity IL-2 receptor, which is sufficient to effect signal transduction upon ligation; however, the physiologic role of this low affinity IL-2R is not known.[151 152] While the IL-2Rα chain is incapable of independently generating intracellular signals, its association with the IL-2Rβ and IL-2Rγ chains forms the high affinity IL-2 receptor.[153 154] Therefore, one function of IL-2Rα is to regulate the sensitivity of activated lymphocytes to IL-2. In humans, the IL-2Rβγ complex can bind IL-2 with intermediate affinity. In contrast, the murine heterodimeric IL-2Rβγ does not show any affinity for IL-2, and expression of IL-2Rα is necessary to complete the functional receptor (IL-2Rαβγ).[155] The IL-2Rα chain has not been found in association with any other receptors and is not known to interact with any ligand other than IL-2.[135] Part of the complex IL-2 receptor signal transduction pathways that contribute to the overall cellular response induce by IL-2 has been defined and reviewed in detail.[144 156 157]

1.4.1.4.2 Regulation of Th cell differentiation by IL-2.

In addition to the prominent role that IL-4 plays on the differentiation of Th2 effector cells, evidences have been provided *in vitro* and *in vivo* that IL-2 is necessary for IL-4 production by $CD4^+$ T cells from normal mice and the development of Th2 responses. Thus, IL-2 was required for IL-4 production by $CD4^+$ T cells from naïve donors polyclonally activated *in vitro*, an effect that did not appear related to a possible IL-2-influenced preferential proliferation or survival of $CD4^+$ T cells with the potential to make IL-4.[85 158 159] Furthermore, priming of $CD4^+$ T cells for Th2 differentiation *in vitro*, although dependent upon IL-4, also required IL-2.[85 86 158-163] An essential role for IL-2 in Th2 cell development *in vivo* is also supported by several experimental results.[98 133 164-167] In this context, Ehrhardt *et*

al.[165] reported that IL-2-deficient mice immunized with TNP-KLH exclusively generated Th1 cells which triggered the inflammatory bowel disease; and reduced IL-4 mRNA expression in $CD4^+$ T cells was observed in IL-2-knockout mice infected with *Salmonella.*[167] In addition, weekly treatment of BALB/c mice infected with *L. major* with anti-IL-2 mAbs resulted in resistance to infection and reduced IL-4 production by specifically stimulated lymph node cells *in vitro.*[98] In contrast to these results, other data, some obtained with IL-2-deficient mice, suggest that Th2 cell development may be possible in the absence of IL-2.[134 168-170] For example, $CD4^+$ T cells from IL-2-deficient DO11.10 TCR-transgenic mice were able to differentiate *in vitro* into Th2 effector cells.[170] Further evidences that IL-2 is not required for the development of Th2 cells are provided *in vivo* by the observations that increased production of the Th2-dependent isotypes IgG1 and IgE can occur in mice deficient for IL-2[168] and that the over-production of these isotypes is lost in IL-2 x IL-4 double deficient animals.[169]

The molecular and cellular mechanisms through which IL-2 acts to promote IL-4 production by $CD4^+$ T cells and the development of Th2 responses have not been established and require further investigations. However, substantial progresses have been made in the understanding of the mechanisms involved in the transcriptional regulation of the IL-2 gene.[171-173]

1.5 $CD4^+CD25^+$ regulatory T cells.

1.5.1 Mechanisms of immune suppression.

The development of autoimmune disease involves a breakdown in the mechanisms that control self vs non-self discrimination. The primary mechanism that leads to self tolerance is thymic deletion of autoreactive T cells, but thymic deletion is not perfect and autoreactive T cells do escape to the periphery. Cells that escape thymic deletion are then subject to mechanisms of peripheral tolerance including T cell anergy[174] and T cell ignorance/indifference.[175] However, anergy can be reversible and ignorant T cell populations have the potential to be activated when their target self-Ags are released into the lymphoid system during the course of an infection or when they are activated by cross-reactive epitopes present on infectious agents.[176] Thus, these "passive" mechanisms for self-tolerance may not be sufficient to completely control potentially pathogenic T cells. Over the past 10 years, evidence has accumulated for an "active" mechanism of immune suppression in which a

distinctsubset of cells suppresses the activation of autoreactive T cells that have escaped the other mechanisms of tolerance.[177]

A variety of organ-specific autoimmune diseases can be induced in rodent strains that are not normally susceptible by interfering with normal T cell maturation or by causing a partial T cell deficiency.[178] In general, a defined subset of T cells from syngeneic healthy donors can prevent the development of autoimmunity on transfer to lymphopenic recipients, indicating that the normal immune system contains immunoregulatory T cells that can prevent the activation of autoreactive T cells.[179] For example, Powrie et al.[180] have shown that colitis can be induced in immunodeficient SCID mice by transfer of the $CD45RB^{high}$ subset of $CD4^+$ T cells from normal mice, but not by the $CD45RB^{low}$ population. The $CD45RB^{low}$ population, when transferred together with the $CD45RB^{high}$ population, completely inhibited development of the disease. Evidence for the existence of regulatory T cells has also been obtained in both the bio-breeding rat and nonobese diabetic (NOD) mouse strains that spontaneously develop autoimmune diabetes.[181 182] $CD4^+$ T cells that express TCRs encoded by endogenous α/β-chain genes are also likely to be responsible for the relative disease resistance of mice that express a transgenic (Tg)[175] TCR specific for a peptide from myelin basic protein.[183]

Studies using two different model systems have demonstrated that a potent $CD4^+$ immunoregulatory T cell population can be defined by expression of the IL-2R α-chain (CD25). In the first model system,[184 185] genetically susceptible mice that were thymectomized on day 3 of life (d3Tx) developed organ-specific autoimmune disease involving one or more organs. The disease process was mediated by $CD4^+$ T cells; however, $CD4^+$ T cells from normal adult mice could inhibit the development of disease in the d3Tx animals if they were transferred by day 14 of life. Furthermore, the inhibitory activity was completely contained within the minor (10%) subset of $CD4^+$ T cells that coexpressed CD25.[186 187] In the second model, when $CD4^+CD25^+$ T cells were depleted from $CD4^+$ T cells isolated from peripheral lymphoid tissues of normal adult mice and the remaining $CD4^+CD25^-$ cells injected into *nu/nu* mice recipients, the recipients developed a high incidence of organ-specific autoimmune disease.[186 188] Again, cotransfer of populations enriched in $CD4^+CD25^+$ prevented the induction of disease by the $CD4^+CD25^-$ population. In addition, $CD4^+CD25^+$ T cells can inhibit the capacity of a cloned line of autoantigen-specific effector cells to transfer disease to *nu/nu* recipients.[189] Thus, the $CD4^+CD25^+$ population can inhibit both the induction and effector function of autoreactive T cells.

1.5.2 Regulatory T cell subsets.

It is has been conceded that several types of regulatory or suppressor cells exist, some of which are induced in response to infectious challenge and some that are considered natural regulators.[190 191] Inducible regulatory T cells (T_{reg} cells) such as T_R1 or T helper type 3 (T_H3) cells can develop from conventional $CD4^+$ T cells that are exposed to specific stimulatory conditions such as the blockade of costimulatory signals, deactivating cytokines or drugs. These cell types have been discussed in several reviews.[190 192 193] Natural T_{reg} cells, however, arise during the normal process of maturation in the thymus and survive in the periphery as T_{reg} cells. This segregation between natural T_{reg} cells and induced T_{reg} cells could prove to be arbitrary, with the relationship between the populations requiring clarification. Nevertheless, natural T_{reg} cells obey defined rules and express a specific set of markers.[191 194] For example, only natural T_{reg} cells constitutively express CD25, the T cell inhibitory receptor CTLA-4 and the glucocorticoid-inducible tumor necrosis factor receptor (GITR). The unique transcription factor Foxp3 is required for the generation of natural T_{reg} cells, and this represents their most specific marker identified so far.[194]

The capacity of naturally occurring T_{reg} cells to inhibit the proliferation of naive T cells *in vitro* requires cell–cell contact;[195] however, *in vivo*, these cells can also function through induction of inhibitory cytokines, such as transforming growth factor-β (TGF-β)[196] and IL-10,[197-201] although in a separate study, these cytokines were shown not to be required for the function of naturally occurring T_{reg} cells.[195 200] Indeed, naturally occurring T_{reg} cells protect lymphopaenic mice from colitis[202-204] and diabetes[196 205] through the actions of IL-10 and/or TGF-β, and they can also inhibit transplant rejection.[206 207] Conversely, the ability of naturally occurring T_{reg} cells to inhibit the immune response to tumours can be damaging to the host.[208 209] Naturally occurring T_{reg} cells have also been shown to regulate both Th1 and Th2 responses,[210 211] as well as immune responses to pathogens such as *Leishmania major*[201] and HIV,[212-214] and to allergens.[215-217] So, although naturally occurring T_{reg} cells can limit an immune response to a pathogen or an allergen, thereby inhibiting immune pathology, they might allow immune evasion and persistent infection by a pathogen. It has recently been suggested that naturally occurring T_{reg} cells might be associated with favourable clinical markers of disease status in HIV infection[213] and might maintain immunity to re-infection with *L. major*.[201]

1.5.3 *In vitro* suppression: The function of interleukin-2.

Soon after it became apparent that $CD4^+CD25^+$ T cells are endowed with suppressor function *in vivo*, an *in vitro* system was established that has been widely used to analyze possible modes of suppression.[195] Typically, this system analyzes the proliferation of nonsuppressive $CD4^+$ and $CD8^+$ effector T cells either alone or in culture together with $CD25^+$ suppressor cells.

In vitro analyses have concluded that $CD25^+$ suppressor T cells are anergic; that is, they do not proliferate in culture when stimulated with antibodies to CD3 or antigens unless supplemented with high doses of interleukin 2 (IL-2).[195] In the absence of exogenous IL-2, stimulated $CD25^+$ T cells suppress the proliferation of $CD4^+$ as well as $CD8^+$ T cells by a reaction that is independent of IL-10 and TGF-β secretion, as has been shown with suppresor T cells from IL-10-deficient and TGF-β-deficient mice, which seem to suppress effectively.[195] However, others have postulated an essential function for cell-bound TGF-β on the basis of inhibition of suppression by antibodies to TGF-β.[218] The suppression of proliferation requires direct cell contact between suppressor and suppressed cells, as suppression does not occur when cells are separated by a permeable membrane. The presence of antigen-presenting cells (APCs) is not required, as suppression occurs in APC-free cultures. In all cases, the suppression requires activation of suppressor T cells by TCR ligands or antibodies to CD3.[195] The target of suppression seems to be transcriptional control of *Il2* in effector cells.[195] A reevaluation of the function of IL-2 has concluded that the initial production of IL-2 by the cells to be suppressed is essential for initiation of the function (and some proliferation) of the suppressor cells.[219] Those *in vitro* experiments did not definitively rule out the possibility that the observed inhibition may often be due to the competitive consumption of IL-2 by suppressor T cells[220] that have much higher expression of the IL-2 receptor and hence favorably compete for IL-2, which represents an essential growth factor for freshly stimulated T cells *in vitro*. Overall, the *in vitro* results indicate that suppression involves direct cell contact between suppressor and effector T cells, targets *Il2* and thereby inhibits T cell proliferation.

With CD25 and Foxp3 as specific molecular markers for detecting and manipulating naturally occurring T_{reg} cells, there is now accumulating evidence that the $Foxp3^+CD4^+CD25^+$ T_{reg} cell population is actively engaged in the negative control of a variety of physiological

and pathological immune responses and can be exploited not only for the prevention or treatment of autoimmune diseases but also for the induction of immunological tolerance to non-self antigens (such as transplantation tolerance), negative control of aberrant immune responses (such as allergy and immunopathology) and enhancement of host defense (such as tumor immunity and microbial immunity).[221]

1.6 Aim of the projects.

Although confirming the pathogenic role of the LACK-specific $CD4^+$ T cells in the expression of the susceptible phenotype, recent studies, using multivalent MHC/LACK peptides and IL-4 reporter mice, have shown that the precursor frequency, the expansion and the IL-4 expression of these cells were similar in susceptible BALB/c and resistant B10.D2 mice.[222] Together, these results suggest that signals other than IL-4 are involved in Th2 differentiation and susceptibility to *L. major.*

In this context, weekly treatment of BALB/c mice with anti-IL-2 mAb resulted in resistance to infection and reduced IL-4 production by specifically stimulated lymph node cells *in vitro.*[98] Furthermore, lymph node cells from BALB/c mice with progressive disease produced more IL-2 in culture than cells from healed C57BL/6 mice and IL-2 synthesis was increased in C57BL/6 rendered susceptible by anti-IFN-γ or anti-IL-12.[223] These results suggest a role for IL-2 in susceptibility to *L. major.* In other experimental systems, studies indicating that the priming of $CD4^+$ T cells for Th2 differentiation, although dependent upon IL-4, also requires IL-2[86 162] have been extended by results demonstrating the direct involvement of IL-2 in Th2 cell differentiation.[224] However, Th2 cell development in the absence of IL-2 was reported.[168 169] Therefore, we initiated studies to directly assess the IL-2 dependence of the early IL-4 response involved in Th2 differentiation and susceptibility to *L. major.*

The possibility of modulating the rapid IL-4 response by treatment with either exogenous IL-12 or IFN-γ[104] suggests that LACK-specific Vβ4-Vα8 $CD4^+$ T cells are not irreversibly committed to IL-4 production. In fact, we have documented the functional plasticity of these cells in terms of cytokines production.[225] Together, these results suggest that these cells are sensitive to regulatory processes.

In the past few years, the concept that subpopulations of T cells were specialized in the suppression of immune responses has been revisited. Considerable attention has been given to a minor subpopulation of $CD4^+$ T cells constitutively expressing CD25, the α-chain of the IL-2 receptor. Both in mice and humans, these cells, named regulatory T cells, have been shown capable of suppressing the proliferation of other T cell populations (reviewed in Refs. 199 and 226).

The present study was undertaken to determine whether or not, following infection of BALB/c mice with *L. major*, the early production of IL-4 by LACK-reactive Vβ4-Vα8 $CD4^+$

T cells and the resulting Th2 responses were subject to the control of regulatory $CD4^+CD25^+$ T cells. The results obtained show that $CD4^+CD25^+$ T cells negatively regulate the magnitude of the early IL-4 response to *L. major* in BALB/c mice as well as the importance of subsequent Th2 cell maturation. These data suggest that $CD4^+CD25^+$ T cells may also regulate harmful immune responses to infectious pathogens.

Materials and methods

2.1 Mice.

Female BALB/c, C57BL/6 and C.B.-17 SCID mice, purchased from IFFA Credo (St. Germain sur l'Arbresle, France) or from Harlan Olac Ltd. (Bicester, UK), were used at 6-8 wk of age. ABLE mice express a transgenic Vβ4-Vα8 TCR recognizing an epitope comprising amino acids 156-173 from the LACK antigen in the context of MHC class II I-A^d molecules.[227] IL-$2^{-/-}$ mice[169] obtained from Dr. A. Abbas (University California, San Francisco, CA) and ABLE mice on a BALB/c background were bred in a barrier-contained facility. IL-2-deficient mice were backcrossed eight generations to ABLE mice and then self-mated. Mice were maintained under pathogen-free conditions in the animal facility of the Swiss Institute for Experimental Cancer Research (ISREC), University of Lausanne (Switzerland).

2.2 Parasites and infection.

L. major LV 39 (MRHO/Sv/59/P strain) were maintained as described.[228] For infection, mice were inoculated s.c. into the hind footpad with $3x10^6$ stationary-phase *L. major* promastigotes in 50 μL DMEM. The lesions were measured weekly with a vernier caliper and compared to the thickness of the uninfected footpad. The number of parasites per lesion was evaluated by limiting dilution analysis.[229]

2.3 Reagents, mAb and treatment of mice.

Mice were injected with 5 μg of recombinant LACK protein in 50 μL DMEM s.c. into the hind footpad.[114] The following mAb were used: biotin-conjugated 1D3 (anti-CD19); PE-conjugated 17A2 (anti-CD3); Cy-Chrome- and PE-conjugated GK1.5 (anti-CD4); FITC-conjugated KT4-10 (anti-Vβ4); PE-conjugated RA3-6B2 (anti-CD45R/B220); FITC-conjugated 53-6.7 (anti-CD8); PE-conjugated H1.2F3 (anti-CD69) and MEL-14 (anti-CD62L), all from Pharmingen (San Diego, CA). 2.4G2 (anti-CD16/32); RR4-7 (anti-Vβ6); S4B6 (anti-IL-2); XMG1.2 (anti-IFN-γ); 145-2C11 (anti-CD3); 2.4G2 (anti-FcR); PC61 (anti-CD25)[230] and 11B11 (anti-IL-4) mAbs were affinity-purified on protein A column from hybridoma culture supernatants. Purified, unlabeled, and FITC-conjugated 5A2 (anti-CD25),[231] was kindly provided by Dr. M. Nabholz (Swiss Institute for Cancer Research,

Epalinges, Switzerland). PE-conjugated PC61 was obtained from Dr. A. Wilson (Ludwig Institute of Cancer Research, Epalinges, Switzerland). CFSE was purchased from Molecular Probes (Eugene, OR). Flow cytometry analysis was performed on a FACScan using CellQuest software (BD Biosciences, Mountain View, CA). Designated mice were treated i.p. with 0.5 mg of anti-IL-2 antibodies 20 h and 4 h before infection with *L. major*. Selected mice received 1 mg of anti-IFN-γ antibodies 18 h before inoculation.[80] Other mice from designated groups received 1 mg of PC61 mAb i.p. 72 h before infection. Some mice were also treated with anti-IL-4 mAb (11B11) i.p. at indicated doses and time points. Purified murine rIL-4 was a gift from Dr. A. D. Levine (Monsanto, St. Louis, MO).

2.4 Fluorescent cell sorting.

Draining popliteal lymph node cells were stained with designated antibodies and sorted into positive and negative populations using a FACScan Plus Flow cytometer (Becton Dickinson). To block non-antigen-specific binding of Ig on FcR, anti-CD16 mAb was used in each staining. The purity of the sorted cell populations expressing the corresponding marker was >98%.

2.5 RNA extraction, competitive and qualitative PCR analysis.

Total RNA was isolated from total popliteal lymph node cells or from sorted cell populations using TRISOL Reagent (Gibco BRL) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on total RNA using a first-strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden). The competitive PCR developed by Reiner *et al.*[232] was performed as described. Results are expressed as the fold increase in mRNA expression in mice infected with *L. major* or injected with the LACK protein as compared with control mice.

2.6 Cell sorting and reconstitution of C.B.-17 SCID mice.

$V\beta4^+$ T cells were removed from spleen cells of naive mice using magnetic cell columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, spleen cells depleted of red blood cells by lysis in Tris-

buffered NH_4Cl solution were first stained with the anti-Vβ4 mAb during 20 min on ice. Following washes, cells were re-suspended in magnetic beads conjugated with goat anti-rat IgG antibodies and theVβ4-depleted population was negatively selected after exposure of positive cells to magnetic field. The same procedure was applied for removal of $V\beta6^+$ T cells or $CD4^+CD25^+$ cells from spleen cells using the anti-Vβ6 mAb or anti-CD25 mAb 5A2 respectively. This procedure generally resulted in the removal of $CD4^+CD25^+$ cells from the spleen cell suspension to background levels of <1.5% of total $CD4^+$ cells, as detected by flow cytometry with subsequent staining using PC61, a second anti-CD25 mAb that binds to a different epitope on the IL-2Rα molecule.[230] [231] In some experiments, depletion was performed with a combination of FITC-labeled 5A2 and anti-FITC microbeads. The efficiency of depletion was similar.

Purification of $V\beta4^+$ $CD4^+$ cells from naive IL-$2^{+/-}$ and IL-$2^{-/-}$ ABLE-transgenic mice was done in two steps. First non-$CD4^+$ T cells were depleted using a cocktail of biotin-conjugated antibodies against CD8, CD45R, DX5, CD11b and Ter-1 and anti-biotin-microbeads according to the manufacturer's instructions (Miltenyi Biotec). Isolation of the highly pure $CD4^+$ T cells was obtained by depletion of magnetically labelled cells. Then, secondary positive selection of $V\beta4^+$ cells in the purified $CD4^+$ T cells was applied. In experiments where SCID mice were adoptively transferred with 10^8 spleen cells depleted in $V\beta4^+$ cells, mice from some groups also received $3x10^4$ transgenic LACK-reactive Vβ4-Vα8 $CD4^+$ T cells.

C.B.-17 SCID mice were bled in the tail vein and PBMC were obtained by Ficoll-Hypaque centrifugation. The PBMC were stained for CD4, CD8, and B220 and screened by flow cytometry. SCID mice that had <1% peripheral B or T cells were reconstituted i.v. with designated numbers of spleen cells from wild-type BALB/c mice. In some experiments, SCID mice were reconstituted with CFSE-labeled spleen cells as above.

2.7 Lymphocyte cultures and detection of cytokines in supernatants.

Draining popliteal lymph node cells ($5x10^6$) were cultured in a final volume of 1 ml in DMEM supplemented with 5% heat inactivated FCS, 216 μg/ml L-glutamine, $5x10^{-5}$ M 2-ME,and 10 mM HEPES at 37°C in an atmosphere of 7% CO2 in the presence or absence of UV-irradiated *L. major* promastigotes ($1x10^6$/ml) or soluble anti-CD3 (2 μg/ml). Supernatants were collected at 48 h from cultures stimulated with soluble anti-CD3 or at 72 h for cultures

stimulated with *Leishmania* Ags, and frozen at -20°C until use. IFN-γ was measured in supernatants by ELISA as described.[233] Mouse rIFN-γ (supernatant of L1210 cells transfected with the murine IFN-γ gene; a gift from Y. Watanabe, Kyoto University, Kyoto, Japan) was used as the standard. The limit of detection of the assay was 10 U/ml. IL-4 was measured by a bioassay using the CTLL-44 cell line (a gift from P. Erb, University of Basel, Basel, Switzerland) as described.[234] Recombinant murine IL-4 secreted by X63Ag-653 cells (a gift from F. Melchers, Basel Institute of Immunology, Basel, Switzerland) was used as the standard. The limit of detection of the assay was 20 pg/ml. For proliferation assays, $5x10^5$ cells were cultured with UV-irradiated stationary phase *L. major* promastigotes ($1x10^6$/ml) or anti-CD3 (0.5 μg/ml) in 200 μl DMEM/5% FCS, and were pulsed with 1 μCi of [^{3}H]thymidine for the final 6 h of culture. Harvested cells were measured for radioactivity using a beta scintillation counter.

2.8 Cell counting.

Cells were either counted using a Neubauer chamber or using the FACS; cell suspensions were resuspended in a volume of 300 μl, and 10^5 bacterial count microbeads (Molecular Probes) were added to each tube and cells were then analyzed by FACS. Gates were assigned to the microbeads and lymphocytes by forward and size scatter characteristics. The ratio of beads to lymphocytes was determined for each specimen from the electronic counts on FACScan.

2.9 Statistics.

Statistical analysis was done using the two-tailed *t* test for unpaired data. The estimation of the frequency of parasites by limiting dilution was calculated by the Taswell method using the program Estimfree.[235]

Results

3.1 LACK-reactive CD4$^+$ T cells require autocrine IL-2 to mediate susceptibility to *Leishmania major.*

3.1.1 Early IL-2 mRNA expression following infection with L. major or injection of LACK.

The results in Fig. 1A confirm the kinetics of IL-4 mRNA expression in response to *L. major* in BALB/c and anti-IFN-γ-treated C57BL/6 mice, with an increase of IL-4 transcripts within the first day of infection.[80 104] As reported, C57BL/6 mice did not exhibit an increase of IL-4 transcripts in response to *L. major* and the kinetics of IL-4 mRNA expression in C57BL/6 mice treated with anti-IFN-γ mAb were different from that of BALB/c mice with a burst at 16 h remaining stable during the first 10 days of infection.[80 104] The increased IL-4 mRNA expression was confirmed by real-time RT-PCR during the first day of infection in BALB/c mice. However, the second wave of IL-4 mRNA from day 5 of infection was higher (data not shown).

A burst of IL-2 mRNA was observed 10 h after infection in draining lymph node cells from all mice tested, which decreased thereafter, reaching the levels of uninfected mice on day 6 (Fig. 1A). The early burst of IL-2 transcription reproducibly preceded the IL-4 mRNA burst in susceptible mice. In a few experiments, similar increases and kinetics of IL-2 mRNA expression were also seen with quantitative real-time RT-PCR (data not shown).

Since previous results have shown that recognition of the LACK antigen drives the early IL-4 response responsible for Th2 cell differentiation in BALB/c mice,[114] the capacity of LACK to elicit IL-2 mRNA transcription was also tested. Results in Fig. 1A show that LACK elicited an early IL-2 mRNA response in BALB/c and anti-IFN-γ-treated C57BL/6 mice. Noteworthy, the LACK-induced IL-2 mRNA expression was an order of magnitude lower in C57BL/6 than in BALB/c and anti-IFN-γ-treated C57BL/6 mice (Fig. 1A). As reported,[80 114] IL-4 transcripts were detected within the first day and decayed over the following 10 days after injection of LACK in either BALB/c or in anti-IFN-γ-treated C57BL/6 mice. No increase in IL-4 mRNA expression was seen after injection of LACK in C57BL/6 mice (Fig. 1A).

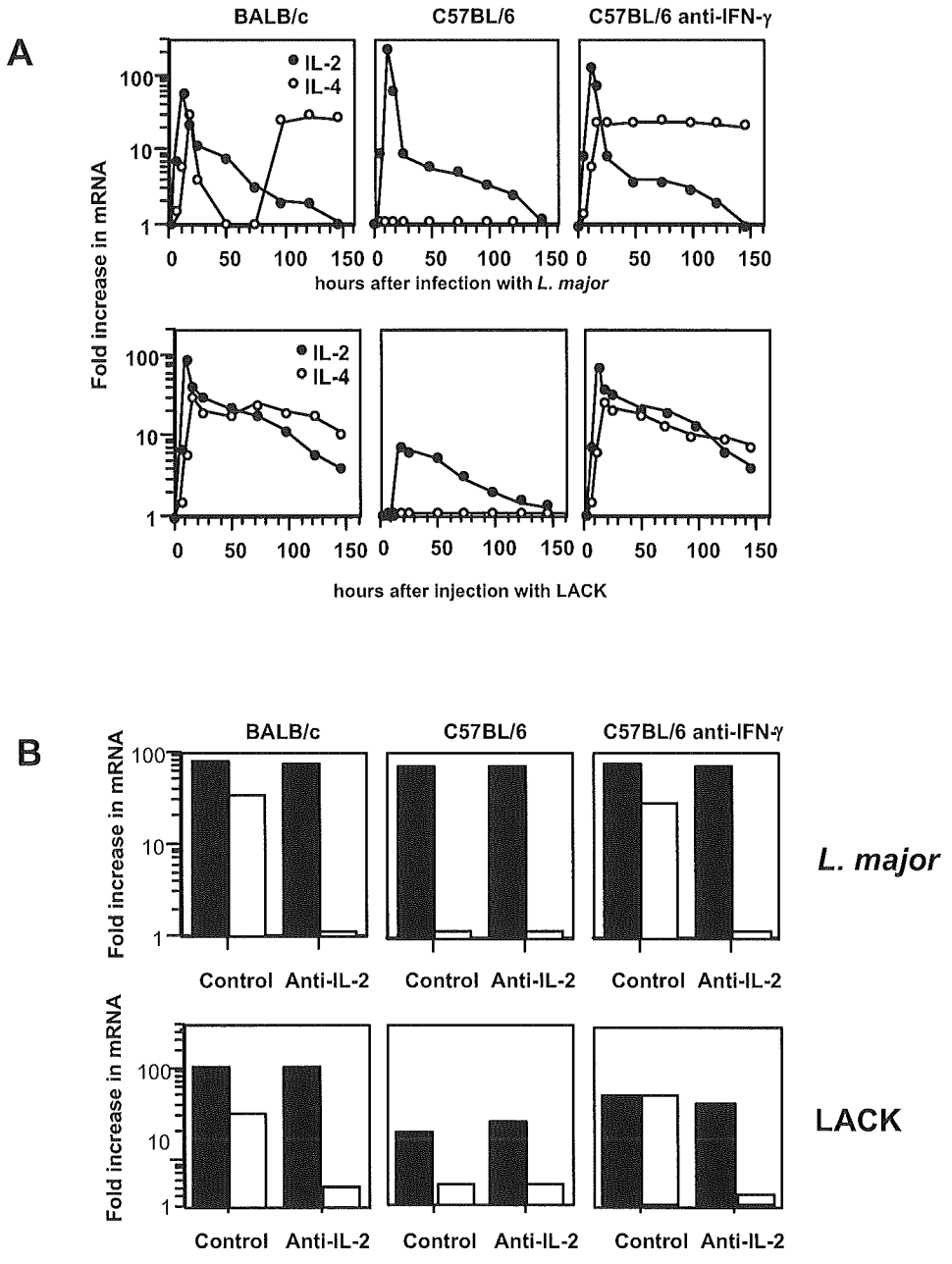

Figure 1. IL-2 is produced in response to infection with *L. major* or injection of LACK and enables the early IL-4 mRNA expression in BALB/c mice and in anti-IFN-γ-treated C57BL/6 mice infected with *L. major*. (A) Kinetics of the IL-2 mRNA expression in popliteal lymph nodes following s.c. injection of $3x10^6$ stationary-phase *L. major* promastigotes or 5 μg of LACK in BALB/c and C57BL/6 mice treated or not with anti-IFN-γ mAb. Various times following injection, mice were killed, RNA extracted from the draining lymph nodes and the relative levels of IL-2 and IL-4 mRNA were determined by competitive RT-PCR. Results are expressed as the fold increase in IL-2 and IL-4 mRNA expression in mice injected with *L. major* or LACK as compared to non-injected mice from the corresponding group. The results are from one of two experiments, which gave the same results. (B) Five-hundred micrograms of anti-IL-2 mAb was administrated i.p. 20 and 4 h prior s.c. injection of $3x10^6$ *L. major* or 5 μg of LACK. Similarly injected mice not treated with anti-IL-2 were used as controls. Mice were killed 16 h after injection, and the relative IL-2 (filled bars) and IL-4 (empty bars) mRNA expression determined in draining lymph nodes. For mice treated with anti-IL-2, results are expressed as the increase in cytokine mRNA expression in treated mice injected with either *L. major* or LACK as compared to mice from the corresponding group treated with anti-IL-2 but not receiving *L. major* or LACK. Results are from one of three experiments, which gave the same results.

3.1.2 *Importance of the early IL-2 response on the rapid IL-4 mRNA expression in susceptible mice.*

Although the early burst of IL-2 transcripts was observed in both resistant and susceptible mice, experiments were designed to assess the importance of the IL-2 produced on the levels of IL-4 mRNA expression in draining lymph nodes of BALB/c and anti-IFN-γ-treated C57BL/6 mice 16 h after either infection with *L. major* or injection with LACK. The administration of 0.5 mg neutralizing anti-IL-2 mAb 20 and 4 h before injection of *L. major* or 5 μg of LACK abrogated the early increase in IL-4 transcripts (Fig. 1B). This did not represent an aberrant kinetic response to *L. major* but rather a markedly attenuated IL-4 response in the absence of IL-2 (data not shown). Treatment with anti-IL-2 mAb had no effect on the burst of IL-2 transcripts (Fig. 1B). Together, these results show that the early IL-4 mRNA increase in response to *L. major*/LACK in susceptible mice requires IL-2.

Furthermore, mice treated with anti-IL-2 mAb at the onset of infection developed a Th1 response and became resistant to infection (data not shown), confirming observations by others of the ability of four weekly administrations of anti-IL-2 mAb or two weekly administrations of anti-IL-2R antibodies to cure susceptible BALB/c mice.[98] The inhibitory effects of anti-IL-2 treatment on Th2 cell maturation and disease progression could be overcome by exogenous IL-4 (1 μg) during the first 64 h of infection (data not shown).

3.1.3 *Cellular origin of the IL-2 mRNA rapidly expressed in response to L. major.*

Since IL-2 has been demonstrated, in other experimental systems, to be produced mainly by activated $CD4^+$ T cells and to a lower extend by activated B cells,[125-127] we analysed, 10 h after infection with *L. major*, the cellular source of the early IL-2 transcripts in draining lymph nodes from BALB/c and C57BL/6 mice treated or not with anti-IFN-γ. In both, BALB/c and C57BL/6 mice, irrespective of treatment with anti-IFN-γ, the increase in IL-2 transcripts occurred mainly in $CD4^+$ T cells, but was not confined to those expressing the Vβ4 TCR chain (Fig. 2A), in contrast to the early IL-4 response only produced by the restricted population of Vβ4-Vα8 $CD4^+$ T cells in susceptible mice. Furthermore, IL-2 transcripts were also detected in B cells ($CD3^-$ $CD19^+$), but at significantly lower levels, in all groups of mice infected with *L. major*. Interestingly, $CD3^-$ $CD19^-$ cells did not contribute to

IL-2 production, indicating that the production of IL-2 by dendritic cells did not account for the observed response.

Analysis of the cellular origin of the early IL-2 mRNA expression induced by injection of LACK revealed that it occurred only in $CD4^+$ T cells that expressed the Vβ4 TCR chain and to a lower degree in B cells from BALB/c and anti-IFN-γ-treated C57BL/6 mice (Fig. 2A). Strikingly, in C57BL/6 mice, injection of LACK resulted in a small IL-2 mRNA increase only in B cells (Fig. 2A). Results in Fig. 2B confirm that the increased IL-4 mRNA transcription in response to infection or injection of LACK occurred only among the $CD3^+$ $CD4^+$ $V\beta4^+$ T cell population in BALB/c and anti-IFN-γ-treated C57BL/6 mice and was not seen in C57BL/6 mice.

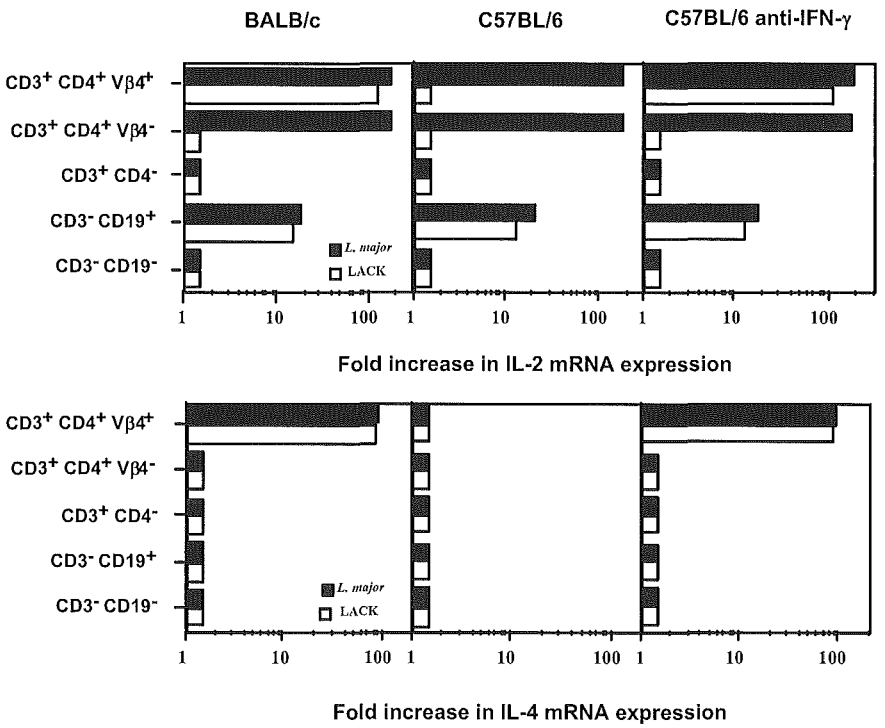

Figure 2. Cellular origin of IL-2 transcripts detected 10 h after infection with *L. major* or injection of LACK. Ten and 16 h after infection with either $3x10^6$ *L. major* promastigotes or injection of 5 μg of LACK, draining lymph node cells of mice from the designated groups were stained with the designated antibodies and sorted into positive and negative subpopulations by flow cytometry for determination of IL-2 and IL-4 mRNA levels. Results are expressed as the fold increase in IL-2 and IL-4 mRNA expression in designated cell population of mice injected with either *L. major* or LACK as compared to the same population in non-infected mice from the corresponding group. These results are from one of two experiments with identical results.

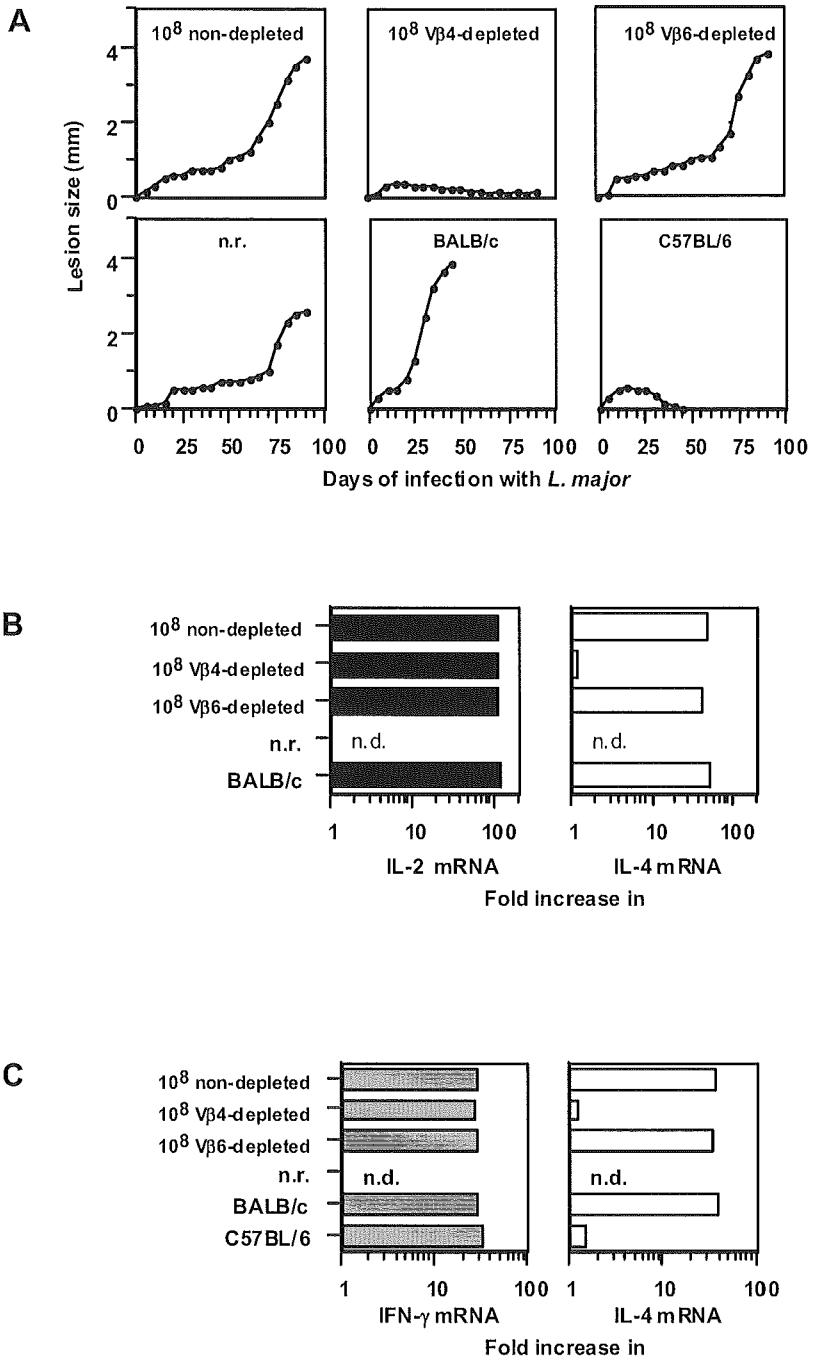

Figure 3. Absence of LACK-specific Vβ4-Vα8 CD4$^+$ T cells prevents the early IL-4 response, progressive disease and Th2 cell maturation in SCID mice reconstituted with 10^8 BALB/c spleen cells and infected with *L. major*. C.B.-17 SCID mice were reconstituted with 10^8 BALB/c splenocytes depleted or not of Vβ4$^+$ CD4$^+$ T cells or Vβ6$^+$ CD4$^+$ T cells. Five days after cell transfer mice were infected with $3x10^6$ *L. major*. Similarly infected unreconstituted SCID mice and BALB/c mice were used as controls. (A) The size of the footpad lesions was monitored in mice from the designated groups. (B) Sixteen hours after infection, the relative levels of IL-2 and IL-4 mRNA were determined in popliteal lymph node cells. Results are expressed as the fold increase in IL-2 and IL-4 mRNA compared with levels in uninfected mice from the corresponding group. (C) The levels of IL-4 and IFN-γ mRNA in draining popliteal lymph node cells were determined 13 wk after infection. Similar results were obtained in two individual experiments; n.d.: not detected; n.r.: not reconstituted.

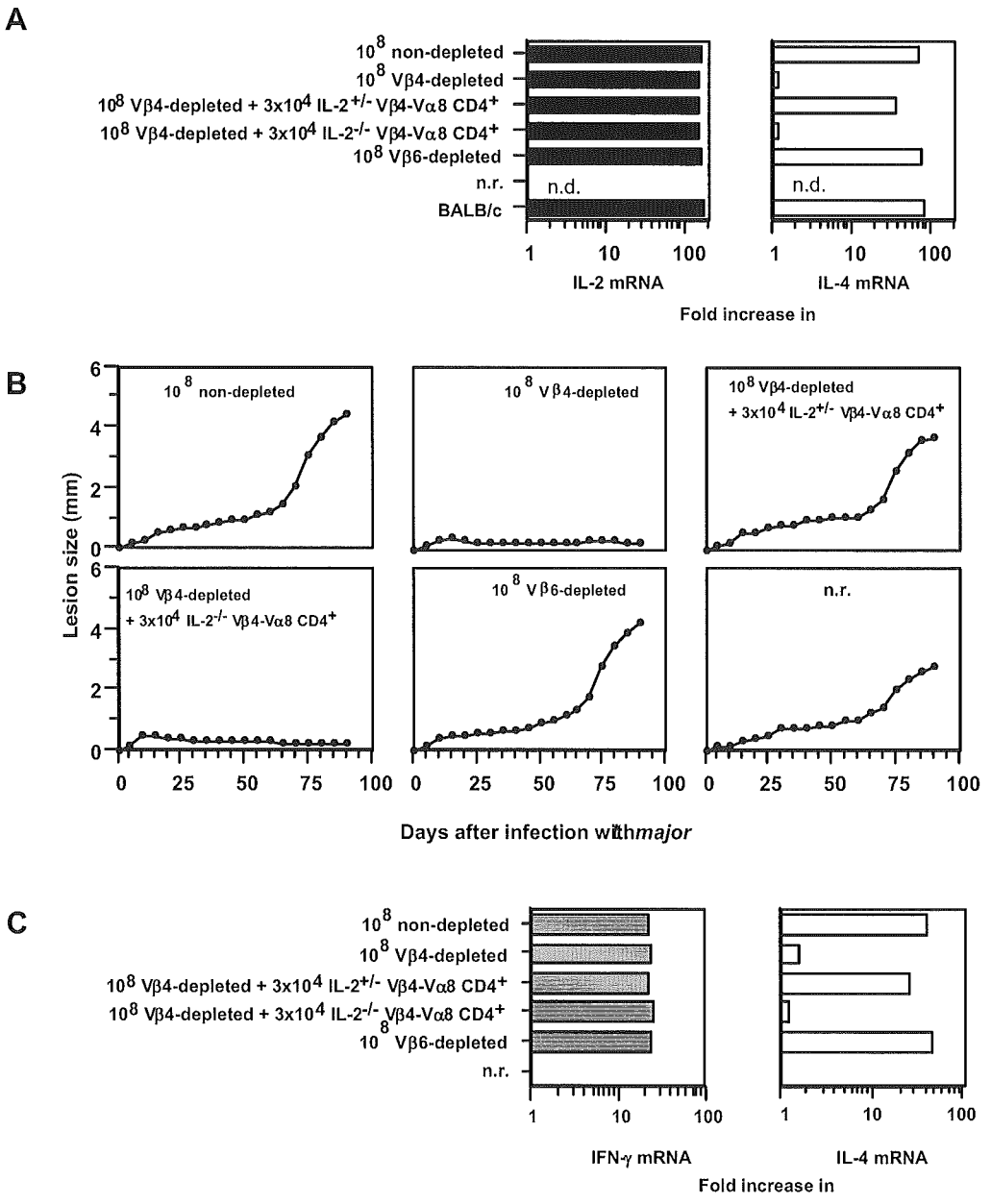

Figure 4. Transfer of IL-2-deficient LACK-reactive CD4$^+$ T cells to SCID mice interferes with the early IL-4 response and directs Th1 cell development and resistance to *L. major*. (A) C.B.-17 SCID mice were adoptively transferred with either 10^8 BALB/c spleen cells or 10^8 V$\beta4^+$ CD4$^+$-depleted spleen cells supplemented or not with LACK-specific V$\beta4$-V$\alpha8$ CD4$^+$ T cells from either IL-$2^{-/-}$ or IL-$2^{+/-}$ ABLE-transgenic mice. Five days after cell transfer, mice were infected with $3x10^6$ *L. major*. Similarly infected SCID mice reconstituted with 10^8 spleen cells depleted in V$\beta6^+$ CD4$^+$ T cells, unreconstituted SCID and BALB/c mice were used as controls. Sixteen hours after infection, the relative levels of IL-2 and IL-4 mRNA were determined in draining lymph nodes. (B) The course of infection (mean size of lesions) was monitored in recipient SCID mice from the designated groups. (C) IL-4 and IFN-γ mRNA levels in draining lymph node cells were determined 13 wk after infection. Similar results were obtained in two individual experiments using four mice in each group; n.d.: not detected; n.r.: not reconstituted.

3.1.4 *Th2 response to L. major reguires IL-2 producing LACK reactive CD4$^+$ T cells.*

Given the various possible sources for the early IL-2 response in BALB/c mice infected with *L. major*, the cellular source of the IL-2 biologically active on the IL-4 response was determined. C.B.-17 SCID mice were adoptively transferred as previously described[3] with either 10^8 spleen cells from naive BALB/c mice or 10^8 BALB/c splenocytes depleted in either Vβ4$^+$ or Vβ6$^+$ CD4$^+$ T cells. Five days later, mice were inoculated with $3x10^6$ *L. major* and lesions monitored. Results in Fig. 3A confirm that SCID mice reconstituted with 10^8 naive spleen cells are unable to control infection as wild-type BALB/c mice. In contrast, SCID mice reconstituted with 10^8 spleen cells depleted of Vβ4$^+$ CD4$^+$ T cells were resistant to infection whereas SCID mice reconstituted with 10^8 spleen cells depleted of the Vβ6$^+$ CD4$^+$ T cells were fully susceptible.

SCID mice reconstituted with spleen cells depleted in Vβ6$^+$ CD4$^+$ T cells like wild-type BALB/c mice and SCID mice reconstituted with 10^8 normal spleen cells produced an early IL-4 mRNA burst (Fig. 3B). Quantification of IL-4 and IFN-γ mRNA expression in lymph node cells 13 wk after infection clearly showed that these mice mounted a specific Th2 response (Fig. 3C). In contrast, SCID mice reconstituted with 10^8 spleen cells depleted in Vβ4$^+$ CD4$^+$ T cells did not mount an early IL-4 response and developed a Th1 response (Fig. 3B, C). Noteworthy, SCID mice reconstituted with 10^8 total spleen cells became resistant to infection when treated with anti-IL-4 mAb (data not shown). All groups of mice were able to mount an early IL-2 response, even SCID mice receiving 10^8 spleen cells depleted in Vβ4 CD4$^+$ T cells, further indicating that the source of IL-2 produced after infection with *L. major* was not restricted to CD4$^+$ Vβ4$^+$ T cells (Fig. 3B). As expected, non-reconstituted SCID mice did not develop IL-2, IL-4 and IFN-γ responses.

Since the early IL-4 mRNA expression was abolished following anti-IL-2 mAb treatment (Fig. 1B) and only CD4$^+$ T cells expressing the Vβ4 TCR chain from susceptible mice transcribed IL-2 and IL-4 mRNA following injection of the LACK protein (Fig. 2), we used this SCID model to test the hypothesis that the IL-2 produced by LACK-reactive Vβ4-Vα8 CD4$^+$ T cells is necessary for IL-4 production by cells from the same subset. Thus, we investigated whether the substitution of wild-type Vβ4 CD4$^+$ T cells by LACK-specific Vβ4-

Vα8 T cells from IL-2-deficient mice would interfere with the early IL-4 response to *L. major*. SCID mice were adoptively transferred with 10^8 spleen cells depleted of $V\beta4^+$ $CD4^+$ T cells and supplemented with $3x10^4$ Vβ4-Vα8 $CD4^+$ T cells (the estimated proportion of Vβ4-Vα8 T cells in 10^8 spleen cells) from either homozygous IL-$2^{-/-}$ or heterozygous IL-$2^{+/-}$ ABLE (LACK-specific TCR-transgenic) BALB/c mice. As controls, SCID mice were reconstituted or not with 10^8 spleen cells or with 10^8 spleen cells depleted in either $V\beta4^+$ or $V\beta6^+$ T cells. Mice from all groups were infected with *L. major* 5 days after cell transfer and the levels of IL-2 and IL-4 mRNA expression were assessed 16 h later.

As shown in Fig. 4A, although SCID mice that received IL-$2^{+/-}$ LACK-specific Vβ4-Vα8 $CD4^+$ T cells or 10^8 spleen cells depleted or not in Vβ6 $CD4^+$ T cells transcribed IL-4 mRNA early after infection with *L. major*, no increase in IL-4 transcripts was detected in the draining lymph nodes of SCID mice adoptively transferred with IL-$2^{-/-}$ LACK-reactive $CD4^+$ T cells or with 10^8 spleen cells depleted in Vβ4 $CD4^+$ T cells. Noteworthy, no significant differences in early IL-2 mRNA expression were observed in all groups of mice infected with *L. major*. These results directly support the hypothesis that the IL-2 produced by LACK-specific Vβ4-Vα8 $CD4^+$ T cells is necessary for the induction of the early IL-4 response.

The course of disease was also monitored in SCID mice reconstituted as above. As shown in Fig. 4B, mice that received 10^8 spleen cells depleted or not in $V\beta6^+$ $CD4^+$ T cells developed progressive lesions whereas SCID mice reconstituted with 10^8 spleen cells depleted in $V\beta4^+$ $CD4^+$ cells controlled their lesions. More interestingly, SCID mice reconstituted with $V\beta4^+$ $CD4^+$ depleted spleen cells supplemented with IL-$2^{-/-}$ LACK-specific $CD4^+$ T cells controlled disease progression, whereas SCID mice reconstituted with spleen cells depleted of $V\beta4^+$ $CD4^+$ cells and supplemented with IL-$2^{+/-}$ LACK-specific $CD4^+$ T cells were fully susceptible. At the time of resolution of the lesions, analysis of the cytokine mRNA expression did not reveal an increased IL-4 mRNA expression in draining lymph nodes from SCID mice reconstituted with cells comprising IL-$2^{-/-}$ LACK-specific $CD4^+$ T cells. In contrast, high levels of IL-4 were detected in SCID mice receiving IL-$2^{+/-}$ LACK-specific $CD4^+$ T cells (Fig. 4C). Thus, healing of lesions in SCID mice receiving IL-2-deficient LACK-specific Vβ4-Vα8 $CD4^+$ T cells was associated with a Th1 response.

To directly assess whether or not the IL-2 produced by cells other than LACK-specific $CD4^+$ T cells was required for the early IL-4 mRNA expression, SCID mice were reconstituted with 10^8 spleen cells depleted in Vβ4 $CD4^+$ cells from either IL-2-deficient or wild-type BALB/c mice together with $3x10^4$ IL-$2^{+/-}$ LACK-reactive Vβ4-Vα8 $CD4^+$ T cells from ABLE mice and inoculated with *L. major* 5 days later. Results in Fig. 5 show that SCID mice reconstituted with either 10^8 IL-$2^{+/-}$ or IL-$2^{-/-}$ spleen cells depleted in Vβ4 cells supplemented with $3x10^4$ IL-$2^{+/-}$ LACK-specific Vβ4-Vα8 $CD4^+$ T cells exhibited a similar burst of IL-4 trancripts in draining lymph nodes. As expected, decrease in IL-2 mRNA expression was observed in infected SCID mice reconstituted with IL-$2^{-/-}$ Vβ4-depleted spleen cells. Together these results strongly suggest that the Th2 response to *L. major* in SCID mice reconstituted with 10^8 spleen cells depends on the ability of LACK-reactive Vβ4-Vα8 $CD4^+$ T cells to produce IL-2.

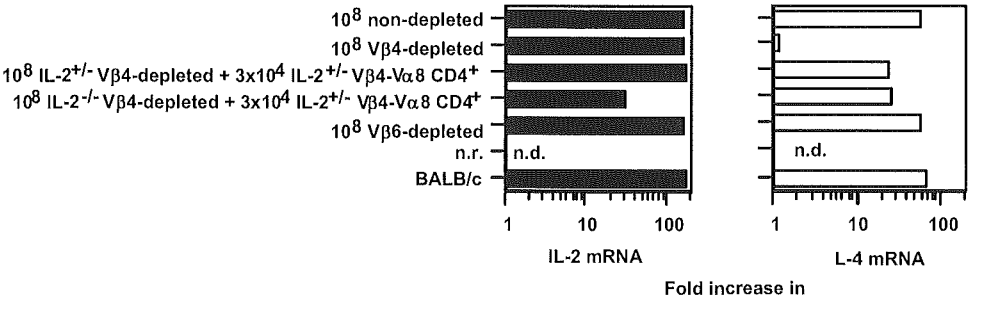

Figure 5. The triggering of an early IL-4 response to *L. major* does not depend upon the IL-2 produced by cells other than LACK-specific Vβ4-Vα8 $CD4^+$ T cells. C.B.-17 SCID mice were adoptively transferred with 10^8 $V\beta4^+$ $CD4^+$-depleted spleen cells from either wild-type or IL-$2^{-/-}$ BALB/c mice supplemented with $3x10^4$ LACK-specific Vβ4-Vα8 $CD4^+$ T cells from IL-$2^{+/-}$ ABLE-transgenic mice. BALB/c mice and SCID mice reconstituted or not with 10^8 spleen cells or 10^8 spleen cells depleted in either $V\beta4^+$ or $V\beta6^+$ $CD4^+$ T cells were used as controls. Mice were infected 5 days after cell transfer and 16 h later the relative levels of IL-2 and IL-4 mRNA were determined in draining lymph nodes. Similar results were obtained in two separate experiments; n.d.: not detected; n..r.: not reconstituted.

3.2 The early IL-4 response to *Leishmania major* and the resulting Th2 cell maturation steering progressive disease in BALB/c mice are subject to the control of regulatory $CD4^+CD25^+$ T cells.

3.2.1 Depletion of $CD4^+CD25^+$ regulatory T cells before infection with L. major exacerbates the development of lesions in BALB/c mice.

To study the role of the $CD4^+CD25^+$ regulatory T cells *in vivo*, BALB/c mice were treated i.p. once with 1 mg of anti-CD25 mAb PC61.[230] Seventy-two hours following such treatment, the proportion of $CD4^+CD25^+$ T cells dropped from around 10% to below 2% of the $CD4^+$ T cell population in lymph nodes (data not shown). The percentage of $CD4^+CD25^+$ T cells within the lymph nodes remained low for over 10 days. The percentage of the $CD4^+CD25^+$ subpopulation in peripheral blood was also reduced to around 2% of the circulating $CD4^+$ T cells (data not shown). This depletion was also long-lasting as reported by others.[208]

Compared with similarly infected control BALB/c mice, mice treated with 1 mg of PC61 mAb 72 h before infection with $3x10^6$ *L. major* promastigotes developed significantly larger lesions (Fig. 6A) that contained higher numbers of parasites (Fig. 6B). The number of parasites measured in lesions of PC61-treated BALB/c mice was consistently significantly higher than that measured in lesions of BALB/c mice in five different experiments (p = 0.019). The development of severe lesions in BALB/c mice depleted of $CD4^+CD25^+$ T cells was correlated with an enhanced IL-4-producing Th2 response. Results in Fig. 6C show that 15 days after infection with *L. major* the amounts of IL-4 transcripts in draining lymph nodes are already five times higher in BALB/c mice depleted of $CD4^+CD25^+$ cells than in similarly infected control BALB/c mice. Comparable results were obtained when supernatants of *L. major*-activated lymph node cells were analyzed for the accumulation of IL-4 (Fig. 6D). This difference in IL-4 production between $CD4^+CD25^+$ cell-depleted and normal BALB/c mice was also observed at later times after infection (data not shown). In most experiments, the level of IFN-γ in supernatants of cultures from draining lymph nodes of mice depleted of $CD4^+CD25^+$ cells was equivalent to, or only slightly higher, than that measured in cultures of lymph nodes from nondepleted BALB/c mice (Fig. 6D).

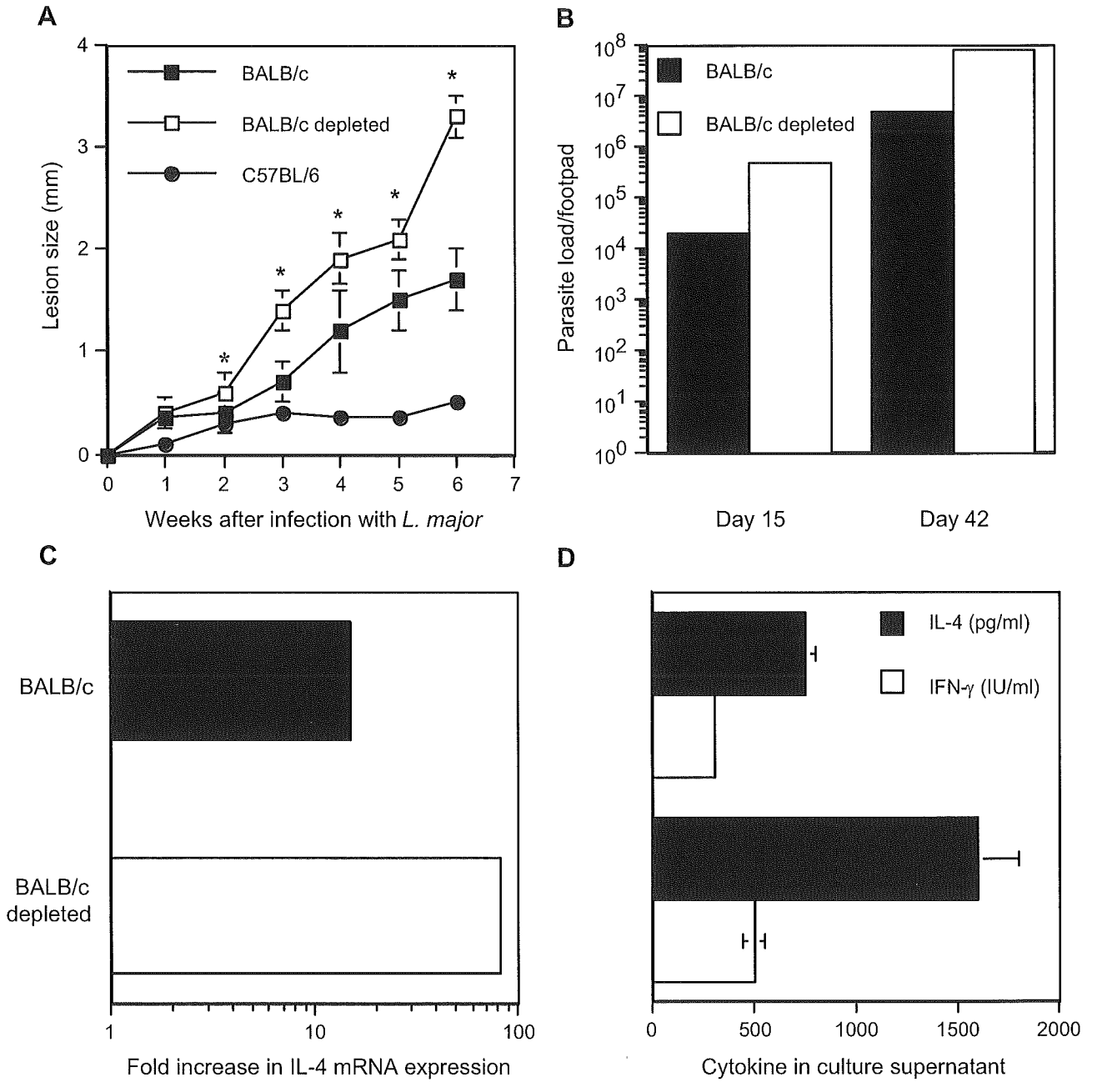

Figure 6. Infection of BALB/c mice depleted of $CD4^+CD25^+$ regulatory cells with *L. major* leads to an exacerbated course of disease and an enhanced Th2 response. (A) BALB/c, C57BL/6, and BALB/c mice depleted of $CD4^+CD25^+$ cells were infected with $3x10^6$ stationary phase *L. major* promastigotes s.c. in the footpads. The course of infection was monitored by weekly measurement of the diameter of footpads with a metric caliper. The mean size of lesions (increase in diameter of footpads due to infection) and SD are shown (four mice per group). Similar results were obtained in three other experiments. *, $p < 0.001$ between $CD4^+CD25^+$ depleted vs nondepleted BALB/c mice. (B) Parasite load in the footpads of mice sacrificed 15 and 42 days after infection with *L. major* was determined by limiting dilution assay. The mean counts of two footpads per group is shown. Data are representative of two experiments. (C) IL-4 mRNA levels in popliteal lymph nodes of BALB/c mice depleted or not of $CD4^+CD25^+$ T cells. BALB/c mice and BALB/c mice depleted of $CD4^+CD25^+$ T cells were infected with $3x10^6$ stationary phase *L. major* promastigotes s.c. in the hind footpads. Control groups were untreated or noninfected BALB/c mice treated with PC61. Fifteen days later, mice were sacrificed, mRNA was extracted from their popliteal lymph nodes and the relative level of IL-4 mRNA was determined by semiquantitative RT-PCR as described in *Materials and Methods* ($n = 4$ mice per group). Results are expressed as fold increase relative to similarly treated, noninfected control mice. The results represent one of three independent experiments. (D) IL-4 and IFN-γ production by lymph node cells of mice 15 days after infection with *L. major*. Popliteal lymph node cells were isolated 15 days after infection with *L. major* and $5x10^6$ cells from mice of each group (three mice per group) were stimulated *in vitro* with 10^6 UV-irradiated *L. major* promastigotes for 72 h. IFN-γ and IL-4 production was evaluated in the supernatant as described in *Materials and Methods*. Results are expressed as mean and SD of triplicate measurements. The data shown are representative of two independent experiments.

Depletion of $CD4^+CD25^+$ T cells in C57BL/6 mice before infection with *L. major* did not alter the course of infection (data not shown). In three independent experiments, no significant difference in the size of lesions between PC61-treated and control C57BL/6 mice was observed, and both groups resolved their lesions. Furthermore, the level of cytokines produced (no IL-4, high level of IFN-γ) in the draining lymph nodes of both groups of mice did not differ. However, parasite load in the footpads of $CD25^+$ T cell-depleted C57BL/6 mice 5 wk after infection was 10 times lower than in control C57BL/6 mice infected with *L. major* simultaneously; 8 wk after infection parasites in the lesions of PC61 treated mice disappeared almost completely (75% of mice with no parasites detectable and 25% of mice with 3–10 parasites/footpad) while C57BL/6 mice not depleted of $CD25^+$ T cells still had $0.5\text{-}6.7\text{x}10^3$ parasites per lesion.

3.2.2 The early IL-4 response to L. major is significantly enhanced in BALB/c mice depleted of $CD4^+CD25^+$ cells.

We have previously documented a burst of IL-4 mRNA expression in draining lymph nodes of BALB/c mice within 1 day of infection with *L. major*.[104] This early IL-4 burst occurs in a restricted population of LACK-reactive $CD4^+$ T cells expressing the Vβ4-Vα8 TCR chains.[106] The causal relationship between this early IL-4 response and subsequent Th2 cell maturation in BALB/c mice was demonstrated.[119]

To investigate whether or not $CD4^+CD25^+$ regulatory T cells control the early IL-4 mRNA response to *L. major*, BALB/c mice treated or not with 1 mg of anti-CD25 PC61 mAb were inoculated, 3 days later, with *L. major* in one hind footpad. Sixteen hours after infection, total mRNA from draining lymph nodes was analyzed for IL-4 mRNA expression using a semiquantitative RT-PCR. Compared with infected mice not treated with PC61 mAb, higher levels (6–10 times) of IL-4 mRNA transcripts were consistently observed in lymph nodes of $CD4^+CD25^+$ T cell-depleted mice (Fig. 7). This increase in IL-4 mRNA was confirmed in five independent experiments where, 16 hr after infection with *L. major*, the level of IL-4 mRNA measured in PC61-treated mice was significantly higher ($p = 0.0001$) compared with that measured in BALB/c mice that were not injected with the mAb. The IL-4 mRNA was detected only in the Vβ4-Vα8 $CD4^+$ T cell population (data not shown). Comparable results were obtained by real-time PCR, 16 h after infection with *L. major* (data not shown). It is

noteworthy that in the absence of infection with *L. major*, treatment of BALB/c mice with the mAb PC61 did not affect the basal level of IL-4 mRNA transcripts. In five independent experiments, no statistically significant difference was observed between the level of IFN-γ transcripts measured in draining lymph nodes of BALB/c mice depleted or not of $CD25^+$ cells 16 h after infection with *L. major* (data not shown).

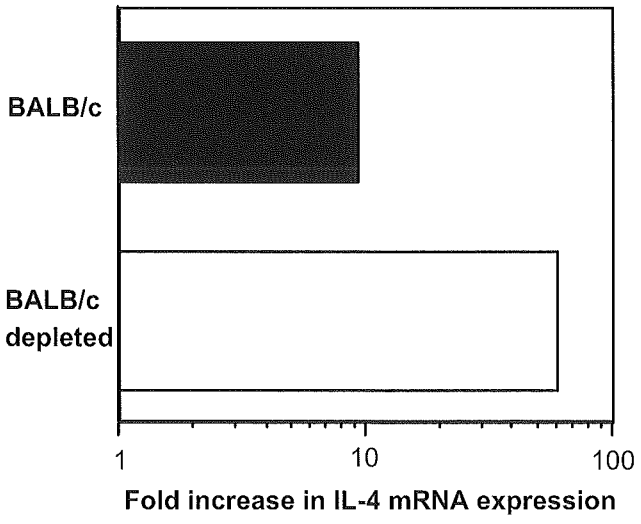

Figure 7. Depletion of $CD4^+CD25^+$ T cells in BALB/c mice enhances the early IL-4 burst observed 1 day after infection with *L. major*. BALB/c mice were treated with 1 mg PC61 i.p. and infected s.c. with $3x10^6$ stationary phase *L. major* promastigotes 72 h later. Control groups consisted of noninfected mice treated with PC61, and mice infected without any prior treatment. Mice (four to five per group) were sacrificed 16 h after infection and mRNA was extracted from popliteal lymph nodes. The relative level of IL-4 mRNA was determined by semiquantitative RT-PCR (as described in *Materials and Methods*). For mice injected with the PC61 mAb, results are expressed as the increase in IL-4 mRNA in popliteal lymph nodes of mice treated with PC61 and infected with *L. major* compared with that measured in noninfected mice similarly treated with the mAb. For untreated mice, the results are expressed as fold increase in IL-4 mRNA in mice infected with *L. major* compared with that in noninfected mice. Results are representative of three independent experiments.

3.2.3 *Demonstration of the inhibitory role of $CD4^+CD25^+$ regulatory cells on disease progression and Th2 cell development using an adoptive cell transfer system.*

In addition to depleting cells from the minor $CD4^+CD25^+$ regulatory subset, treatment of mice with the anti-CD25 mAb PC61 could also lead to depletion of other $CD4^+$ T cells induced to express CD25 following activation by *L. major* Ags. To circumvent this potential problem, we directly tested the regulatory potential of $CD4^+CD25^+$ cells using an adoptive cell transfer system originally described by Mitchell *et al.*[3] and subsequently by others.[26 236 237] Reconstitution of syngeneic *nu/nu* or SCID mice with 10^7 spleen cells from naive BALB/c mice was demonstrated to render these otherwise highly susceptible immunocompromised mice resistant to infection with *L. major*. These reconstituted SCID mice were shown to develop polarized Th1 response to infection. Conversely, reconstitution of SCID mice with 10^8 spleen cells from naive BALB/c mice was shown to restore the susceptible phenotype characterized by the development of unhealing lesions. These reconstituted SCID mice developed polarized Th2 differentiation following infection with *L. major*.

Therefore, to study the role of $CD4^+CD25^+$ regulatory cells in Th cell maturation following infection with *L. major*, SCID mice were adoptively transferred with either 10^7 spleen cells obtained from normal naive BALB/c mice, or 10^7 spleen cells depleted in $CD25^+$ cells by MACS sorting. Five days later, all mice were infected with $3x10^6$ *L. major* into the hind footpad and the development of lesions was monitored. Results in Fig. 8A confirm that SCID mice reconstituted with 10^7 naive BALB/c spleen cells are capable of controlling infection. In contrast, mice reconstituted with spleen cells depleted in $CD25^+$ cells developed progressive lesions (Fig. 8A). Estimation of the numbers of viable parasites in lesions by limiting dilution analysis substantiated these findings because 10 wk after infection, the parasite burden was 3–5 log higher in lesions of SCID mice reconstituted with $CD25^+$ cell-depleted spleen cells compared with mice reconstituted with unseparated spleen cells (Fig. 8B). Susceptibility of these mice to *L. major* was correlated with ultimate Th2 cell development because after stimulation with *L. major in vitro* their lymph node cells produced elevated amounts of IL-4 and reduced amounts of IFN-γ (Fig. 8C).

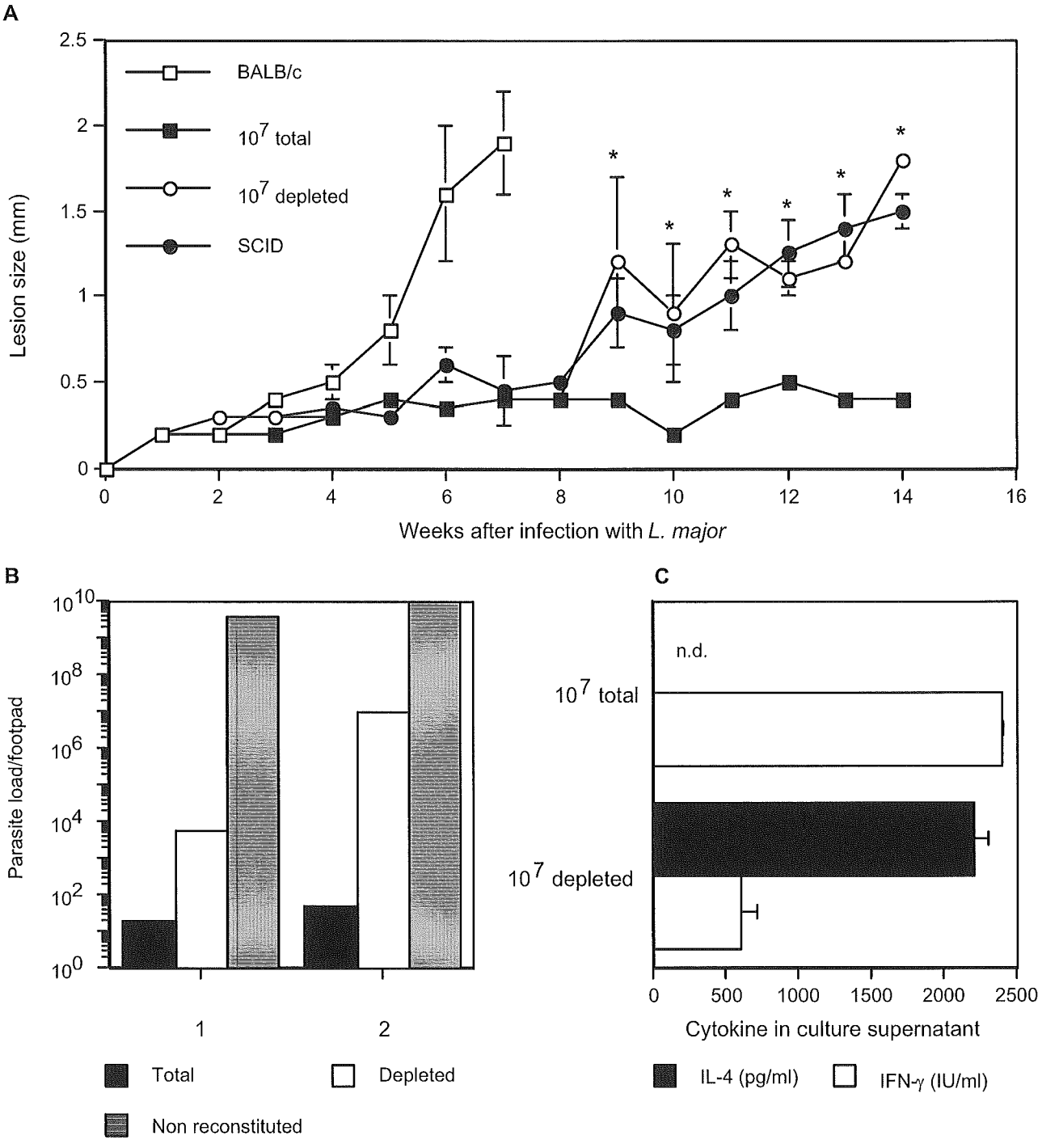

Figure 8. SCID mice reconstituted with 10^7 spleen cells from syngeneic BALB/c mice depleted of $CD4^+CD25^+$ T cells mount a polarized Th2 response to *L. major* infection and develop a progressive disease. (A) Evolution of lesion in SCID mice reconstituted with BALB/c spleen cells depleted of $CD4^+CD25^+$ T cells. SCID mice were reconstituted with 10^7 total spleen cells or 10^7 spleen cells depleted of $CD4^+CD25^+$ T cells as described in *Materials and Methods*. Nonreconstituted SCID and BALB/c mice were used as controls. Each group of mice was infected with $3x10^6$ stationary phase *L. major* promastigotes and the course of infection was monitored by measuring the diameter of the footpads weekly with a metric caliper. The mean size of lesions (increase in diameter of footpads due to infection) and SD for three to five mice per group is shown. Similar results were obtained in three other experiments including three to seven mice per group. *, $p < 0.001$ between the size of lesions in SCID mice that received spleen cells depleted vs not depleted of $CD4^+CD25^+$ regulatory T cells. (B) Parasite burden in the footpads of SCID mice reconstituted with BALB/c spleen cells depleted or not of $CD4^+CD25^+$ T cells 10 wk after infection with *L. major*. SCID mice were reconstituted with 10^7 total spleen cells or BALB/c splenocytes depleted of $CD4^+CD25^+$ regulatory T cells and infected 5 days later with $3x10^6$ stationary phase *L. major* promastigotes in the footpads. Nonreconstituted SCID mice were similarly infected as control. Ten weeks later, mice were sacrificed and the parasite burden in their footpads was determined by limiting dilution analysis as described in *Materials and Methods*. The results are the mean counts from two footpads per group in two separate experiments. (C) Cytokine production in lymph node cells from SCID mice

reconstituted with spleen cells depleted of $CD4^+CD25^+$ T cells and infected with *L. major*. Popliteal lymph node cells were isolated 10 wk after infection and $5x10^6$ cells from each group of reconstituted mice (three mice per group) were stimulated *in vitro* with 10^6 UV-irradiated *L. major* promastigotes for 72 h. IFN-γ and IL-4 production was evaluated in the supernatants as described in *Materials and Methods*. Results are expressed as mean of three measurements. The data shown are representative of three independent experiments. n.d., not detectable.

It is noteworthy that reconstituted SCID mice were infected with *L. major* 5 days after adoptive cell transfer, a time when significant repopulation of lymph nodes with donor cells had occurred. Using flow cytometry and establishing a ratio of 10^5 bacterial count microbeads to lymphocytes, an equivalent number of cells was measured in the lymph nodes and spleens of SCID mice reconstituted with $CD25^+$-depleted or undepleted spleen cells 5 days after reconstitution (data not shown).

3.2.4 *Demonstration of the inhibitory role of $CD4^+CD25^+$ regulatory cells on the early IL-4 response to L. major in BALB/c mice using an adoptive cell transfer system.*

The requirement for LACK-reactive Vβ4-Vα8 $CD4^+$ T cells and the IL-4 they produce, within one day of infection with *L. major* for subsequent Th2 cell development and expression of a susceptible phenotype in BALB/c mice, has been demonstrated.[114 121] Therefore, experiments were designed to determine whether the susceptible phenotype of mice reconstituted with $CD25^+$ cell-depleted spleen cells and the resistant phenotype of SCID mice reconstituted with 10^7 BALB/c spleen cells were correlated with the expression of an early IL-4 mRNA burst in response to *L. major* or lack thereof, respectively. Five days after reconstitution with either 10^7 total or CD25-depleted spleen cells, SCID mice were infected with $3x10^6$ *L. major* and 16 h later IL-4 transcripts were quantitated in their draining lymph node cells by RT-PCR. The lymph nodes of SCID mice that received CD25-depleted BALB/c spleen cells showed a rapid increase in IL-4 mRNA similar in magnitude to that observed in BALB/c mice simultaneously infected with *L. major*. In contrast, no increase in IL-4 mRNA expression was observed in lymph node cells of mice that received an equivalent number of BALB/c spleen cells not depleted in $CD4^+CD25^+$ regulatory T cells (Fig. 9). These results show that in this model system, as in BALB/c mice, an early IL-4 burst precedes Th2 cell maturation. Experiments aimed at identifying the cellular origin of the rapid IL-4 mRNA burst in SCID mice reconstituted with $CD25^+$ cell-depleted spleen cells have revealed that it occurred in $CD4^+$ T cells that express the Vβ4 TCR chain (data not shown).

Because use of anti-CD25 mAb is likely depleting all $CD25^+$ T cells including the small percentage (<2%) of $CD4^-CD25^+$ T cells, experiments were performed to directly assess the suppressive role of $CD4^+CD25^+$ T cells on the early IL-4 mRNA burst. FACS-sorted $CD4^+CD25^+$ T cells ($2.8x10^5$) isolated from BALB/c spleen were added to spleen cells depleted of $CD25^+$ T cells by MACS and 10^7 cells were injected i.v. into SCID mice. Five days later, mice were injected with $3x10^6$ *L. major* promastigotes and IL-4 mRNA was measured in their draining lymph nodes 16 h later. No significant increase in IL-4 mRNA was detectable in the draining lymph nodes of these reconstituted SCID mice when compared with similarly reconstituted uninfected SCID mice. However, SCID mice that received 10^7 CD25-depleted spleen cells with no inclusion of $CD4^+CD25^+$ cells showed an increase of 38 times in IL-4 mRNA as compared with similarly reconstituted SCID mice that were not infected (data not shown). These results show that $CD4^+CD25^+$ T cells are indeed regulating the early IL-4 mRNA burst observed 16 h after parasite inoculation.

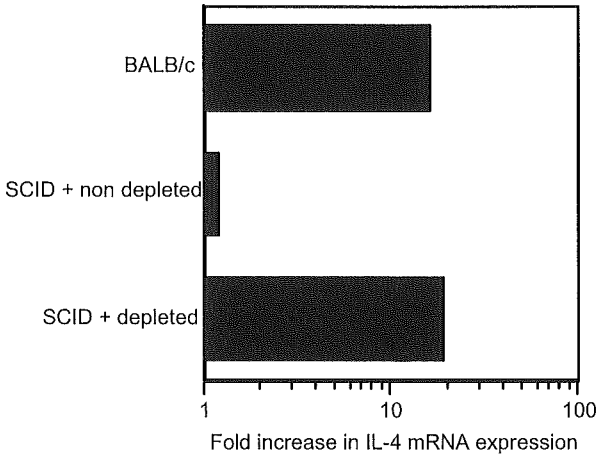

Figure 9. Draining lymph node cells from SCID mice reconstituted with BALB/c spleen cells depleted of $CD4^+CD25^+$ T cell and infected with *L. major* show an early IL-4 burst similar to that observed in BALB/c mice 16 h after infection. SCID mice were reconstituted with 10^7 BALB/c splenocytes depleted or not of $CD4^+CD25^+$ T cells *in vitro* and infected or not with $3x10^6$ stationary phase *L. major* promastigotes in the footpad. Four mice per group were sacrificed 16 h after infection and mRNA was extracted from their popliteal lymph nodes. The relative levels of IL-4 mRNA were determined by semiquantitative RT-PCR (*Materials and Methods*). Results are expressed as fold increase relative to the noninfected similarly treated control mice. This is a representative experiment of five.

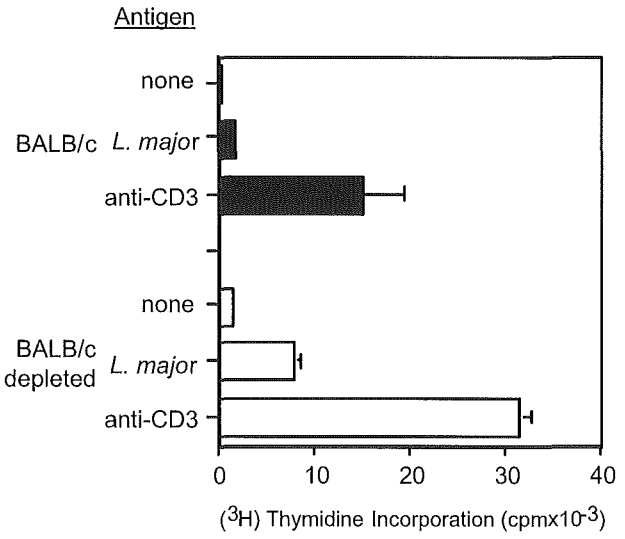

Figure 10. Absence of $CD4^+CD25^+$ regulatory T cells leads to enhanced proliferation of BALB/c lymph node cells in response to antigenic restimulation *in vitro*. BALB/c and BALB/c mice depleted of $CD4^+CD25^+$ T cells by i.p. administration of 1 mg of PC61 mAb 72 h before infection were injected with $3x10^6$ stationary phase *L. major* promastigotes s.c. in the hind footpads (four mice per group). Draining lymph node cells were removed at 15 days after infection and $5x10^5$ cells were stimulated with 0.5 μg anti-CD3 or $2x10^5$ UV-irradiated *L. major* for 48 or 72 h, respectively. [^{3}H]Thymidine was added for the last 6 h of culture. Shown are the mean and SD of scintillation cpm for triplicate cultures. Data are representative of two separate experiments.

3.2.5 Depletion of $CD4^+CD25^+$ cells leads to enhanced proliferation of $CD4^+$ T cells following infection of mice with L. major.

Once activated, $CD4^+CD25^+$ cells are capable of inducing cell cycle arrest of activated $CD4^+$ T cells in an Ag-nonspecific manner.[238 239] In most of our experiments with BALB/c mice, the total lymphocyte count from the popliteal lymph nodes of $CD25^+$ depleted mice was higher than that from nondepleted mice for the same duration of infection (data not shown). Therefore, we tested whether draining lymph node cells from CD25-depleted and nondepleted BALB/c mice infected with *L. major* differed in their proliferative capacity to specific stimulation *in vitro*. As shown in Fig. 10, cells from CD25-depleted mice showed a higher rate of thymidine incorporation in response to *L. major* or anti-CD3 in culture. This was also observed in nonrestimulated cells as a higher background. Surprisingly, this difference was still apparent 5 wk after infection, at least for lymph node cells stimulated with anti-CD3, suggesting that the state of activation of $CD4^+$ cells in the periphery has been altered by the absence of the $CD4^+CD25^+$ T cells *in vivo* during the initial infection with *L. major*.

No preferential expansion of $V\beta4^+$ cells was found when cultured cells were stained and tested by flow cytometry (data not shown), suggesting that the state of activation rather than the preferential expansion of Vβ subsets leads to the increased proliferation observed in lymph node cell cultures from mice depleted of $CD4^+CD25^+$ T cells.

Upon stimulation with *L. major in vitro*, the proliferative response of lymph node cells obtained 10 wk after infection with *L. major* from SCID mice reconstituted with 10^7 $CD25^+$ T cell-depleted splenocytes was clearly higher than that observed in lymph node cells of similarly infected SCID mice reconstituted with the same number of total spleen cells (Fig. 11).

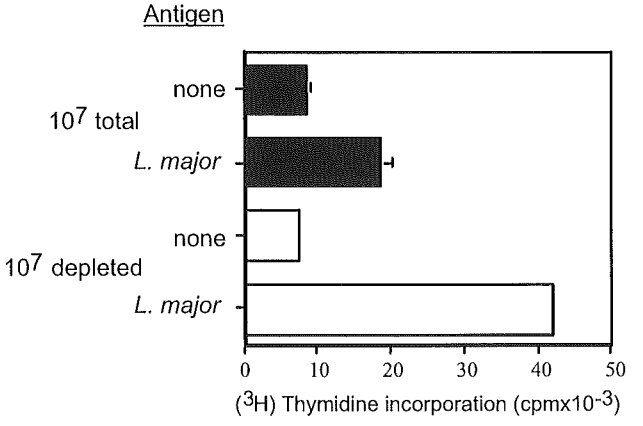

Figure 11. Increased *in vitro* proliferative response to *L. major* stimulated lymph node cells isolated from infected SCID mice reconstituted with $CD4^+CD25^+$-depleted spleen cells. Thymidine incorporation of cells from SCID mice reconstituted with spleen cells depleted or not of $CD4^+CD25^+$ T cells and subsequently infected with *L. major*. Draining lymph node cells were removed 10 wk after infection and $5x10^5$ cells were restimulated *in vitro* with $2x10^5$ UV-irradiated *L. major* for 72 h. [^{3}H]Thymidine was added for the last 6 h of culture. Shown are the mean and SD of scintillation cpm for triplicate cultures. Data are representative of two separate experiments.

3.2.6 *Neutralization of IL-4 prevents Th2 cell maturation and susceptibility to L. major in SCID mice reconstituted with 10^7 BALB/c spleen cells depleted of $CD4^+CD25^+$ T cells.*

Treatment of BALB/c mice with anti-IL-4 mAb at the initiation of infection is capable of redirecting protective Th1 cell development resulting in resistance to *L. major*.[25 105 106] Similarly to BALB/c mice, infection with *L. major* of SCID mice reconstituted with 10^7 BALB/c spleen depleted of the $CD4^+CD25^+$ regulatory T cell population results in a rapid burst of IL-4 mRNA expression in draining lymph node cells (Fig. 9). Experiments were then designed to determine whether the Th2 response developing in these mice was also instructed by the IL-4 produced as a result of this early IL-4 mRNA burst. SCID mice reconstituted with 10^7 BALB/c spleen cells depleted of $CD25^+$ cells were infected with $3x10^6$ *L. major* and treated or not with 1 mg of anti-IL-4 mAb 11B11 at the onset of infection. Control groups included similarly infected nonreconstituted SCID mice, SCID mice reconstituted with 10^7 unseparated spleen cells, and BALB/c mice treated or not with anti-IL-4 mAb. Monitoring the development of lesions in mice from these various groups clearly showed that, similarly to BALB/c mice, treatment with anti-IL-4 renders SCID mice reconstituted with 10^7 spleen cells devoid of $CD25^+$ T cells fully resistant to *L. major* (Fig. 12A). Furthermore, the numbers of parasites recovered after culture *in vitro* of footpad tissues, removed 12 wk after infection, confirmed that parasite growth was controlled in these mice ($2x10^3$ *L. major*/footpad lesion vs $3x10^8$ in control mice not treated with anti-IL-4 mAb). Resistance to infection was correlated with the ultimate development of Th1 responses 12 wk after infection. Results in Fig. 12B show that SCID mice reconstituted with spleen cells free of $CD25^+$ cells and treated with anti-IL-4 mAb exhibited a >10-fold decrease in the amounts of IL-4 transcripts in their draining lymph node lymphocytes compared with similarly infected SCID mice not treated with anti-IL-4 mAb. As previously observed, it is noteworthy that the responses of the mice from the various groups could not be discriminated on the basis of the amounts of IFN-γ transcripts. Comparable results were obtained when supernatants of specifically activated lymph node cells were analyzed for the accumulation of IL-4 or IFN-γ (data not shown).

Importantly, the anti-IL-4 treatment did not affect the proliferation rate of transferred $CD4^+$ T cells in reconstituted SCID mice as assessed by determining the CFSE profile in draining lymph node cells 15 days after transfer of CFSE-labeled spleen cells, i.e., 10 days after infection with *L. major* (data not shown).

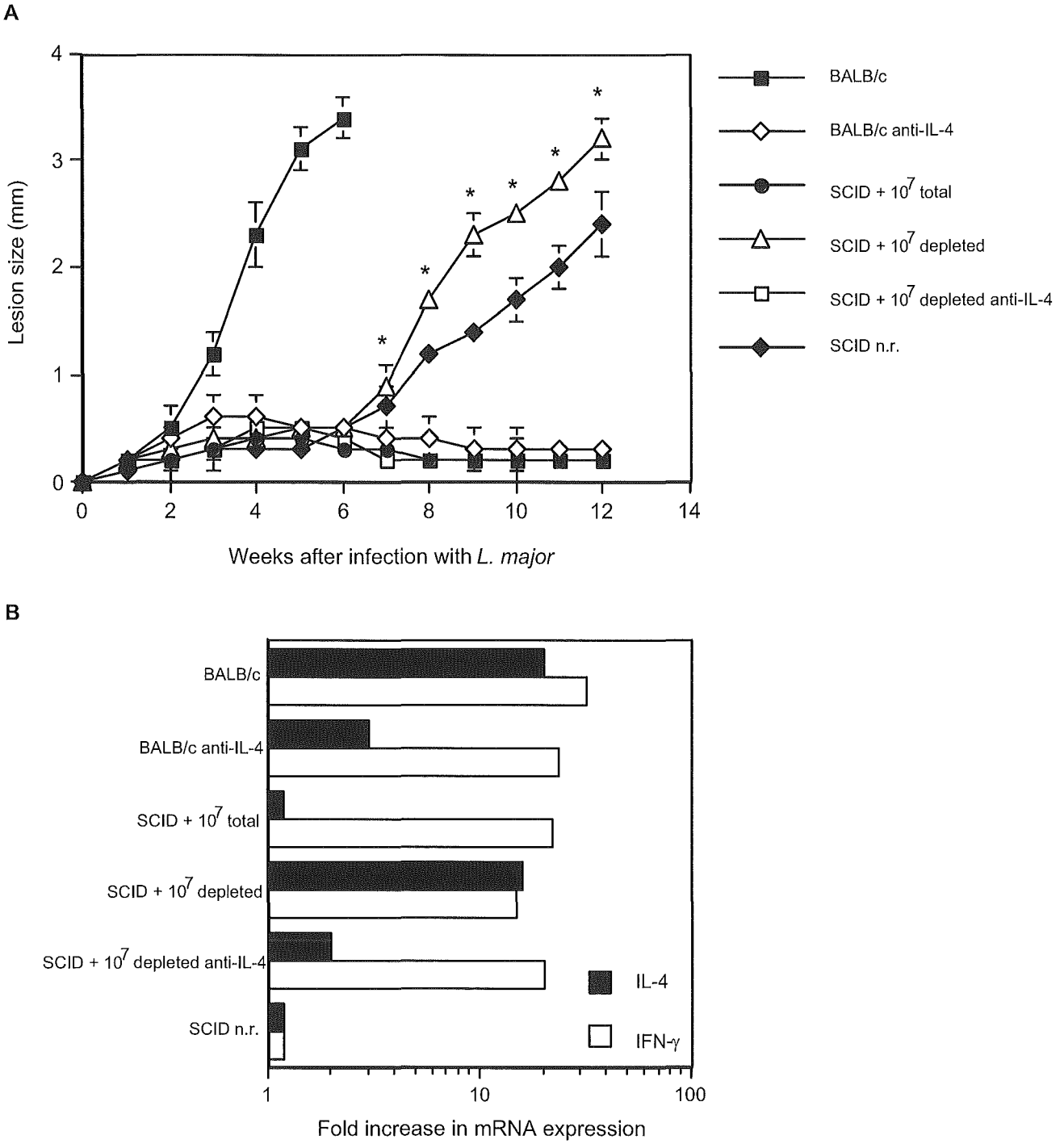

Figure 12. Neutralization of IL-4 abrogates the Th2 response of SCID mice reconstituted with $CD4^+CD25^+$ T cell-depleted splenocytes following *L. major* infection and leads to control of lesions. (A) SCID mice were reconstituted with 10^7 spleen cells depleted or not of $CD4^+CD25^+$ regulatory T cells and infected with $3x10^6$ *L. major* promastigotes 5 days later. A group of mice reconstituted with depleted cells was treated with 1 mg of anti-IL-4 mAb i.p. at the time of infection. Lesion size was monitored with a metric caliper. Data are from five mice per group. n.r., SCID mice nonreconstituted. *, $p < 0.00001$ between SCID + 10^7 depleted and SCID + 10^7 depleted anti IL-4. (B) mRNA was isolated from the lymph nodes of mice 12 wk after infection with *L. major*. cDNA was prepared from each group, and semiquantitative PCR was performed for IL-4 and IFN-γ as described in *Material and Methods*. Results are expressed as fold increase in mRNA of mice infected with *L. major* compared with mice similarly treated but not infected. n.r., SCID mice nonreconstituted.

3.2.7 *The early IL-2 response to L. major is significantly enhanced in BALB/c mice depleted of $CD4^+CD25^+$ cells.*

The suppressive capacity of the $CD4^+CD25^+$ population likely results, at least in part, from its ability to inhibit IL-2 transcription and IL-2 production in the target T cell population.[240] Interestingly, we have previously documented that triggering of the early IL-4 response subject to the control of regulatory $CD4^+CD25^+$ T cells required autocrine IL-2.

To investigate whether or not $CD4^+CD25^+$ regulatory T cells control the early IL-2 mRNA response to *L. major*, BALB/c mice treated or not with 1 mg of anti-CD25 PC61 mAb were injected s.c., 3 days later, with *L. major* into the hind footpads. Draining popliteal lymph nodes were removed at various times after infection for RNA extraction and IL-2 or IL-4 mRNA levels were quantified by semiquantitative RT-PCR. The results in Fig. 13 confirm the kinetics of IL-2 and IL-4 mRNA expression in response to *L. major* in BALB/c mice, with a early burst of IL-2 transcription reproducibly preceded the IL-4 mRNA burst. Compared with infected mice not treated with PC61 mAb, higher levels of early IL-2 mRNA transcripts were consistently observed in lymph nodes of $CD4^+CD25^+$ T cell-depleted mice (Fig. 13B). The significant increase in the level of IL-4 mRNA measured in PC61-treated mice compared with that measured in BALB/c mice that were not injected with the mAb confirmed previous results (Fig. 13A). It is noteworthy that in the absence of infection with *L. major*, treatment of BALB/c mice with the mAb PC61 did not affect the basal level of IL-2 and IL-4 mRNA transcripts.

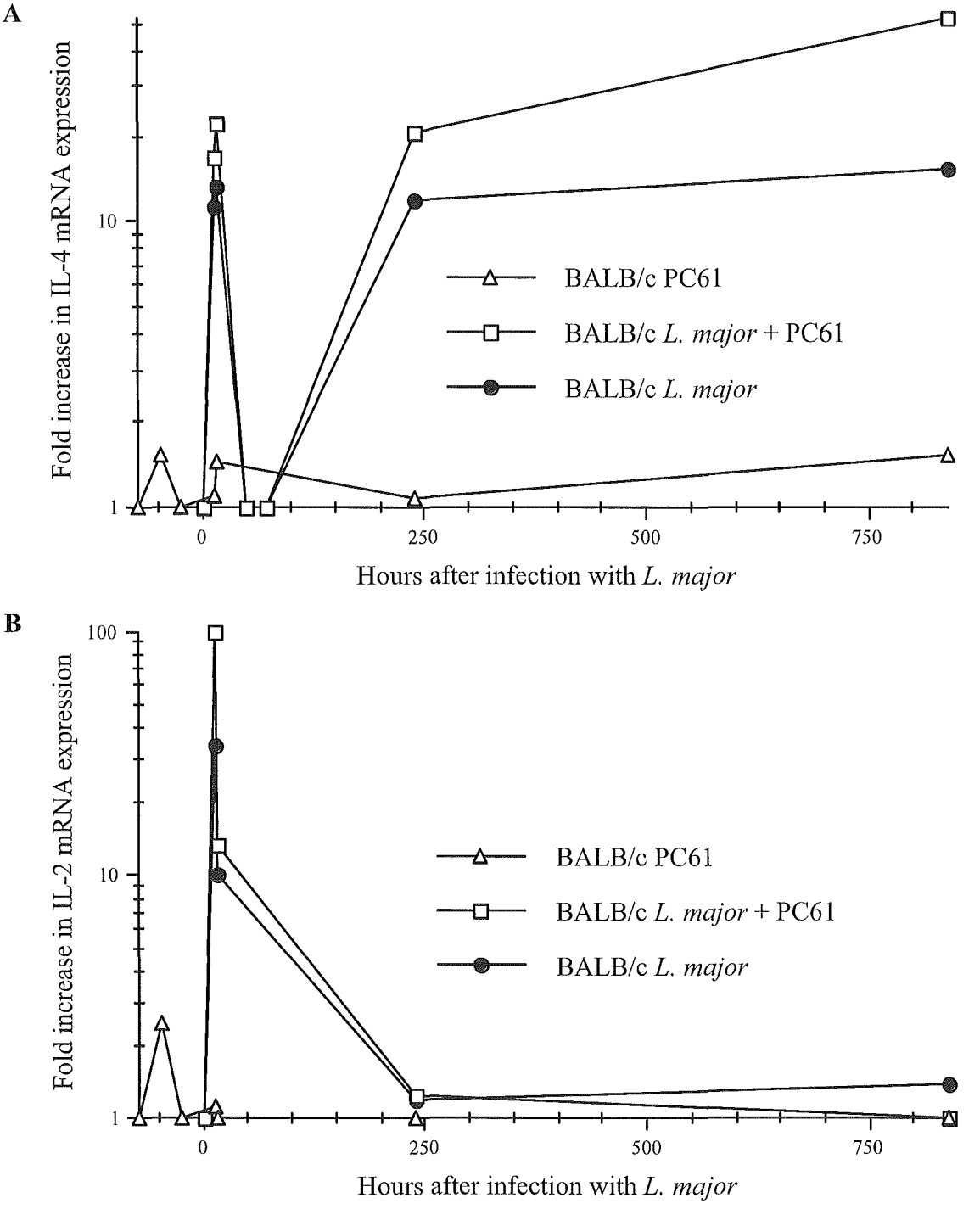

Figure 13. Depletion of $CD4^+CD25^+$ T cells in BALB/c mice enhances the early IL-2 burst observed 1 day after infection with *L. major*. BALB/c mice were treated with 1 mg PC61 i.p. and infected s.c. with $3x10^6$ stationary phase *L. major* promastigotes 72 h later. Control groups consisted of noninfected mice treated with PC61, mice infected without any prior treatment and noninfected untreated mice. Various times following infection mice were killed and mRNA was extracted from popliteal lymph nodes. The relative level of IL-4 (A) and IL-2 (B) mRNA was determined by semiquantitative RT-PCR. For mice injected with the PC61 mAb and infected, results are expressed as the increase in IL-2 and IL-4 mRNA in popliteal lymph nodes of mice treated with PC61 and infected with *L. major* compared with that measured in noninfected mice similarly treated with the mAb. For mice only injected with the PC61 mAb, the results are expressed as fold increase in IL-2 and IL-4 mRNA in mice treated with PC61 and compared with untreated and noninfected mice. For untreated mice, the results are expressed as fold increase in IL-2 and IL-4 mRNA in mice infected with *L. major* compared with that in noninfected mice.

Discussion

4.1 LACK-reactive CD4$^+$ T cells require autocrine IL-2 to mediate susceptibility to *Leishmania major*.

In this report, we document a burst of IL-2 mRNA expression peaking in draining lymph node cells of BALB/c, C57BL/6 and anti-IFN-γ-treated C57BL/6 mice around 10 h after infection with *L. major*. This rapid IL-2 production appears necessary for expression of the burst of IL-4 transcripts that occurs 16 h after infection and plays a role in Th2 cell development and susceptibility to *L. major* in BALB/c and anti-IFN-γ-treated C57BL/6 mice.[80 104] Indeed, neutralisation of IL-2 redirected Th1 cell maturation and resistance to *L. major* in otherwise susceptible mice through interference with the generation of an early IL-4 response. That only the IL-2 produced by LACK-reactive Vβ4-Vα8 CD4$^+$ T cells is essential for IL-4 production by the same subpopulation of cells represents a prominent finding of this study.

The mechanisms by which IL-2 favours disease progression are not known. Particularly, it could be that the effect of IL-2 in this model system results from the ability of this cytokine to promote T cell growth or to stimulate IL-4 production by CD4$^+$ T cells. Our present data strongly suggest that the main function of the early IL-2 produced following infection with *L. major* is to allow the initial IL-4 production to exceed the threshold required for Th2 lineage commitment.

The IL-2 dependence of the early IL-4 response by LACK-reactive Vβ4-Vα8 CD4$^+$ T cells in susceptible mice could also proceed from an IL-2-driven enhanced proliferation and survival of these cells. This hypothesis seems unlikely given the rapidity of IL-4 production following infection with *L. major* or injection of LACK[104] and our unpublished observation that CFSE-labelled Vβ4-Vα8 CD4$^+$ T cells from ABLE mice adoptively transferred to either normal or anti-IL-2-treated BALB/c mice proliferated similarly following injection of recipient mice with *L. major* or LACK. The IL-2 requirement for IL-4 production by primed or naive CD4$^+$ T cells using other experimental systems was also not correlated with preferential proliferation or survival of IL-4-producing cells.[85 158] Remarkably, in other systems, the effect of IL-2 on Th2 cell development was recently related to its capacity to stabilize the accessibility of the IL-4 gene.[224]

The expression of an early IL-4 response in BALB/c mice infected with *L. major* unlikely results from an excessive production of IL-2, since a similar burst of early IL-2 mRNA expression is also observed in draining lymph nodes of infected resistant C57BL/6 mice (Fig. 1). The fact that the IL-2 produced in C57BL/6 mice did not induce IL-4 expression in LACK-specific Vβ4-Vα8 $CD4^+$ T cells could result from differences in responsiveness of these cells to IL-2 between resistant and susceptible mice.[241] This hypothesis appears unlikely since it implies that neutralisation of IFN-γ in C57BL/6 mice increases the IL-2 responsiveness of $CD4^+$ T cells.

The observation that up-regulation of IL-4 expression by LACK-reactive Vβ4-Vα8 $CD4^+$ T cells depends upon the cellular source of IL-2, which differs between BALB/c and C57BL/6 mice, is the prominent finding of our study. Following infection with *L. major*, $CD4^+$ T cells, presumably expressing a large repertoire of Vβ TCR chains ($V\beta4^+$ and $V\beta4^-$), rapidly expressed IL-2 transcripts in susceptible BALB/c or anti-IFN-γ-treated C57BL/6 mice as well as in resistant C57BL/6 mice. Remarkably, the injection of LACK induced an early IL-2 response in susceptible BALB/c and anti-IFN-γ-treated C57BL/6 mice but failed to induce IL-2 mRNA expression in $V\beta4^+$ $CD4^+$ T cells in resistant C57BL/6 mice. The demonstration of a rapid IL-2 response by $V\beta4^+$ $CD4^+$ T cells in C57BL/6 mice treated with anti-IFN-γ mAb combined with previous data documenting a rapid IL-4 response to *L. major* by LACK-reactive Vβ4-Vα8 $CD4^+$ T cells in C57BL/6 mice treated with anti-IFN-γ mAb[80] indicate that autocrine production of IL-2 was required for IL-4 expression in LACK-reactive Vβ4-Vα8 $CD4^+$ T cells.

The identification of LACK-reactive Vβ4-Vα8 $CD4^+$ T cells as the source of the IL-2 required for IL-4 expression in the same cells came from experiments performed in mice from the BALB/c background. Reconstituted C.B.-17 SCID mice where the only cells genetically unable to produce IL-2 were LACK-reactive $CD4^+$ T cells failed to generate an early IL-4 response and were resistant to infection. Conversely, reconstituted SCID mice where Vβ4-Vα8 $CD4^+$ T cells are the only cells capable of IL-2 production mount an early IL-4 mRNA response, showing that the IL-2 produced by cells other than LACK-specific Vβ4-Vα8 $CD4^+$ T cells was not involved in the regulation of this early IL-4 mRNA expression after infection. Together these results strongly indicate that IL-4 production by LACK-reactive Vβ4-Vα8

$CD4^+$ T cells and manifestations of their pathogenic role in disease progression are contingent on autocrine IL-2.

The reason why only the IL-2 produced by LACK-specific $CD4^+$ T cells plays a role in the expression of IL-4 mRNA by the same Vβ4-Vα8 $CD4^+$ T cells is not known. It appears that cytokines exert their activity locally rather than at a distance.[242] In this context, it has been elegantly shown that, after activation, on the one hand the IL-2Rα chain co-polarizes with the TCR at the site of the immunological synapse[243] and, on the other hand, IL-2 secretion also occurs toward the immunological synapse.[244] Accordingly, it is possible that only autocrine production of IL-2 by LACK-specific Vβ4-Vα8 $CD4^+$ T cells would provide sufficient levels of this cytokine at the site where its receptors are concentrated.

Thus, the two types of signalling by cytokines - autocrine and paracrine - appear to coexist during the early response of susceptible mice to infection with *L. major*. First, IL-2 is produced by LACK-specific $CD4^+$ T cells and steers these cells to IL-4 production. Second, as already proposed, the IL-4 produced by LACK-specific $CD4^+$ T cells might serve to allow the spread of IL-4 production to $CD4^+$ T cells of specificities for other antigens from *L. major*.[222 245] It is of interest that the IL-4 receptors do not appear to co-localize with the TCR at the site of the immunological synapse.[243]

The nature of the cellular and molecular events upstream the induction of IL-2 production by LACK-reactive cells in susceptible mice is not known. The results presented in this report indicate that this rapid IL-2 response is down-regulated by IFN-γ in resistant C57BL/6 mice. Thus, it is possible that an early production of IFN-γ in resistant mice could account for the inability of Vβ4-Vα8 LACK-reactive cells to produce IL-2. Since cells from the innate immune system, *i.e.* NK cells, can produce IFN-γ, it would thus be possible that differences in NK cell activation and IFN-γ production following infection with *L. major* between resistant and susceptible mice explain the observed difference in the induction of the early IL-2 response. This hypothesis is rendered unlikely by recent results from our laboratory clearly showing that NK cells from resistant and susceptible mice exhibit similar increase in IFN-γ mRNA expression early following infection with *L. major* (unpublished observation).

In conclusion, the results reported here provide evidence that the IL-2 produced early during infection of susceptible mice with *L. major* by LACK-specific Vβ4-Vα8 $CD4^+$ T cells plays a crucial role in promoting Th2 maturation and disease progression. Furthermore, the inability of LACK-specific Vβ4-Vα8 $CD4^+$ T cells to express an IL-4 mRNA burst in response to *L. major* in resistant C57BL/6 mice appears to stem from the incapacity of these cells to produce IL-2.

4.2 The early IL-4 response to *Leishmania major* and the resulting Th2 cell maturation steering progressive disease in BALB/c mice are subject to the control of regulatory $CD4^+CD25^+$ T cells.

In this report, we show that compared with normal BALB/c mice, BALB/c mice depleted of $CD4^+CD25^+$ regulatory T cells three days before infection with *L. major* develop significantly more severe lesions that contain higher numbers of parasites. This enhanced disease progression was correlated with the faster development of robust IL-4-producing $CD4^+$ T cell responses. In the absence of $CD4^+CD25^+$ regulatory T cells, the early IL-4 transcriptional burst seen in BALB/c mice within 1 day of infection with *L. major* was significantly enhanced. Inhibition of the activation of MHC class II-restricted parasite-specific $CD4^+$ T cells due to the persistence of the anti-CD25 PC61 depleting mAb is unlikely to account for the observed effects. Indeed, SCID mice reconstituted with BALB/c spleen cell populations, depleted of $CD25^+$ regulatory T cells *in vitro*, also exhibited increased levels of IL-4 transcripts in the draining lymph nodes 1 day following infection with *L. major*, enhanced disease progression and Th2 cell development.

Studying the requirement for IL-2 signaling on the progression of lesions in BALB/c mice infected with *L. major*, others have reported that the biweekly administration of anti-IL-2Rα (CD25) mAb PC61 during the first 4 wk of infection renders BALB/c mice resistant.[98] In these experiments, it is likely that persistent blocking of the IL-2R and/or depletion of $CD4^+$ T cells, that are induced to express the IL-2Rα chain following specific activation, preferentially interfered with the expansion of recently activated $CD4^+$ Th2 cells because IL-2 signaling has been reported to be required for the establishment and maintenance of Th2 responses.[98 246 247] Interestingly, in this study restricting the administration of the anti-IL-2Rα PC61 mAb to the first day of infection with *L. major* either did not modify or sometimes exacerbated disease progression.[98] Because we show in this study that an interval of 3 days after the injection of PC61 mAb is required for the maximal depletion of $CD25^+$ T cells, it is likely that when the anti-CD25 mAb is administered the day of infection, the exacerbating effect on disease progression varies depending upon the numbers of $CD25^+$ regulatory T cells available. In this context, at least *in vitro*, the degree of suppression mediated by $CD4^+CD25^+$ regulatory T cells has been demonstrated to be proportional to the numbers of regulatory T cells.[238]

Even in the absence of antigenic stimulation, the results in this report show an increased proliferation and repopulation of lymph nodes by adoptively transferred $CD4^+$ T cells in syngeneic SCID recipients when the $CD4^+CD25^+$ population was removed from the spleen cell inoculum before transfer. That $CD25^+$ regulatory cells regulate the size of the peripheral lymphoid compartment is also supported by several other observations. Thus, a shortage of $CD25^+$ regulatory T cells has been reported in IL-2- or IL-2Rα-deficient mice that exhibit a dysregulation of both the size and the content of their peripheral lymphoid compartment resulting in autoimmunity.[135] [248] Similarly, the activated/memory $CD45RB^{low}CD4^+$ T cell population, containing natural regulatory T cells, was clearly shown to limit the peripheral expansion of naive $CD45RB^{high}CD4^+$ T cells when both $CD4^+$ T cell subpopulations were transferred into syngeneic Rag-$2^{0/0}$ recipients.[249] Furthermore, depletion of $CD25^+$ cells *in vivo* with the PC61 mAb led to an increased expansion of adoptively transferred C57BL/6 spleen T cells in syngeneic nude mice.[135]

In contrast to naive classical $CD4^+$ T cells that need to be activated to express CTLA-4, $CD4^+CD25^+$ regulatory cells have been shown to express CTLA-4 constitutively.[203] [205] [250] Blockade of CTLA-4 using anti-CTLA-4 mAb has been reported to decrease the suppressive capacity of $CD25^+$ regulatory T cells *in vitro*[250] and to interfere *in vivo* with the ability of these cells to control intestinal inflammation.[203] [250] It is presently not known whether the CTLA-4 molecules on $CD25^+$ regulatory T cells prevent interaction between the CD28 molecules on target cells with the B7 (CD80/CD86) molecules on APC[250] or, alternatively, cross-linking the CTLA-4 molecules on $CD25^+$ cells results in TGF-β production.[251] In this context, treatment with anti-CTLA-4 mAb has been shown to exacerbate disease progression and to lead to enhanced Th2 responses in BALB/c mice infected with *L. major*[252] effects similar to those reported in this study following depletion of $CD4^+CD25^+$ regulatory T cells. Using different antigenic systems, other studies have shown that mice expressing a transgenic TCR on a CTLA-$4^{-/-}$ background preferentially develop Th2 responses and conversely that signaling through CTLA-4 inhibits Th2 maturation.[253] Thus CTLA-4 would limit the magnitude of Th2 differentiation. Therefore, the similarities between the effects observed either in the absence of $CD4^+CD25^+$ regulatory T cells or following the blockade of CTLA-4 support the hypothesis that CTLA-4 may play a role in the suppressive activity of $CD4^+CD25^+$ regulatory T cells.

The suppressive capacity of the $CD4^+CD25^+$ population likely results, at least in part, from its ability to inhibit IL-2 transcription and IL-2 production in the target T cell population.[240] As a result, these responder cells fail to proliferate and undergo cell cycle arrest at the G_0/G_1 phase.[240] Whether this inhibition, dependent on cell contact between the regulatory and the responder cells, is mediated by other cytokines, in soluble or membrane-bound forms, is still a matter of debate (reviewed in Refs. 199 and 254). In this context, we show in this study that up to 5 wk after infection with *L. major*, the draining lymph node cells of either BALB/c mice depleted of $CD4^+CD25^+$ regulatory T cells or SCID mice reconstituted with spleen cells depleted in regulatory T cells *in vitro* exhibited significantly enhanced proliferative responses upon specific restimulation *in vitro*. Likewise, following infection with *L. major*, enhanced $CD4^+$ T cell proliferation was also observed *in vivo* in SCID recipients of CSFE-labeled $CD4^+CD25^+$-depleted BALB/c spleen cell populations. This increased proliferative capacity likely results from enhanced IL-2 production in mice lacking $CD25^+$ regulatory cells because we have detected, soon after infection with *L. major*, higher levels of IL-2 transcripts in draining lymph node cells of BALB/c mice depleted of $CD25^+$ T cells as compared with normal BALB/c mice.

Cell cycling favors cytokine gene expression. Although entry into the S phase is necessary for the expression of the IFN-γ and IL-4 genes,[227 255] there is a controversy regarding possible differences in the number of cell divisions required for naive $CD4^+$ T cells to differentiate toward either IFN-γ-producing Th1 cells or IL-4-producing Th2 cells.[256] In the absence of $CD4^+CD25^+$ regulatory T cells, results presented in this study show that donor $CD4^+$ T cells proliferate more intensively in response to *L. major in vitro*. Combined with the present demonstration of the inhibitory role of $CD4^+CD25^+$ regulatory T cells on Th2 cell maturation following infection with *L. major*, these results could indicate that the magnitude of the Th2 response is dependent upon the proliferation rate. However, more likely is that the enhanced IL-4 production by Vβ4-Vα8 $CD4^+$ T cells during the first day of infection with *L. major* observed in the absence of $CD4^+CD25^+$ regulatory T cells could account for the subsequent development of magnified Th2 responses. Such a hypothesis is strongly supported by the results in this report showing that neutralization of the IL-4 produced during the early stage of infection with *L. major* led to a significant inhibition of Th2 cell development in SCID mice reconstituted with syngeneic spleen cells depleted of $CD4^+CD25^+$ regulatory T cells without affecting the proliferation rate of the transferred $CD4^+$ T cells. Therefore, we

believe that an excessive production early after infection of the IL-4, necessary for instructing further Th2 cell development, by LACK-reactive Vβ4-Vα8 $CD4^+$ T cells is a major consequence of the depletion of $CD25^+$ regulatory T cells in this experimental system. The requirement for LACK-reactive Vβ4-Vα8 $CD4^+$ T cells and the IL-4 they produce during the first day of infection with *L. major* for subsequent Th2 cell maturation and disease progression in susceptible BALB/c mice has been firmly established.[106 119 121] The mechanism by which $CD25^+$ regulatory T cells down-regulate IL-4 production by LACK-specific Vβ4-Vα8 $CD4^+$ T cells is not known. However, given our findings that this early IL-4 response to *L. major* is regulated by autocrine IL-2 (see chapter 3.1), it is possible that a $CD25^+$ regulatory T cell-mediated inhibition of IL-2 gene transcription by LACK-specific $CD4^+$ T cells could affect IL-4 production by the same Vβ4-Vα8 $CD4^+$ T cells. The enhanced early IL-2 response to *L. major* in BALB/c mice depleted of $CD4^+CD25^+$ cells support this hypothesis.

Although anergic in terms of proliferation, $CD4^+CD25^+$ regulatory T cells have been reported to express their suppressive activity at concentrations of Ags significantly lower than those necessary for activation of other (naive) T cells.[238] In this context, results from elegant experiments indicated that the development of $CD4^+CD25^+$ regulatory T cells requires higher avidity of their TCR for MHC class II/self-peptides than other (naive) T cells.[257] The LACK Ag of *L. major* is the *Leishmania* homolog of mammalian RACK1 and there is some degree of homology between the two proteins, particularly within the region of the immunodominant $I\text{-}A^d$ epitope of LACK eliciting the rapid IL-4 response by Vβ4-Vα8 $CD4^+$ T cells in BALB/c mice.[114 116] It is thus tempting to speculate that some $CD4^+CD25^+$ regulatory T cells expressing TCR with high avidity for a peptide in mammalian RACK1 are positively selected in the thymus and readily activated in the periphery to exert suppression following injection of LACK. The mechanism by which regulatory $CD4^+CD25^+$ T cells suppress IL-4 production by LACK-reactive cells remains elusive. However, if regulatory $CD25^+$ T cells and Vβ4-Vα8 $CD4^+$ T cells recognize their specific epitope on the same APC, the possibility that activated regulatory $CD25^+$ T cells renders this APC unable to provide costimulatory signals necessary for IL-2 transcription in Vβ4-Vα8 $CD4^+$ T cells is attractive. In this context, some observations already strongly suggest that the $CD25^+$ suppressor T cell population acts on APC,[226] and suppress naive T cells by inhibition of a common pathway leading to IL-2 secretion[258] as long as both T cells are of the same antigen specificity[258-260] and are contacting the same APC.[258]

Noteworthy, depletion of $CD25^+$ T cells in C57BL/6 mice before infection with *L. major* had no significant effect on the resolution of lesion; however, the transient absence of $CD25^+$ regulatory T cells resulted in reduced parasite numbers within lesions and suppressed almost totally the parasite reservoir remaining normally in clinically cured C57BL/6 mice. $CD4^+CD25^+$ regulatory T cells have been reported to produce IL-10 *in vivo*.[197 261] Because IL-10 is a cytokine important in controlling the residual parasites in clinically cured C57BL/6 mice,[262] depletion of regulatory T cells which last for over a month, could result in loss of IL-10 and thus reduce the number of residual parasites within the lesions. In BALB/c mice, depletion of $CD4^+CD25^+$ T cells leads to the opposite phenomenon, i.e., increased parasite growth. Thus in strains of mice susceptible to infection with *L. major*, $CD4^+CD25^+$ T cells act very early on cells responsible for driving Th2 differentiation, regulating early IL-4 secretion, but in resistant strains developing Th1 responses following infection, $CD4^+CD25^+$ T cells may produce the IL-10 found necessary for the persistence of parasites in clinically cured animals.[262] Additional experiments are needed to further decipher the mechanism of action for $CD4^+CD25^+$ T cells in strains of mice resistant to infection with *L. major*.

In summary, in this study we have shown that regulatory T cells, highly efficient in controlling self-reactive effector T cells and preventing autoimmunity, are also able to restrain the development of detrimental Th2 responses to an intracellular parasite in genetically susceptible hosts. Defining the fine specificities of $CD25^+$ regulatory T cells, understanding the cellular and molecular mechanisms involved in suppression and their role in controlling pathologies induced by specific Th subsets remain important issues that deserve further studies. This knowledge could ultimately lead to the design of new strategies for manipulating the development of effector responses to the host's benefit.

5 Conclusions.

The murine model of infection with *L. major* has not only allowed to validate *in vivo* the existence of functionally distinct $CD4^+$ T cell subpopulations, but has demonstrated their crucial role on the outcome of infectious diseases. This model is now revealing itself as a powerful tool to understand the cellular and molecular mechanisms operating in the selective maturation of peripheral effector $CD4^+$ T cells *in vivo*. A thorough definition of these mechanisms is a prerequisite for the rational development of efficient immunoprophylactic and immunotherapeutic measures applicable to humans. Information gained by studying this disease, which afflicts mainly the developing world, has no doubt significantly added to our understanding of critical issues related to the T cell response. It is important that this knowledge foments the development of novel interventional strategies for the prevention and treatment of this and other serious infection diseases. In general, the results accumulated have clarified certain aspects of Th cell differentiation. Continued work in this area should also provide critical information as to how to direct immune responses to the type of effector function that would be most useful in eliminating or preventing a given type of infectious disease, in diminishing immunologic tissue damage in autoimmunity, and in mounting effective anti-tumor immunity.

6 Acknowledgements.

I thank Prof. Thierry Calandra, who have kindly accepted to evaluate this scientific work. I would like to express my gratitude to Prof. Jacques Louis for offering me the invaluable opportunity to perform my thesis in his laboratory, for his constant support to these projects, for all what I learned from him, and for his kindness and confidence throughout that period. I am grateful to Prof. Pascal Launois, Dr. Fabienne Tacchini-Cottier, Dr. Abraham Aseffa and Dr. H. Robson MacDonald for their kind help and understanding. I would like also to express my thanks to all members of the group for their friendship and help.

This work was supported by grants from the Swiss National Science Foundation, the UBS Optimus Foundation, Switzerland, the Deutsche Forschungsgemeinschaft (RO 764/8-1) and by the Institut Pasteur.

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