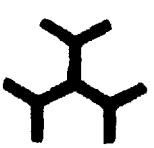
# REGULATION OF IMMUNE INFLAMMATION NETWORKS

Thesis submitted to **Jawaharlal Nehru University** in partial fulfillment of requirement for the degree of **Doctor of Philosophy**

*Varanasi Vineeth*



*Immunobiology Laboratory-II*
*National Institute of Immunology*
*New Delhi*
*India*
*2007*

राष्ट्रीय प्रतिरक्षाविज्ञान संस्थान
NATIONAL INSTITUTE OF IMMUNOLOGY

# CERTIFICATE

This is to certify that the thesis titled "**Regulation of Immune Inflammation Networks**" submitted by **Varanasi Vineeth** in partial fulfilment of the degree of **Doctor of Philosophy** from Jawaharlal Nehru University embodies the work done by the candidate under my guidance at the National Institute of Immunology, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

Satyajit Rath, MD (Supervisor)
Staff Scientist VII
National Institute of Immunology
New Delhi

अरूणा आसफ अली मार्ग, नई दिल्ली - 110067 फैक्स : 91-11-26162125, 26177626 तार : IMMUNOLOGY
Aruna Asaf Ali Marg, New Delhi - 110067 Fax : 91-11-26162125, 26177626 Grams : IMMUNOLOGY
दूरभाष (Telephones) EPABX : 26717121 - 45 & 26717010 - 19

*To*

*Amma & Nannagaru*

# Acknowledgements

*It is with great pleasure that I thank the many people who made this work possible.*

*I express my heartfelt gratitude to my guide Dr.Satyajit Rath for being an enduring and inspirational teacher. I thank my DC members Dr.Vineeta Bal, Dr.Anna George, Dr.Ayub Qadri and Dr.Balachandran Ravindran for their constructive and helpful suggestions during the course of my work.*

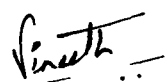
*I gratefully acknowledge the National Institute of Immunology for providing all the financial and infrastructural support. All the technical staff of the small animal facility, where I spent large periods of my working days, have been very helpful and greatly facilitated my work. I am very appreciative of all the technical staff of Immunobiology group for their help and assistance.*

*Special thanks to Shuchi and Anupriya for being ever supportive and caring. I must thank Divya, Ashutosh and Smita for making lab a very stimulating and fun place. It has been a pleasure to be in Immunobiology laboratories with Shipra, Hridesh, Priya, Hamid, TK, Bharti, Gautam, Abhishek and Ritu. I also thank all the past members of the Immunobiology group for all their help, and must apologize for not mentioning them personally.*

*I will forever be in awe of the Himalayas for keeping me sane and for the calming influence they had on me, and will always be glad they were not very far from NII.*

*I am deeply indebted to my wife, Subhashini, for being there in all the good and not so good times, and gladly sharing them both with me. Thank you for being you.*

*Lastly, and most importantly of all, I must thank my parents for their unwavering support and encouragement in all my endeavors, and keeping faith in me. This thesis would not have been possible without them.*



*Vineeth*

# Table of Contents

# Abbreviations

| | |
|---|---|
| *(-/-)* | *knockout* |
| *+* | *positive* |
| *%* | *percent* |
| *-* | *negative* |
| *α* | *alpha* |
| *β* | *beta* |
| *γ* | *gamma* |
| *μg/ml* | *microgram per milliliter* |
| *μl* | *microlitre* |
| *μM* | *micromolar* |
| *$^0$C* | *degree celcius* |
| *APC* | *antigen presenting cell* |
| *BMDC* | *Bone marrow derived-dendritic cell* |
| *bp* | *base pair* |
| *BTK* | *Bruton's tyrosine kinase* |
| *CD* | *cluster of differentiation* |
| *DC* | *dendritic cell* |
| *DLC* | *differential leukocyte count* |
| *DNA* | *deoxyribose nucleic acid* |
| *ELISA* | *enzyme-linked immunosorbent assay* |
| *FCS* | *fetal calf serum* |
| *Fig.* | *figure* |
| *FITC* | *fluorescein isothiocyanate* |
| *g/l* | *gram per litre* |
| *h* | *hours* |
| *HRP* | *Horse Radish Peroxidase* |
| *Ig* | *immunoglobulin* |
| *IL* | *interleukin* |
| *iNOS* | *inducible nitric oxide synthase* |

| | |
|---|---|
| *TLC* | *total leukocyte count* |
| *TNF* | *tumor necrosis factor* |
| *Vβ* | *variable chain-β* |
| *WT* | *wildtype* |
| *XID* | *X-linked immunodeficiency* |
| *XLA* | *X-linked agammaglobulinemia* |

# *Introduction*

Disease susceptibility is determined by the complex interplay between host genetic factors and environmental factors. Many inherited host susceptibility factors have been observed to show association with disease phenotypes, such that individuals within a population cohort respond differently to a specific infection. Differences in susceptibility to disease can also be observed at the level of populations, such that marked differences in disease susceptibility are seen between population groups of different ethnicities residing in the same geographical region (Modiano et al., 1996).

Among the many approaches to identifying disease susceptibility loci, have been the identification of disease predisposition against specific infectious agents in individuals with rare monogenic mendelian conditions (Fischer, 2001). Many of these rare mendelian disorders involve loci that are core regulators of the immune response mediating multiple overlapping functions across many cell lineages of the immune system leading to clinical disorders that are characterized by generalized immunodeficiency. Therefore, majority of the monogenic susceptibility loci regulate protection against a wide spectrum of infectious diseases and explain immunodeficiency disorders rather than individual susceptibilities to specific infections.

Family studies of such mendelian disorders have identified associations of mycobacterial infection susceptibility to mutations in the interferon-γ receptor 1 (IFN-γR1) and with the interleukin-12 (IL-12) deficiency or a mutation in the IL-12 receptor gene. Further, single locus susceptibility to mycobacterial infections have also been identified in family based studies. Such studies offer insights into the molecules, in this case IFN-γ or IL-12, that are crucial for immunity against specific pathogens. Still, it is unlikely for such

highly penetrant mutations to survive evolutionary selection pressure and to account for differences in disease susceptibility in the general population. More common alleles of these susceptibility loci, with subtler effects on gene function, might effectively explain the variation that underlies differences between individuals in susceptibility to major pathogens.

It is also precisely due to this multigenic and heterogeneous distribution of susceptibility loci that human studies on disease gene identification has proved difficult to achieve. Conversely, major gene effects can be identified in mouse models of infection, where effective genetic control can be exercised, allowing the use of mutant mice to identify host genes contributing to susceptibility to infections (Bedell et al., 1997a; Bedell et al., 1997b). Some genes critical for protection against individual infectious agents have also been identified by mouse studies and when applied to human populations, show consistent polymorphism in suffering individuals (Carrington et al., 1999; Casanova and Abel, 2004; Fortier et al., 2005; Mira et al., 2004; Pan et al., 2005).

One such extensively studied polymorphism involves an inactivating mutation of the gene encoding Bruton's tyrosine kinase (BTK) causing X-linked immunodeficiency (XID) in mice and X-linked agammaglobulinemia (XLA) in humans. Most of the effects related to a lack of functioning BTK in the XID mouse model, however, have been subtle and quantitative in nature unlike the human disease counterpart. *Btk* is a member of the *Btk/Tec* family of non-receptor tyrosine kinases and a point mutation in any subdomain of *Btk* can result in XLA, characterized by a block in B-cell development at the pre-B cell stage, resulting in a severe deficit of B-cells and serum immunoglobulin (Ig). Both a

naturally occurring point mutation in *Btk*, which results in murine X-linked immunodeficiency (XID), and the inactivation of the murine *Btk* gene result in a phenotype that is significantly milder than XLA. There is no simple correlation between the type of mutation and the severity of disease. Further, XID mice with simultaneous depletion of T cells or inactivation of CD40 or TEC result in increased disease severity mimicking that seen in human XLA, invoking the likelihood that the XLA phenotype is dependent on genetic context based interactions between the B and T cell lineage. All the above studies however involve modulations in molecules that have multiple roles across cell lineages. Therefore, in the present study, a model of complete T cell ablation induced by introducing a TCR β chain deficiency in the XID mice was studied to prove the T cell intrinsic nature of the phenotype rescue observed in XID.

In myeloid cells, BTK functions as a key signaling molecule engaged on receptor activation of various surface receptors like cytokine receptors, death receptors, and Toll-like receptors (TLRs), all of which have an important role in regulating the inflammatory immune response executed by myeloid cells, both in mice and humans. Previous work has demonstrated that *Btk* deficient XID myeloid cells, macrophages and neutrophils, showed compromised generation of effector molecules and XID macrophages displayed poorer induction of the Rel family of NF-κB transcription factors (Mangla et al., 2004; Mukhopadhyay et al., 2002). With this background, the role for *Btk* in modulating eosinophil effector functions was studied. Since many of the NF-κB transcription factors are known to have functionally redundant roles, the relative significance of the deficient induction of c-Rel, a Rel family NF-κB transcription factor, on effector functions in XID macrophages was further defined using a c-Rel$^{-/-}$ mouse model.

Engulfed pathogens are targeted to the endo-lysosomal compartments to undergo degradation and processing into peptide fragments for presentation on major histocompatibility complex II (MHCII) molecules. Intracellular trafficking of these vesicles is regulated by association with small molecular GTPases of the Rab family. LYST, a lysosomal transport protein of unknown function, has also been associated with regulating this process of lysosomal maturation. Mutation in the *Lyst* gene is associated with an immunodeficiency syndrome, Chediak-Higashi Syndrome (CHS), associated with defects in both myeloid effector and antigen presentation functions (Ward et al., 2000). The murine model for the *Lyst* mutation, the bg/bg mouse also displays a similar phenotype. Compromised neutrophil functions exhibited in *Lyst* mutant bg/bg mice have been shown to result in recurrent bacterial infections (Takeuchi et al., 1986). Given the lysosomal function defects, the *Lyst* mutant macrophages would be expected to be poorer at clearing intracellular infections. The functional consequence of the *Lyst* mutation in the bg/bg mice on clinical course of leishmaniasis, caused by obligate intracellular parasites of the *Leishmania* species was analyzed using the model system of *Leishmania major* (Lm) induced cutaneous leishmanial infection.

Multiple genes have been implicated in familial clustering of many chronic human diseases, and while these polygenic factors are essential, they are by no means sufficient to induce disease (Tisch and McDevitt, 1996). Environmental factors, particularly viral infections, have been shown to be involved in disease induction or progression (Singh et al., 1998). Both the mouse and human genomes carry endogenous genome-integrated retroviruses. These retroviruses represent the germline integrants of exogenous retroviruses, which subsequent to inactivating mutations remain fixed in the genome.

These germline integrants may have been preserved in the genome for their ability to provide the host with new functions, such as protection from exogenous viruses and fusogenic activity (Goff, 1996; Stoye and Coffin, 2000). Many of these proviral insertions code for superantigen proteins (SAgs) that associate MHCII molecules (15) and stimulate T cells bearing particular T cell receptor V-beta (TCRVβ) segments leading to activation and/or deletion of these T cells (Janeway, 1991). Human endogenous retroviruses (HERVs) have been shown to be associated with a number of autoimmune states (Bengtsson et al., 1996; Clausen, 2003; Nakagawa et al., 1997), while endogenous murine tumor viruses (MTVs) are reported to mediate susceptibility to both infections (Bhadra et al., 2006; Gorgette et al., 2002) and tumors (Schirrmacher et al., 1998). While most of the proviral integrants are transcriptionally inactive, studies in both mice and humans have shown reconstitution of transcriptional activity in the presence of subsequent active viral infections. Transcriptional activity of an endogenous integrant HERV-K18 has been shown to be reconstituted on viral infection (Stauffer et al., 2001; Sutkowski et al., 2001). Similarly, surrogate retroviral sequences have been shown to be competent at restoring replication competency of dormant MTVs in mice (Lee and Bieniasz, 2007). Reactivation of these dormant sequences could impact immune responses by modulating the activity of gene sequences near the viral integration site. While the mechanisms involved in these associations have not been elucidated as yet, they appear not to be restricted to the T cell deleting functions of the encoded SAgs. With this background, the effect of MTV integrations on leishmanial disease progression was examined in two closely related genetically resistant strains of mice.

Developments in modern genetics and genomics have contributed to our understanding of the pathogenic processes that underlie major infectious diseases by allowing a more systematic study of the genetic influences. Genetic analyses of the kind utilized in the present study, using comprehensive knowledge of mammalian biology can enable new insights in understanding the interplay between the complex functional and regulatory processes involved in determining susceptibility to disease states.

# Review of Literature

# *Review of literature*

The evolutionary design of the vertebrate immune system has been shaped by constant and intimate interactions with microbial flora. Microbe-vertebrate host interactions are dynamic, with both the host and the microbe bringing their repertoire of survival mechanisms to bear. The eventual outcome can be deleterious for either the host or the microbe, as happens in acute infections, or result in an apparent steady-state co-existence for long periods of time, as happens in chronic infectious diseases. Even in such an apparent compromise, the interaction can lead to disease at times when the host immune status is modified.

In order to deal with a wide variety of potential microbial interactions, the immune system uses two lines of defense. The first line of defense consists of cells of the myeloid lineage, which acts rapidly on contact with pathogen and depends on broad based pathogen pattern recognition. This then alerts and activates the highly specialized lymphoid response, which depends on specific target-based pathogen recognition resulting in a highly specific response, with eventual preservation of pathogen recognition in the form of a long-lasting memory response. These two categories of responses are separate but highly interdependent; with the generation of the lymphoid response being dependent on instruction by the myeloid cells, and the eventual pathogen clearance being affected by the lymphoid cells making use of the highly effective effector responses of the myeloid cells. Given the combination of complexity and specificity in this mosaic of host-microbe interaction, it is not surprising that some of the genes controlling these wings of immune defense are key regulators of cellular signaling responses and have global effects on multiple cell lineages involved in the immune response, while others have pathogen specific modulatory effects.

Receptor ligand interactions, whether in myeloid lineage cells subsequent to ligation of pattern recognition receptors (PRRs), or in lymphoid cells of the B and T lineage subsequent to T cell receptor (TCR) and B cell receptor (BCR) ligation, result in activation of intracellular signaling intermediates. One of the first events subsequent to receptor ligation, conserved across both these cell lineages, is the activation of receptor associated and non-receptor associated protein tyrosine kinases (PTKs) (Schmidt et al., 2004). While the receptor associated tyrosine kinases are intrinsically associated with the cytoplasmic domains of cell surface receptors, the non-receptor tyrosine kinases undergo signal induced association, and both transduce ligand-induced intracellular signals by tyrosine phosphorylation.

## *1. Bruton's tyrosine kinase*

The Tec family is the second largest of the cytoplasmic non-receptor associated tyrosine kinases and is composed of five mammalian members: Btk, Bmx, Itk, Tec and Txk (Smith et al., 2001). Bruton's tyrosine kinase (BTK) is expressed only in the hematopoietic lineage, and is found in all hematopoietic cells with the exception of T lymphocytes and plasma cells (Smith et al., 1994). It has multiple signaling outcomes regulating both effector functions and development of the various cell lineages it is present in, including B cells and myeloid cells. Structurally, in addition to the Src homology (SH) domain, which it shares with other Tec kinases; BTK has a characteristic pleckstrin homology (PH) domain located at the N-terminus of the molecule. The PH domains bind to membrane phospholipids, phosphatidyl inositol-3, 4, 5-triphosphate (PIP3) in case of BTK, which serves to translocate BTK to the membrane in response to phosphatidylinositol 3-kinase (PI3K) activation and directly regulates its signaling

function (Lindvall et al., 2005). Membrane recruitment of BTK is a crucial step towards phosphorylation and activation. Abrogation of membrane recruitment due to a mutation (R28C) in the PH domain results in X-linked immunodeficiency in mice (Mohamed et al., 1999).

## *1.1 BTK in B cells*

Membrane ligation of BCR by various cytokine receptors such as those for IL-3, IL-4 and IL-6 (Lindvall et al., 2005), or by PRRs such as TLR2 and TLR4 (Horwood et al., 2006) leads to recruitment of BTK as a downstream signaling molecule in B cells. Activated Btk then phosphorylates phospholipase C-γ2 (PLC-γ2) (Rodriguez et al., 2001) that hydrolyses $PIP_3$ into inositol triphosphate (IP3) and diacyl glycerol (DAG) resulting in calcium mobilization and activation of protein kinase C (PKC) (Miller and Berg, 2002). Further downstream, BTK signalling plays an important role in degradation of inhibitory protein of the transcription factor nuclear factor-κB (NF-κB), IκBα, resulting in transcriptional activation.

### *1.1.1 Role of BTK in B cell development*

BTK plays a crucial signaling role in B cell development, such that a lack of its functioning in XLA (X-linked agammaglobulinemia) results in greatly reduced numbers of mature circulating B cells (Conley, 1985), severe agammaglobulinemia (Campana et al., 1990) and bone marrow B cell development arrest at the pre/pro-B cell stage (Cancro et al., 2001). By comparison, the defect manifested in the murine XID or even the BTK deficient *Btk*$^{-/-}$ mice is more subtle, with a reduction in circulating mature B cells to half the normal numbers, normal bone marrow B cell development. This is associated with a

peripheral B cell maturation block at the immature to mature B cell transition in the spleen (Kerner et al., 1995; Khan et al., 1995), resulting in an increased frequency of immature $IgM^+$ B cells and lower frequency of mature $IgD^+$ B cells (Scher et al., 1975a; Scher et al., 1975b). Also, while serum IgM and IgG3 levels show reduction, the levels of other Ig isotypes remain unaltered (Scher et al., 1975a; Scher et al., 1975b).

However, T cell depletion in XID mice manifests with a severe B cell developmental arrest and serum immunoglobulin deficiency that closely resembles human XLA, as shown by introducing the XID defect into a FOXN1-null *nude* phenotype (Wortis et al., 1982) or by marrow reconstitution in surgically thymectomised irradiated adult XID mice (Sprent and Bruce, 1984), indicating a role for the presence of T cells in mediating B cell development in the absence of BTK. No mechanisms mediating this role have as yet been demonstrated or proposed.

Tec, the founding member of the Tec kinase family, is expressed in T and B cells, in myeloid cells, and in liver (Sprent and Bruce, 1984) and is activated in response to BCR (Kitanaka et al., 1998) or anti-CD19 (Kitanaka et al., 1998) ligation, and by stimulation of the cytokine receptors for IL-3, IL-6 (Takahashi-Tezuka et al., 1997), stem cell factor (Tang et al., 1994). Though analysis of *Tec*-deficient mice did not reveal any defect in B and T cell lineages, *Tec/Btk* double deficient mice show an accumulation of pro/pre-B cells (Ellmeier et al., 2000), the stage where developing B cells become dependent on BCR signaling for further differentiation into $IgM^+$ B cells, suggestive of a role for Tec in regulating B cell development in the absence of BTK. Though members of the Tec family have been demonstrated to participate in both cytokine receptors (Yang et al., 2000) as well as pre-BCR mediated signaling events (Su and Jumaa, 2003), it is

unclear whether Tec kinase is activated in response to pre-BCR formation or in response to cytokines of T cell origin in B lineage cells.

Surface CD40 on B-cells can also modulate B-cell fate. Thus, while surface immunoglobulin (sIg) cross-linking results in programmed cell death, concomitant CD40 co-stimulation suppresses the same. The ligand for CD40, CD40L, is expressed in activated T cells (Foy et al., 1996), and in bone marrow stromal cells (Abe et al., 2002). XID or *Btk*$^{-/-}$ mice with an engineered CD40 deficiency display depletion of serum Ig of all isotypes, with a substantive peripheral B cell developmental arrest at the immature to mature B cell transition (Khan et al., 1997; Oka et al., 1996). From these studies, there emerges a potential but unexplored role for cross-talk between the circulating T cells and the developing B cells in the bone marrow, possibly involving CD40-CD40L interactions and Tec as the signaling intermediates.

### *1.1.2 Role of Btk in B cell effector functions*

BTK has been shown to be a key intermediate in signaling pathways controlling proliferation, differentiation and immunoglobulin secretion by B cells. BTK deficient murine B cells proliferate poorly on stimulation *in vivo* with either thymus dependent antigens (Khan et al., 1995) or Type II T-independent antigens (Alugupalli et al., 2007) resulting in a compromised IgM and IgG3 secretion in the primary antibody response, but with generation of re-stimulation competent memory B cells, which respond normally in a subsequent antigenic recall (Ridderstad et al., 1996; Ridderstad and Tarlinton, 1997). XID B cells have also been shown to be unresponsive to CD38 signaling (Santos-Argumedo et al., 1995) and show decreased proliferation and Ig secretion on CD40 and BCR co-ligation (Hostager et al., 2003). One of the explanations for the poor

proliferative responses in XID B cells is the poorer generation and maintenance of intracellular calcium response following BCR ligation (Rigley et al., 1989), alluding to the role of BTK in regulating intracellular calcium stores (Fluckiger et al., 1998).

Cellular adhesion and trans-endothelial migration, induced by chemokine gradients, form the earliest inflammatory responses undertaken by immune cells. BTK, through its PH domain, interacts with filamentous actin and small GTPases of the Rho family such as Cdc42 and Rac1 resulting in lamellipodia formation and membrane ruffling, thereby affecting cell motility (Nore et al., 2000). Consistent with this role, BTK deficient murine B cells exhibited compromised adhesion and migratory response in response to chemokines, SDF1 and CXCL13 (de Gorter et al., 2007).

## *1.2 BTK in myeloid cells*

BTK is expressed in various myeloid lineage cells including mast cells, macrophages, granulocytes, and dendritic cells. Activation of BTK is seen subsequent to stimulation through various cytokine receptors, integrin receptors and PRRs such as TLRs.

### *1.2.1 Role of BTK in myeloid cell development*

BTK deficiency has been shown not to result in defects in the development of mast cells (Hata et al., 1998) or dendritic cells (Sochorova et al., 2007). However, mature polymorphonuclear neutrophil granulocytes (PMNs) and monocytes have been reported to be substantially lower in the XID mouse marrow, along with significantly lower numbers of total granulocyte lineage cells in the XID (Mangla et al., 2004). Whether this reflects a developmental defect in XID granulopoiesis, or is simply the result of altered

granulopoiesis because of changes in the lifespan of peripheral BTK-deficient PMNs is not yet clear.

### *1.2.2 Role of BTK in myeloid cell effector functions*

Mast cells are crucial mediators of allergic reactions and act by secreting cytokines and inflammatory mediators such as histamines and leukotrienes, contained in preformed cytosolic granules, released by degranulation, subsequent to cross-linking of high affinity IgE receptors (FcεRI) (Schmidt et al., 2004). XID and *Btk*$^{-/-}$ mice display poor anaphylactic reactions, degranulation, histamine release, and show a severe compromise in cytokine production and release of IL-2, IL-6, GM-CSF and TNFα (Hata et al., 1998). Compared to the rapid release of mediators, the late phase reactions requiring *de novo* synthesis of secreted mediators are more severely impaired in BTK-deficient mast cells as is $IP_3$ generation and $Ca^{2+}$ release, subsequent to receptor ligation (Hata et al., 1998; Kawakami et al., 2000). These data suggest that the major target of BTK signaling during mast cell activation is likely to be induction of transcription.

Dendritic cells from XLA patients show impaired IL-6 and TNF-α production following stimulation with TLR8 agonist ssRNA, but not in response to stimulation of TLR1/2, 2/6, 3, 4 and 5 (Sochorova et al., 2007). Lipopolysaccharide (LPS) induced generation of effector molecules, reactive oxygen intermediates (ROI) (Mangla et al., 2004) and reactive nitrogen intermediates (RNI) (Mukhopadhyay et al., 1999), as well as pro-inflammatory cytokines, TNF-α and IL-1β, is compromised in XID macrophages. Compromised induction of inducible nitric oxide synthase (iNOS), the enzyme responsible for RNI generation, results in the poorer RNI generation (Mukhopadhyay et al., 1999). Further, induction of transcription factors of the NF-κB family is also

defective, with poor induction of p-50 and lack of induction of p-65 and c-Rel members, seen on LPS stimulation (Mukhopadhyay et al., 2002). These defects result in poorer microbial clearance, microfilaria (Mukhopadhyay et al., 1999) or *Escherichia coli* (Mukhopadhyay et al., 1999), *in vitro*, and decreased susceptibility to inflammatory conditions *in vivo*, such as experimental autoimmune encephalomyelitis (EAE) or dextran sulfate sodium (DSS)- induced colitis (Mangla et al., 2004).

Consistent with these data, macrophages from XLA patients also show compromised TNF-α induction on LPS stimulation and TLR2 ligation (Horwood et al., 2003; Horwood et al., 2006). XLA monocytes display poorer chemotaxis and phagocytic functions (Amoras et al., 2003), as well as nuclear translocation of NF-κB (Jefferies et al., 2003).

PMNs from XID mice show compromised RNI and ROI induction, poorer bacterial clearance *in vitro*, and poorer chemotaxis and recruitment in the absence of any defect in phagocytosis (Mangla et al., 2004). Inhibition of BTK function in human PMNs has been reported to lead to reduced neutrophil adhesion, migration and ROI generation {Gilbert, 2003 #93}. XID macrophages and PMNs show an enhanced tendency to undergo apoptotic cell death in response to inflammatory signals such as TLR ligands and pro-inflammatory cytokines (Mangla et al., 2004).

## 2. *Host-Pathogen interactions*

The interplay between a potential host and the pathogen begins on first contact with the pathogen, and is continued through the activation of the process of immune inflammation.

## *2.1 Initiation of inflammation*

Recognition of specific structural motifs of the invading pathogens, pathogen associated molecular pattern (PAMPs), by phylogenetically conserved cell surface pattern recognition receptors (PRRs), present on macrophages and dendritic cells, forms the first point of contact with pathogens (Janeway and Medzhitov, 2002). Central to the organization of an inflammatory response subsequent to pathogen recognition is the recruitment of immune effector cells, achieved by the secretion of signaling molecules known as chemokines (chemoattractant cytokines) (Premack and Schall, 1996). Chemokines are heparin binding small protein molecules of 7-10 kDa, divided into three distinct families based on the position of the amino-terminal cysteine residues that are present in all of these molecules (Strieter et al., 1996). Of these, CXC family members are specific mediators of neutrophil migration, CC family members mediate monocyte, eosinophil and lymphocyte migration, while the C family primarily recruit lymphocytes (Schluger and Rom, 1997). Additionally, specific signaling initiated on pathogen contact differentially controls the expression of one or more of the families of chemokines (Schluger and Rom, 1997). Acute bacterial infections are characterized by the preferential induction of CXC chemokines such as IL-8, resulting in a predominantly neutrophilic infiltrate (Mizgerd et al., 1996). *Mycobacteria*, which are macrophage resident intracellular pathogens, expectedly induce secretion of CC family members, monocyte chemotactic protein (MCP) and macrophage inflammatory protein (MIP), to induce macrophage infiltration (Friedland et al., 1993). Further, induction of T cell recruitment, proliferation (Taub et al., 1996) and Th-1 (by MCP) and Th-2 (by MIP-1α) differentiation (Karpus et al., 1997), has also been reported. Primed helper-T cells then act to amplify the immune response by secreting inflammatory cytokines.

## *2.2 Eosinophils*

Eosinophils, a specific lineage of granulocytes, so named because of their eosinophil granule content, are multifunctional leukocytes recruited in response to CC chemokines (RANTES and eotaxin), as well as Th2 cytokines such as IL-4, IL-5, and IL-13, and modulate protection against parasitic helminth infections and mediate allergic diseases (Rothenberg and Hogan, 2006).

### *2.2.1 Eosinophil development*

Eosinophils develop in the bone marrow form a common precursor they share with basophils (Boyce et al., 1995), under the influence of three classes of transcription factors; GATA-1 (a zinc finger transcription factor), PU.1 (an ETS family member) and C/EBP members (CCAAT/enhancer-binding protein family), of which GATA-1 is the most important for eosinophil lineage specification (McNagny and Graf, 2002; Nerlov and Graf, 1998; Nerlov et al., 1998). Eosinophils possess a high affinity palindromic GATA site, which is present in the GATA-1 promoter region and also in the regulatory regions of eosinophil specific genes such as eotaxin receptor, chemokine receptor-3 (CCR3) and IL-5 receptor alpha (IL-5α). Activation of this palindromic GATA site results in the eosinophil specific expression of these genes (Du et al., 2002; Yu et al., 2002; Zimmermann et al., 2000). Three cytokines, IL-3, IL-5 and GM-CSF, share a common receptor beta chain, and regulate eosinophil development by providing proliferation and differentiation signals and are under transcriptional control of GATA-1. Of these, IL-5 is the most proficient at inducing eosinophil differentiation and release form the marrow (Collins et al., 1995; Sanderson, 1992), as well as their exit from

peripheral circulation, consequently, over-expression of IL-5 in mice results in profound eosinophilia (Tominaga et al., 1991).

### *2.2.2 Eosinophil functions*

Eosinophils have been identified to serve as major effector cells by releasing cytotoxic granules containing cationic proteins such as major basic protein (MBP), eosinophilic cationic protein (ECP), and eosinophil peroxidase (EPO), and lipid mediators such as leukotrienes and platelet activating factor, resulting in tissue damage and heightened inflammatory responses (Rothenberg and Hogan, 2006). Receptor activation of eosinophils leads to targeted release of specific granule vesicles (Dvorak et al., 1991), which are relocated to the plasma membrane by the formation of docking complexes composed of N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (SNAREs) located on the vesicle (v-SNAREs) and the target membrane (t-SNAREs) (Logan et al., 2002). Release of chemokine RANTES, but not cationic proteins, on IFN-γ stimulation of eosinophils, is an example of such a specific granule release (Lacy et al., 1999). ECP and MBP, besides exerting non-specific toxicity on a variety of tissues, have specific regulatory effects such as inhibition of immunoglobulin synthesis and T cell proliferative responses, and induction of mast cell degranulation, in case of ECP (Venge et al., 1999), and induction of basophil and mast cell degranulation, in case of MBP (Piliponsky et al., 2001; Zheutlin et al., 1984). EPO catalyzes halides ($Cl^-$, $Br^-$, $I^-$) and nitric oxide to reactive oxygen and reactive nitrogen metabolites ($NO_2$), which lead to oxidative stress and induce apoptosis and tissue necrosis (MacPherson et al., 2001; Wu et al., 1999). Eosinophils, under normal conditions are mostly resident in the intestinal lamina propria where they survive for up to 2 weeks (Mishra et al., 1999), and are

recruited to sites of inflammation under the influence of eosinophil specific chemokines of the eotaxin family and Th2 cytokines.

### *2.2.3 Immunomodulatory effects of eosinophils*

MBP induces IgE independent mast cell degranulation, eicosanoid production and release of cytokines such as IL-18, TNF-α and GM-CSF (Piliponsky et al., 2002). Eosinophils also produce nerve growth factor (NGF), an important cytokine promoting mast cell survival and activation (Bullock and Johnson, 1996). Eosinophils have also been reported to process and present microbial and parasitic antigens, and modify Th1/Th2 commitment of the ensuing CD4 response by secreting a range of polarizing cytokines, notably IL-4 and other Th2 cytokines such as IL-5 and IL-13 (MacKenzie et al., 2001). Recent reports have indicated the ability of eosinophils to preferentially activate effector T cell proliferation (van Rijt et al., 2003), which, when examined in context of the ability of primed eosinophils to track to T cell rich paracortical zones of lymph nodes (Shi et al., 2000), implies a role for these cells in mediating an amplification of an ongoing effector T cell response. The three types of eotaxins reported, eotaxin-1, 2 & 3, all signal via the same eosinophil specific chemokine receptor, CCR3 (Daugherty et al., 1996), and have been reported to be sequentially induced on inflammation in epithelial cells, fibroblasts as well as infiltrating macrophages and eosinophils (Rothenberg and Hogan, 2006). Of these, the earliest acting eotaxin-1 & 2, in conjunction with IL-5 which activates a STAT-6 (signal transducer and activator of transcription-6)-dependent signaling pathway, together resulting in induction of tissue eosinophilia and Th2 skewed immune responses (Zimmermann et al., 2003).

### *2.2.4 Comparative functions of eosinophil and neutrophils*

Eosinophils and neutrophils constitute are the earliest responding cells recruited to sites of pathogen entry, and mediate rapid pathogen elimination utilizing pre-formed granule proteins stored in rapidly mobilizable vesicular organelles. In addition, neutrophils recognize microbial PAMPs (Hayashi et al., 2003) and initiate vigorous phagocytosis, whereas eosinophils depend on a less efficient antibody-dependent phagocytosis (Sanderson and de Souza, 1979) and largely mediate pathogen killing by granule exocytosis. Specialized granule structures of the neutrophils have well demarcated functions in effecting microbial killing. The azurophilic and specific granules mediate killing intracellularly by fusing with the phagosome, while the specific granules are also released by exocytosis and mediate microbial elimination in the extracellular milieu (Theilgaard-Monch et al., 2006). Neutrophils have also been demonstrated to generate extracellular fibres, called neutrophils extracellular traps (NETs), which physically trap bacteria on which the NET resident granule proteins and cathepsins then act to secure microbial killing (Brinkmann et al., 2004). The NETs have thus been seen to represent an efficient method to restrict bacterial spread at the site of infection and form a efficient mechanism to mediate focused lytic activity. In addition to the immediate killing and degradation of pathogens, activated eosinophils and neutrophils synthesize a host of chemokines and cytokines, which act to regulate the recruitment and function of other effector cells such as macrophages, T cells and other granulocytes including themselves (Theilgaard-Monch et al., 2004; Zimmermann et al., 2003). Whereas, eosinophils mediate the induction of a Th2 skewed response (MacKenzie et al., 2001), neutrophils secrete Th1 cytokines such as IL-12, IFN-γ, and chemokines active on Th1 cells such as

MIG (monokine induced by interferon-γ) and MIP-1α, resulting in a distinct IFN-γ biased Th1 response (Bliss et al., 2000; Chen et al., 2001).

## *2.3 Phagocytosis*

Phagocytosis is the phylogenetically conserved process of ingestion of particulate ligands including microbes and cell debris > 1μm in size, mainly mediated by phagocytic leukocytes. Phagocytosis result in routing of ingested pathogen to the lysosomes where microbial death is mediated by hydrolytic enzymes and free radicals, and directs the microbial antigens to compartments containing MHC class II (MHCII) for processing and presentation to lymphocytes. Thus, phagocytosis serves a dual role: an immediate immune effector function as well as a bridge between the innate myeloid and acquired lymphoid responses.

### *2.3.1 Receptor recognition and signaling events*

Pathogen recognition by myeloid cells is mediated by PRRs such as mannose receptors, NOD-like receptors (NLR) or Toll-like receptors, of which TLRs have been the most extensively characterized. They are expressed on the cell surface (TLRs 1 to 6 and TLR11) or intracellularly on endosomal membranes (TLR3, 7, 8 and 9), and binding of respective ligand rapidly transduces signals initiating NF-κB activation or cytokine secretion, especially type-I interferons (Akira, 2006). Cell surface resident TLRs specialize in ligands on microbial cell wall molecular motifs enabling extracellular bacterial recognition, while endosomal TLRs sample a variety of viral and bacterial nucleic acids effecting recognition of intracellular pathogens. Accordingly, TLR2, along with TLR1 & 6 recognize cell wall peptides of gram-positive bacteria and mycoplasma,

whereas TLR4 recognizes LPS present on gram-negative bacteria. TLR3 recognizes viral dsRNA, TLR7 & 8 recognize viral ssRNA, and TLR9 is the receptor for bacterial and viral CpG DNA repeat motifs and non-nucleic acids such as hemozoin (Akira, 2006). Recognition of PAMPs by the cognate TLR ligand results in receptor association of TIR (Toll/interleukin-1 receptor) domain containing adapters, MyD88, TIRAP (Toll-interleukin-1 receptor domain-containing adaptor protein), TRAM (toll-like receptor adaptor molecule-1) and TRIF (TIR domain-containing adapter inducing IFN-beta), and depending on the TLR that has been ligated, various combinations of these adaptors are recruited to mediate distinctive responses (Akira and Takeda, 2004). All the families of TLRs (except TLR3) utilize a MyD88 dependent pathway, where MyD88 activation by phosphorylation results in recruitment of members of IRAK (IL-1 receptor-associated kinase) family, which through subsequent phosphorylation events activate the NF-κB transcription factors resulting in expression of inflammatory cytokines TNF-α (tumor necrosis factor-α), IL-6 and IL-1β (Akira and Takeda, 2004; Kawai et al., 1999). In addition, TLR engagement, in a MyD88 independent pathway induce activation of the members of the transcription factor family interferon regulatory factor (IRF), mainly IRF-3 resulting in induction of IFN-β and IFN inducible genes (Kawai et al., 2001; Yamamoto et al., 2003). TLR4, 5 and 9 ligand engagement results in induction of Th1 associated cytokines such as IL-12, similarly TLR2 ligands induce Th2 responses characterized by IL-10 secretion (Dillon et al., 2004; Redecke et al., 2004). Thus, signaling events subsequent to pathogen recognition, not only induce specific effector myeloid responses, but also modulate the course of the lymphoid response.



### *2.3.2 Phagosome formation and maturation*

Actual physical pathogen engulfment involves signaling machinery independent of pathogen recognition by PAMPs and include Fc receptors (Bharadwaj et al., 2001), receptors for components of the complement pathway (Zaffran et al., 1998) and scavenger receptors (Thomas et al., 2000), and mediates uptake of both microbial pathogens and apoptotic cell debris. Signaling through these receptors results in the activation of PI3K (Phosphoinositide-3 kinase) and phospholipase C, which generates $IP_3$ (inositol triphosphate) and DAG (diacyl glycerol) by hydrolysis of $PIP_2$ (phosphatidylinositol-4, 5-bisphosphate), which subsequently initiate calcium recruitment and activation of PKC mediated signaling pathways. $PIP_2$ plays a crucial role in mediating the massive cytoskeletal remodeling needed for pathogen uptake, by associating with members of the Rho family of GTPases, including Rho (Hackam et al., 1997), Rac1 and Cdc42 (Castellano et al., 2000; Cox et al., 1997), which mediate focal F-actin assembly at the periphagosomal region to initiate phagocytosis. ARF6, a member of the ARF family of GTPases, augments Rac functions and serves as a co-factor in inducing $PIP_2$ generation at the plasma membrane (Honda et al., 1999; Zhang et al., 1999). Together they induce actin filament based pseudopodia extension of the plasma membrane around the pathogen, resulting in engulfment and membrane closure mediated by several unconventional myosins (Titus, 1999), resulting in the internalization of the phagosome. These newly formed phagosomes undergo sequential fusion with endosomes and ultimately with lysosomes to effect degradation and killing of the microbe, a process termed phagosome maturation (Vieira et al., 2002). During the process of maturation, the phagosome recruits various signaling and effector molecules, undergoing increasing

acidification, culminating in the low pH lysosomes, pH sensitive acid hydrolases eventually act to ensure microbial degradation.

Though phagocytosis of microbes and apoptotic cells is mediated by the same phagocytic machinery, qualitatively different cellular responses ensue, due to specific pathogen recognition by TLRs. More importantly, microbial uptake is not mediated by TLRs, but rather by these phagocytic receptors which by themselves do not induce inflammation (Taylor et al., 2005), but can be transcriptionally induced on TLR activation (Doyle et al., 2004). Further, TLRs can also be recruited to phagosomes, and in a MyD88 and p38 MAPK (mitogen-associated protein kinase) dependent fashion, induce increased microbial uptake and phagosomal trafficking kinetics resulting in enhanced clearance (Blander and Medzhitov, 2004; West et al., 2004). Though the exact molecular mechanisms for this modulation have to be defined, they have been speculated to involve regulation of V-ATPases (vacuolar-ATPase) which mediate phagosome acidification, association of Rab GTPases which mediate vesicular trafficking, and increasing phagosome movement on the microtubule assembly resulting in enhanced phagosomal maturation (Blander and Medzhitov, 2006).

### *2.3.3 Microbicidal functions of phagosomes*

Subsequent to pathogen recognition and internalization, pre-formed flavocytochrome $b_{558}$, the core component of the NADPH oxidase system is recruited to the phagosome membrane where it forms a channel to direct electrons generated from cytosolic NADPH oxidation onto oxygen contained in the phagosome vacuole, thereby generating superoxide radicals ($O_2^-$)(Segal, 2005). Reactive oxygen- species/intermediates (ROS/ROI) are generated as reaction products from superoxides and include hydroxyl,

hydrogen peroxide, ozone, halides and superoxide itself, which are further converted into hypochlorous acid by superoxide dismutase (Chapman et al., 2002). These ROS species play an important role in pathogen clearance and the dysfunction of the NADPH oxidase system causes CGD (chronic granulomatous disease), characterized by profound susceptibility to bacterial and fungal infections and failure to clear anaerobic infections. Reactive nitrogen intermediates (RNI), including nitric oxide (NO) and its reaction products generated by constitutive and inducible expression of NO synthase enzymes also mediate important microbicidal effector functions (James, 1995). Accumulated NO gets converted to highly reactive peroxynitrite ($ONOO^-$) radicals by phagosomal superoxide and affects microbicidal functions. Inducible nitric oxide synthase (iNOS) levels are also transcriptionally enhanced by TLR ligation or Type I interferon, prominently IFN-γ, resulting in conversion of L-arginine to L-citrulline and NO. RNIs produced in this fashion are important effector molecules in immune response against bacterial, fungal, helminth and protozoan infections (James, 1995).

## *3. Endo-lysosomal trafficking*

### *3.1 Rab and small GTPases*

Phagosomal maturation proceeds with sequential fusion of endosomal vesicles, resulting in loss of cell surface markers associated with the engulfed portion of the plasma membrane which now forms the phagosome bilipid membrane, and acquisition of markers of early endosomes, late endosomes and lysosomes (Desjardins et al., 1994; Pitt et al., 1992). Phagosome maturation involves multiple transient fusion events with endosomal vesicles, referred to as 'kiss and run' fusion, allowing for acquisition of

proteolytic and microbicidal substances by the phagosome, but preventing complete fusion with the endosomes (Desjardins, 1995; Desjardins et al., 1997). Such phagosomal fusion events with early endosomes are regulated by Rab5, Rab4 and Rab11 and late endosomal fusion is regulated by Rab7 and Rab9. Inactive GDP form of Rab proteins associate with the vesicle and dissociate by rapid phosphorylation to the GTP-bound form, to ensure the transient nature of the fusion. Prolonged fusion, induced by the GTP-state arrest of Rab5 results in formation of giant endosomes, and has been reported to interfere with impaired clearance of intracellular parasite *Leishmania donovani* (Duclos et al., 2000). Proton pump V-ATPase and lipid raft-associated domains (Dermine et al., 2001) associate with the phagosome during its interaction with early endosomes. Also, lipid raft domains play a facilitatory role in actin accumulation and fusion with late endosomes (Kjeken et al., 2004) as well as in recruitment and assembly of cytoslic domains of the NADPH oxidase complex on the phagosomal membrane (Shao et al., 2003; Vilhardt and van Deurs, 2004).

## *3.2 Lysosomal transporter - LYST*

LYST (lysosomal trafficking regulator), belongs to a family of proteins characterized by the presence of a BEACH (Beige and Chediak-Higashi syndrome) and WD-domains in their C-terminal region and two members, LYST and Alfy, have been reported in *Dictyostelium* to be associated at different stages of endosomal trafficking (Cornillon et al., 2002).LYST has been shown to be associated with microtubules (Faigle et al., 1998) and shows a cytosolic distribution (Perou et al., 1997). However, despite the availability of the complete sequence of the *Lyst* gene, the precise function of LYST has not been characterized. Most studies have therefore focused on analysis of functional

defects due to mutations in the *Lyst* gene, resulting in an immunodeficiency syndrome Chediak-Higashi syndrome (CHS) in humans and the mouse model for CHS, the *Beige* (bg/bg) (Barbosa et al., 1996). CHS presents as an autosomal recessive disorder, characterized by partial albinism, recurrent bacterial infections and the accumulation of giant intracellular granules, which include lysosomes, melanosomes, cytolytic granules and platelet dense granules. The *Lyst* mutation results in enlarged lysosomal compartments and reduced cytotoxicity due to defective granule exocytosis, evident in cytotoxic T lymphocytes (Baetz et al., 1995), NK cells (Haliotis et al., 1980; Roder et al., 1980) and granulocytes (Gallin et al., 1974). Early endosomes and trans-golgi network (TGN) are the main sources of membrane protein trafficked to the late endosomes. In CHS, molecules normally routed to the lysosomes from these two sources, via the late endosomes, are found to accumulate in early endosomes. This indicates a block in the vesicular transport between early and multivesicular late endosomes (Faigle et al., 1998). Multivesicular late endosomes, formed by fission of large portions of early endosomes mature by extensive material exchange with TGN and eventually fuse with pre-existing multilaminar lysosomes to form mixed compartments. These compartments undergo sorting and lysosomes are retrieved back. It is the missorting of endosomal proteins during the early to late endosomal transit that is likely to result in the consequent perturbation of lysosomal identity and functions (Faigle et al., 1998). Such a missorting could explain the relative lack of lysosomal membrane markers and the abundance of endoplasmic reticulum proteins in the CHS lysosomes. This could also account for the low levels of cathepsins, which are routed in membrane bound form from early endosomes, and are active at the low pH found in lysosomes (Nishi and Forgac, 2002). Cathepsin L (in mice) (Nakagawa et al., 1998) and cathepsin S (in humans) (Bania et al.,

2003) are necessary for proteolysis of the invariant chain of MHC II to CLIP. Therefore defective cathepsin transport due to missorting in CHS results in impaired peptide loading, and in conjunction defective transport of peptide-MHC II complexes to the cell surface, leads to defective MHC II mediated antigen presentation (Faigle et al., 1998; Huynh et al., 2004). Cathepsin G also has important microbicidal functions and its impaired function, especially in neutrophils, might account for the defective bacterial clearance observed in CHS (Gallin et al., 1974; Ganz et al., 1988).

Defects in phago-lysosomal function of the kind exhibit in the CHS and bg/bg has major consequences on effective pathogen clearance, especially in infections with intracellular pathogens, as evidenced by increased susceptibility of bg/bg mice to *Leishmania donovani* infection (Kirkpatrick and Farrell, 1982).

## 4. *Leishmania major*

Digenetic intracellular protozoan parasites of the genus *Leishmania* cause a chronic parasitic disease with a wide range of clinical presentations: cutaneous, mucocutaneous and visceral, with the involvement of multiple host and pathogen factors, genetic and environmental, in determining clinical disease outcome (Lipoldova and Demant, 2006). Extracellular infective stage, flagellated promastigotes of the *Leishmania* parasite are transmitted to human hosts during a blood-meal by infected phlebotome sandflies, which act as the invertebrate hosts. The parasites then gain access and are taken up by the host phagocytes, neutrophils, macrophages, monocytes and dendritic cells, and convert into the phagocyte resident aflagellated amastigote form. Amastigotes reside and multiply in host cell lysosomes, eventually rupturing the cell to be released and phagocytosed by uninfected cells. Since macrophages are both circulatory and tissue

resident in nature, infected macrophages can gain access to lymphoid organs, spleen and lymph nodes to set up a visceral infection, or take up residence in the dermis or mucosal tissue resulting in a localized cutaneous or the diffuse mucocutaneous form of leishmaniasis. Since the host macrophages are the major immune effector cells mediating pathogen elimination, the *Leishmania* parasite has evolved a variety of mechanisms to subvert macrophage function. Consequently modulation of macrophage function by acquired immune responses mediated largely by the CD4 subset of T lymphocytes plays a major role in affecting parasite clearance.

## *4.1 Pathogen uptake and residence in phagocytes*

*Leishmania* parasites lack the ability to actively invade host cells and depend on phagocytic uptake by host macrophages involving complement receptors (CR1 and CR3) and pattern recognition receptors such as mannose/fucose receptors (Alexander and Russell, 1992). Phagocytosed parasites are then rapidly trafficked to and fuse with lysosomes to form the phagolysosomal residence site of the parasite, referred to as the parasitophorous vacuole (Courret et al., 2002). Phagocytosed promastigotes are highly susceptible to the action of acid hydrolases, hence rapid conversion to the hydrolase-resistant amastigote is critical for parasite survival (Dermine et al., 2000; Desjardins and Descoteaux, 1997). Therefore, rapid transport of the phagocytosed parasites to the lysosomal compartments is necessary to ensure elimination. Macrophage effector molecules, NO and ROI are also essential for parasite killing. Mice deficient for the enzyme iNOS are unable to control a *Leishmania* infection and macrophages derived from these mice are incapable of affecting a clearance of parasites in culture (Wei et al., 1995). Mice deficient in ROI generation however clear the infection after an initial period

of increased susceptibility (Murray and Nathan, 1999), indicating that ROIs play a somewhat less significant role in parasite clearance. Induction of IL-12, a dominant inducer of IFN-γ secretion by Th1 cells, results in enhanced clearance due to the ability of the secreted IFN-γ to induce iNOS and hence NO production in the macrophages, consequently, DCs derived from IL-12 deficient mice show compromised *Leishmania* clearance ability (Berberich et al., 2003).

*Leishmania* parasites have evolved multiple mechanisms to evade the early host effector responses. *Leishmania* evade the lytic effects of the membrane attack complex (MAC) of the complement system composed of complement factors C5b-C9, by the surface expression of glycosylated lipophosphoglycan (LPG) which prevents attachment of MAC on surface of infective metacyclic promastigotes (McConville et al., 1992). Surface expression of proteinase gp63 further enhances this protection by mediating cleavage of C3b component into an inactive form iC3b (Brittingham et al., 1995), which opsonises the parasite for CR3 mediated uptake by macrophages. In addition to ensuring the targeting of parasite to its cellular residence of choice, ligation of CR3 is of added advantage to the parasite as no respiratory burst activity is induced on CR3 activation (Mosser and Edelson, 1987). Since the promastigotes are vulnerable to lysosomal hydrolases, they retard endosomal maturation and endo-lysosomal fusion by an LPG mediated mechanism involving inhibition of protein kinase C (PKC) activity (Desjardins and Descoteaux, 1997). PKC also plays a key role in the induction of ROIs, consequentially respiratory burst activity is also inhibited (Olivier et al., 1992). Consistent with these roles of LPG, *Leishmania* deficient in LPG expression showed poorer survival within macrophages after infection (Spath et al., 2000). *Leishmania* have also been reported to prevent the induction of cytokines involved in anti-inflammatory

responses (IL-1β and TNF-α) (Hatzigeorgiou et al., 1996) and T cell activation (IL-12) (Piedrafita et al., 1999; Weinheber et al., 1998). Further, *Leishmania* induce overproduction of both IL-10 and TGF-β from infected murine macrophages resulting in uncontrolled parasite replication and non healing lesions (Barral et al., 1993; Kane and Mosser, 2001).

## *4.2 Antigen presentation and induction of Leishmania-specific T cell responses*

Processing and presentation of parasite peptides on MHCII to CD4 T cells is essential for controlling a primary infection with *Leishmania* (Locksley et al., 1993). While presentation on MHCI to CD8 cells does not seem to be essential in controlling a primary infection in β2-microglobulin deficient mice (Wang et al., 1993), other studies have found CD8 T cell functions to be important in controlling infection in a low-dose parasite challenge in mice (Belkaid et al., 2002) and in induction of optimal IFN-γ responses in primed CD4 T cells (Herath et al., 2003).

*Leishmania* infection has been reported to impair association of MHCII molecules with the parasitophorous vacuole, resulting in poor antigen presentation to CD4 T cells (Antoine et al., 1999). In addition, down-regulation CD80, an important co-stimulatory molecule involved in MHCII-TCR interaction, has also been observed in *Leishmania* infected macrophages (Saha et al., 1995). Ligation of surface CD40 on infected macrophages leads to activation of p38 MAPK signaling, resulting in increased activation of iNOS and improved parasite clearance (Awasthi et al., 2003); while impaired CD40-40L co-stimulation is observed in leishmania infected macrophages (Kamanaka et al., 1996; Soong et al., 1996).

Many human diseases, including infectious diseases have now been understood to develop predominantly in genetically predisposed individuals, with multiple genes of low-penetrance determining the disease susceptibility (Pharoah et al., 2002). However, since mapping of genes with such low-penetrance in humans would involve studies requiring the participation of a large number of subjects, in addition to correction for environmental and lifestyle factors, the more feasible approach of genetic analyses on disease-equivalent animal models has been utilized (Bedell et al., 1997a; Bedell et al., 1997b). These models have been useful in leishmaniasis in which robust immunological and genetic components are involved in determining disease outcomes.

## *4.3 T cell response quality and Leishmania clearance*

Infection of human hosts commonly leads to the development of localized cutaneous lesions, which resolve spontaneously with the generation of a sustained life-long immunity to re-infection. This phenotype is also shared by most laboratory mouse models as well as the natural rodent reservoirs of infection. However, certain strains, notably the BALB/c, develop systemic disease with progressive lesions, and are thought to mimic more systemic forms of the human disease such as visceral leishmaniasis (kala-azar) or diffuse mucocutaneous leishmaniasis (Sacks and Noben-Trauth, 2002). The genetic predisposition for resistance or susceptibility in mouse models of infection has been correlated with the dominance of an IL-12/IFN-γ driven Th1 response resulting in cure (in most inbred strains) and an IL-4 dominant Th2 response causing disease (BALB/c), respectively. While this paradigm has made a major contribution to understanding the principles of control of infectious agents, it also has major limitations in the context of understanding the genetic basis of human leishmaniasis. Firstly, the Th2

biased responses in the BALB/c mice do not seem to be disease specific, but are induced globally in every infection or immunization scenario (Hsieh et al., 1995). Secondly, the C57BL/6 mice clear a *Leishmania major* infection rapidly and the BALB/c mice succumb to a rapidly fatal leishmanial infection (Howard et al., 1980). Both scenarios are very unlike the human clinical leishmanial outcome, which results in most instances in chronic, persistent but indolent lesions, with prospects of future reactivation in disease. Thus, while this model contributes to elucidating the genetic regulation of the Th1/Th2 balance, additional putative loci that might impart qualitative regulation on the disease outcome in humans, and the genetic targets of such regulators are still poorly understood (Lipoldova and Demant, 2006). In light of this limitation of the system, this rather simplistic model has been challenged in recent times. With emergence of data on the multiple players involved in the cytokine regulation and the mechanism of acquired resistance to leishmaniasis, and in context of the newly discovered functions of Th3 and Th17 subsets of helper T-helper cells, further complexities are likely to be discovered.

### *4.3.1 Th1 responses*

Control of a leishmanial infection requires the T-cell dependent activation of macrophages to attain a microbicidal state capable of restricting parasite replication. Experiments using various knockout mice have shown that the minimal requirement for effecting a cure in a leishmanial infection are the presence of CD4 T cells (Holaday et al., 1991; Moll et al., 1988; Varkila et al., 1993), peptide presentation on MHCII, Th1 inducer IL-12 and induction of Th1 cytokine IFN-γ (type 1 cytokines), activation of macrophage microbicidal enzyme iNOS and the resultant synthesis of NO. Consistent with this model of type 1 cytokine responses, mice deficient in type 1 cytokines, IL-12

(Mattner et al., 1996) or IFN-γ (Wang et al., 1994), cytokine receptors such as IFN-γ receptor (Swihart et al., 1995), transcription factors such as T-bet (Szabo et al., 2002) or STAT-4 (Signal transducer and activator of transcription protein-4) (Stamm et al., 1999) and co-stimulatory molecule interactions CD40-CD40L (Campbell et al., 1996; Kamanaka et al., 1996), have all been shown to exhibit increased disease susceptibility.

IL-12 plays a central role in the orchestration of a curative, type 1 cytokine response. Myeloid cells including macrophages and dendritic cells are the major sources of IL-12 production, as proved by the inability of primed Th1 T cells from healed wild-type mice to transfer immunity to IL-12 deficient mice (Park et al., 2000). As mentioned previously, macrophages infected with *Leishmania* exhibit impaired IL-12 production, hence DCs including tissue resident Langerhan's cells (LC), have been identified as the predominant IL-12 secreting cell type, both *in vitro* (Konecny et al., 1999; von Stebut et al., 1998) and *in vivo* (Belkaid et al., 2000; von Stebut et al., 2000). LCs have also been reported to transport *Leishmania* from the site of infection to the draining lymph node (Moll et al., 1993) leading to T- cell priming in the presence of secreted IL-12, thereby playing an early role in determining the eventual T-helper response. Natural killer (NK) cells have also been identified as sources of IL-12, but are not indispensable for induction of an anti-leishmanial Th1 response as demonstrated by efficient IL-12 dependent IFN-γ responses induced in CD4 T cells and efficient lesion resolution in NK cell deficient mice (Satoskar et al., 1999; Wakil et al., 1998). Naïve CD4 T cells have low levels of IL-12 receptor expression, which are upregulated on cognate TCR ligation. In addition, early secretion of IFN-γ by DCs and NK cells leads to activation of transcription factor T-bet, initiating a commitment of responding T cells towards a Th1 response and upregulation of IL-12 receptor (Afkarian et al., 2002; Lighvani et al., 2001). Upregulation of and

signaling through the IL-12 receptor and IL-18 receptor, then activates transcription factor STAT-4 leading to commitment to Th1 responses (Ouyang et al., 1999; Yang et al., 1999). In addition to augmentation of IFN-γ secretion, both IFN-γ and IL-12 signaling pathways have been reported to participate in silencing of the Th2 locus (Manetti et al., 1993; Seder et al., 1993) by mediating suppression of key Th2 transcription factor GATA-3 (Ouyang et al., 2000), resulting in inhibition of IL-4 responses.

### *4.3.2 Th2, Th3 and Tr1 responses*

Th2 lineage commitment is dependent on the induction of Th2 lineage transcription factor GATA-3 by the IL-4/STAT-6 signaling pathway (Kurata et al., 1999; Ouyang et al., 1998) and TCR-CD28 mediated GATA-3-independent pathway (Rodriguez-Palmero et al., 1999). The resultant IL-4 production results in diminished IFN-γ expression and stabilizes GATA-3 induction by chromatin remodeling (Lee et al., 2000; Ouyang et al., 2000). Lowered Th1 responses, eventually lead to inhibition of nitrite generation, resulting in enhanced parasite survival.

Activation of a distinct subset of IL-4 secreting CD4 T cells, possessing Vβ4Vα8 TCR recognizing the *leishmanial* LACK antigen (*Leishmania* homologue of receptors for activated C kinase), has been shown to be induced very early in infection in the BALB/c mice and was believed to confer disease susceptibility. This was based on the observation that infected Vβ4 deficient BALB/c mice mounted Th1 biased CD4 T cell response, resulting in improved parasite clearance (Himmelrich et al., 2000; Launois et al., 1997). Recent evidence has, however, demonstrated similar levels of activated LACK-CD4 T cells in resistant and susceptible mouse strains, early in infection (Stetson et al., 2002). Further, generation of Th2 cytokines in resistant mice by administering IL-4 (Chatelain et

al., 1992) and anti-IL-12 antibodies (Hondowicz et al., 1997), early in infection, did not result in a sustained Th2 response and eventually still led to clinical cure.

Therefore, though IL-4 mediated Th2 response has been shown to result in non-healing leishmanial infection in mouse strains such as the BALB/c, these studies indicated that IL-4 was not the sole and sufficient factor regulating disease susceptibility, and that other cytokines such as IL-13, IL-10 and TGF-β (inducers of Th3 and Tr1 responses) might contribute in determining the disease phenotype. Studies using BALB/c mice deficient for IL-4 and IL-4-receptor-α chain (IL-4Rα) (Noben-Trauth et al., 1999) have reported partial improvement in the former strain and marked improvement in the later. However, mice with double deficiency of IL-4 and IL-13, whose receptor shares its α-chain with the IL-4-receptor, showed an even greater degree of resistance than either of the parental knockout mice (Matthews et al., 2000). TGF-β has also been reported to suppress Th1 responses and macrophage activation. Treatment of infected mice with anti-TGF-β antibodies has been reported to result in enhanced resolution of lesions, in spite of no observable effect on the levels of IFN-γ or IL-4. This improvement in clearance has been speculated to involve activation of macrophage effector molecules, leading to the enhanced levels of NO detected in the lesions after antibody treatment (Li et al., 1999). IL-10 has also been attributed with similar properties of Th1 and macrophage function suppression (Moore et al., 2001). IL-4Rα deficient mice show enhanced resistance on suppression of IL-10 function, indicating a role for IL-10 in conferring disease susceptibility (Noben-Trauth et al., 2003). Enhanced IL-10 levels have also been associated with human cases of chronic localized leishmaniasis, post-kala-azar dermal leishmaniasis (PKDL) and visceral leishmaniasis. IL-10 is produced by a wide variety of cells including macrophages, B cells, DCs and regulatory T cells (T-Regs), and the exact

source of IL-10 over-production in leishmanial infection has been a subject of much debate. Early studies have shown production of IL-10 on macrophages stimulated *in vitro* with LPS and opsonised *Leishmania*, resulting in suppression of IL-12 and TNF-α production (Kane and Mosser, 2001). However, as anti-CD4 treatment mediated depletion of CD4 T cells (Kropf et al., 1997) in IL-4 deficient mice BALB/c mice resulted in rapid control of infection, CD4 T cells were implicated as the crucial source for IL-10 *in vivo*. This view was further strengthened by the high levels of IL-4 and IL-10 mRNA expression levels reported in CD4 T cells from BALB/c mice (Reiner et al., 1994). IL-10 and Fcγ-receptor deficient mice have been reported to produce lower levels of IL-10 and show enhanced lesion resolution (Buxbaum and Scott, 2005). Recent studies have demonstrated production of IL-10 by both conventional $CD25^{-}CD4$ T cells and $CD25^{+}CD4$ regulatory-T cells in a mouse model of chronic leishmanial infection (Anderson et al., 2007; Jankovic et al., 2007). Further, antigen specific IFN-γ secreting Th1 cells at the lesion site were the major contributors to this IL-10 response, and depletion of the T-Reg population, led to exacerbated disease characterized by overproduction of Th2 cytokines including IL-10 (Anderson et al., 2007; Jankovic et al., 2007).These data suggest that antigen specific Th1 cells induced early in infection in the setting of a strong inflammatory response also participate in a feedback control of inflammatory responses in chronic infection, by secreting the IL-10 as a suppressive cytokine.

### *4.3.3 Th17 responses*

Recent work has identified a novel T-helper subset of CD4 T cells (Th17) that are pro-inflammatory in nature and have been implicated in the pathogenesis of autoimmune

diseases. Th17 cells predominantly secrete IL-17 (hence referred to as Th17), in addition they also secrete TNF-α and IL-6 (Langrish et al., 2005). Th17 cells induce many pro-inflammatory cytokines and chemokines leading to tissue infiltration and uncontrolled inflammation. Antibody mediated blockade of IL-17 has been reported to improve the clinical outcome in adjuvant-induced arthritis (Bush et al., 2002) and experimental autoimmune encephalomyelitis (EAE)(Komiyama et al., 2006). Naive T cells activated in the presence of IL-6 and TGF-β have been shown to differentiate into Th17 T cells (Veldhoen et al., 2006). Further expansion of the Th17 response is thought to require the presence of an IL-12 family cytokine, IL-23. Several cytokines have been reported to enhance or attenuate Th17 differentiation or expansion (Bettelli et al., 2007). Cytokines such as IL-1 and TNF, and co-stimulatory molecules ICOS (inducible co-simulator)(Park et al., 2005) and OX40 (Nakae et al., 2003) enhance the generation of Th17 cells. IL-27 expression, on the other hand, negatively regulates Th17 differentiation (Hunter, 2005). The differentiation of Th17 cells is independent of Th1 and Th2 transcription factors, STAT-4 and STAT-6 (Park et al., 2005), and has been identified to be regulated by a separate transcription factor, RORγt (Akimzhanov et al., 2007). Th17 T cells have now been implicated in disease exacerbation in various autoimmune diseases, previously believed to be mediated by Th1 cells (Langrish et al., 2005; Nakae et al., 2003). Ironically, the Th1 cells in such diseases have now been shown to be involved in disease protection due to the inhibitory effect of IFN-γ on Th17 induction (Krakowski and Owens, 1996; Park et al., 2005; Tran et al., 2000; Willenborg et al., 1996). Further, IFN-γ, IL-27 mediated Th17 suppression requires active signaling through a STAT-1 mediated pathway (Batten et al., 2006).

Recent correlational studies on the lesion progression and parasite persistence using the Leishmania amazonensis (*L amaz*) model of infection indicate to interesting possibilities on the modulatory effects of these cytokines. The Lm resistant C57BL/6 mice have been reported to be sensitive to *L amaz* induced cutaneous leishmaniasis, characterized by progressive lesions in the presence of a robust Th1 response, and the lack of IL-4 over-production (Ji et al., 2002). A recent study using two strains resistant (TR) and susceptible (TS) to induction of oral tolerance, has shown increased disease susceptibility in the TR but not in the TS strain on *L amaz* infection (Tavares et al., 2006). Cytokine analysis showed low IL-10 and TGF-β responses, greater lesion size and a paradoxically low lesion parasite burden in the TR mice, with the TS mice showing the converse phenotype. On induction of oral tolerance with low dose of antigen, TR mice showed a regression of lesion and a surprising exacerbation of lesion on immunization with high dose of antigen. The TS strain did not reveal any modification of the phenotype on immunization with either dose of antigen. In view of the known role of IL-10 as an immunosuppressive cytokine, it is possible that the exacerbated lesions seen in the TR strain could be due to the lack of this cytokine, and conversely, higher IL-10 levels in the TS strain could explain lower lesion size and increased parasite persistence in accordance with the NO suppression induced by IL-10. Since induction of oral tolerance leads to increase in the levels of suppressive cytokines IL-10 and TGF-β (Weiner et al., 1994), this could be a reason for the decreased lesion size seen in TR mice immunized at lower antigen doses. Paradoxically, the higher TGF-β levels, could also have led to the induction of Th17 responses at the higher antigen dose, resulting in further exacerbation of disease. Though the role of IL-17 producing T cells in leishmanial infections has not yet been properly addressed, it is likely to play a role in acute exacerbations in disease

after long periods of latency as seen in human cases of PKDL and mucocutaeous leishmaniasis, possibly brought on by a compromise in the IL-17 inhibitory IFN-γ/Th1 responses. Studies to dissect the possibility of the involvement of such operant mechanisms could help better understand human clinical disease exacerbations.

## *5. Immune modulations by retroviruses*

Viruses have been long characterized to induce immune response modulations to improve their infectivity as well as survival. Modulations mediated by retroviruses have come into focus due to the HIV (human immunodeficiency virus) mediated AIDS (acquired immunodeficiency syndrome) pandemic. Other retroviruses, including MMTV (murine mammary tumor viruses) have also been extensively studied and characterized to modulate host immune responses. Retroviruses, integrate into the host genome to gain replication sufficiency, and in the process pose the inherent risk of not only causing modulations in host gene responses, but in the event of inactivating mutations to the viral template the integrant replication deficient viruses, gain vertical transmission and continue to mediate effects on the host genome across subsequent generations.

HIV viral protein Nef (negative factor) participates in down-regulation of surface MHC (Cohen et al., 1999; Stumptner-Cuvelette et al., 2001) and co-stimulatory molecules (Chaudhry et al., 2005) to prevent recognition of virus infected cells by the immune system. In addition, Nef induces secretion of inflammatory cytokines to cause recruitment and activation of T cells (Swingler et al., 1999), which are otherwise resistant to infection in the resting state, thereby facilitating its transmission to uninfected cells. MMTVs, members of beta-retrovirus family, infect mammary tissue in female mice and

are undergo milk borne vertical transmission to the pups, where they track to the intestinal lymphoid tissue to infect B lymphocytes and dendritic cells (Baribaud et al., 1999; Golovkina et al., 1999). The v-SAg (viral encoded superantigen) then binds to MHCII on these infected cells, resulting in activation of large populations of CD4 T cells due to ligation of particular TCR-variable-β regions (TCR Vβ)(Janeway, 1991). The cytokines secreted by these CD4 cells leads to activation of bystander cells, which then become susceptible to MMTV infection, in a similar fashion to that observed with HIV (Baribaud et al., 1999; Maillard et al., 1998). At later stage in infection, these v-SAg reactive T cells eventually undergo deletion (Acha-Orbea and Palmer, 1991).

Some of these retroviruses incur inactivating mutations, rendering them replication deficient, and remain genome integrant. Such genome integrant retroviruses have been estimated to occupy almost 8% of the human genome and up to 6-8 MMTV integrant proviral sequences (*mtv*) have been identified in most inbred mouse colonies (Kozak et al., 1987). Some of these *mtv* sequences however retain their ability to encode for SAgs, which now mediate complete thymic deletion of reactive subsets, rendering the host T cell repertoire deficient in certain TCR Vβ subsets, and since this deletion occurs at negative selection, it affects both the CD4 and CD8 T cell compartments (Tomonari et al., 1993). Such a loss of TCR Vβ subset, mediated by the *mtv-7* locus, has been associated with conferring resistance to cerebral malaria in mice due to the deletion of the causal Vβ 8.1 subset (Gorgette et al., 2002). MMTV integrations have also been reported to mediate susceptibility to tumors (Scianimanico et al., 1999), graft-versus host disease (GVHD) (Allen et al., 2000) and bacterial infections with *Vibrio cholerae* (Bhadra et al., 2006), and in all these instances, MMTV integrations have been reported to increase disease susceptibility independent of their T cell deletion function. Studies on a model of GVHD

with *mtv-7* integrations have demonstrated that the development of disease was independent of *mtv-7* mediated deletion, and have speculated the role of it's modulation on surface so-stimulatory molecules, CD48 (a CD2 ligand involved in regulating the Th1/Th2 balance) in this case, which are in linkage with the *mtv-7* locus (Allen et al., 2000). In another study, an *mtv*-null mouse model was found to be resistant to both MMTV and *V cholerae* infections, both of which did not posses any shared antigenic epitopes (Bhadra et al., 2006). Further, susceptibility to both infections could be reconstituted in the *mtv*-null mice by an endogenous provirus lacking the coding sequences for the immunodominant SAg protein (Haak-Frendscho et al., 1994). Therefore, these data appear to be suggesting a deletion function independent mechanism for immune modulation by such proviral integrations.

Many of the retroviral integrants are believed to have been positively selected during evolution due to their ability to protect against viral infections as demonstrated in recent study where changes in an immune protein have been shown to confer protection against ancient retroviruses which presumably plagued our primate ancestors (Kaiser et al., 2007). On the flip side, however, these very changes might have rendered us susceptible to newer retroviruses, including HIV (Kaiser et al., 2007), or other MMTVs in case of mice (Bhadra et al., 2006). Reactivation of ancient retroviruses is a further possibility arising out of recent studies which demonstrated this possibility using surrogate viral sequences to restore transcriptional activity and replication competency in integrant replication deficient proviruses (Lee and Bieniasz, 2007; Stauffer et al., 2001; Sutkowski et al., 2001).

# *Materials and methods*

# Materials and Methods

# *1. Reagents*

## *1.1 Culture media used:*

All the cell culture experiments were carried out in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, USA) supplemented with 5 mM HEPES, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 1.35 g/l sodium bicarbonate (all from Sigma-Aldrich, USA), 10% heat inactivated FCS (Biological Industries, Israel) and 10 μg/ml gentamycin (Hi Media, India)[Complete medium].

*Leishmania major* promastigotes were cultured in DMEM supplemented with 10% heat inactivated FCS (Biological Industries), 0.05 mM adenosine, 0.05 mM xanthine, 5 mg/ml hemin (dissolved in 0.05 M NaOH), 3 mg/ml BSA, 5mM HEPES, 2 mM L-glutamine, 10 mg/ml gentamycin and 55 μM β-mercaptoethanol (all from Sigma-Aldrich)[Modified DMEM].

## *1.2 Phosphate-buffered saline (PBS) (1X):*

| | | |
|---|---|---|
| KCl | 0.2 g/l | |
| $KH_2PO_4$ | 0.2 g/l | |
| NaCl | 8.0 g/l | |
| $Na_2HPO_4$ | 11.5 g/l | pH adjusted to 7.2-7.4 |

## *1.3 Hanks balanced salt solution (HBSS) (1X):*

A 10X stock of Hank's balanced salt solution (HBSS) (Biological Industries) was diluted to 1X in 0.035% sodium bicarbonate solution in sterile water, and used for washing the cells.

### *1.4 Chemicals:*

Reagents used in the study include dimethyl sulphoxide (DMSO), paraformaldehyde, sodium azide, trinitrobenzene sulphonic acid (TNBS), sodium azide, tween-20, saponin , 2', 7'- dichloroflurescein diacetate (DCF-DA), monensin, sulfanilamide, N-(1-Napthyl) ethylenediamine dihydrochloride, carrageenan iota, lipopolysaccharide (LPS; *Salmonella typhosa*) (all from Sigma-Aldrich), Syto 13 (Molecular Probes, USA), dextan T-500 (Amersham Biosciences, USA). Recombinant granulocyte-macrophage colony stimulating factor (GM-CSF), recombinant mouse IFN-γ (Pepro-Tech Inc., USA). Restriction enzymes, DNA polymerase, dNTPs and DNA ladder (New England Biolabs, USA).

### *1.5 Protein antigens used:*

Maleylated form of chicken egg ovalbumin (Sigma-Aldrich) was used as an antigen to induce ovalbumin specific T cell responses, *in vivo* or *in vitro*.

### *1.6 Reagents used for flow-cytometry:*

Reagents used to stain mouse specific markers were biotin, fluorescein, phycoerythrin (PE), PE-Cy5 or PE-Texas red coupled antibodies directed against B220, CD4, CD8, CD43, CD44, CD62L, CD69, CD90.2 (Thy1.2), Gr-1, IFN-γ, IgM, IgD, TCRVβ3, TCRVβ6, TCRVβ4, TCRVβ10, TCRVβ14 and Siglec-F (All from BD Pharmingen, USA). Labeled secondary detection reagents used included streptavidin-PE, streptavidin-PE-Cy5, and streptavidin-PE-Texas Red (BD Pharmingen).

## *1.7 Reagents used for ELISA:*

For assaying IFN-γ/IL-10 levels in culture supernatants, Mouse IFN-γ/IL-10 ELISA (BD Opt EIA, BD Pharmingen) kits were used. They had the following reagents:

Capture antibody: anti-mouse IFN-γ/IL-10 monoclonal antibody, diluted in coating buffer.

Detection antibody: Biotinylated anti-mouse IFN-γ/IL-10 monoclonal antibody.

Enzyme reagent: Avidin-horseradish peroxidase conjugate

Standard: Recombinant IFN-γ/IL-10

In addition, $H_2O_2$ (E. Merck) and o-phenylenediamine (Sigma-Aldrich) dissolved in substrate buffer were used for developing the ELISA and 2N $H_2SO_4$ (Qualigens, GSK Pharmaceutical Ltd, India) was used for terminating the reaction.

***Coating buffer (0.1M, pH 9.5)***

0.2 M $Na_2CO_3$

0.2 M $NaHCO_3$

8 ml of 0.2M $Na_2CO_3$ and 17 ml of 0.2 M $NaHCO_3$ were mixed and volume made up to 100ml with deionised water.

***Citrate-phosphate buffer (substrate buffer), pH 5.0***

0.1M citric acid

0.1M $Na_2HPO_4$

51.5 ml of 0.1M citric acid and 48.5 ml of 0.2 M $Na_2HPO_4$ were mixed and used as substrate buffer.

### *1.8 Brewer's thioglycollate broth:*

4 g of Brewer's thioglycollate medium (Hi Media) was dissolved in 100 ml of water by boiling. It was then sterilized by autoclaving and stored in dark for at least 2 weeks before use.

## *2. Methods*

### *2.1 Parasite preparation and infection:*

*Leishmania major* (Lm) strain MHOM/Su73/5ASKH (a kind gift of Dr. Bhaskar Saha, National Centre for Cell Sciences, India) was a cultured at 25°C in modified-DMEM. Stationary phase promastigotes were collected from *in vitro* culture and washed with PBS. Parasite number was estimated by counting 2% PFA-fixed parasites at appropriate dilution, using a hemocytometer.

Mice were infected subcutaneously in the hind footpad with $5x10^6$ promastigotes re-suspended in 50µl of PBS (Gray et al., 2006). The clinical progression of the infection was monitored by measuring increase in footpad thickness in comparison to the contralateral uninfected footpad using a dial-gauge micrometer (Mitutoyo, Japan).

### *2.2 Ex-vivo cell preparation:*

Peritoneal exudate cells (PECs) were induced by intraperitoneal (i.p) injection of 4% Brewer's thioglycollate broth. At 16 h (for granulocytes) or 72 h (for macrophages) post-injection, mice were euthanized by cervical dislocation and cells harvested by peritoneal wash with 10 ml of ice cold 0.9% saline. Macrophages were further purified by 1 h of plastic adherence at 37°C, and removal of non-adherent B cells.

These adherent cells were then used as peritoneal macrophages for all assays. Resident peritoneal cells were similarly obtained from the peritoneum in the absence of thioglycollate broth instillation.

Spleen and lymph nodes were dissected out from mice euthanised by cervical dislocation. These tissues were teased between a pair of frosted glass slides to obtain single cell suspensions. Spleen cells were subjected to RBC lysis with Gey's buffer, following which cells were suspended in complete medium.

### *2.3 Bone marrow-derived dendritic cell (BMDC) and Bone marrow-derived macrophage (BMDM) culture:*

Bone marrow cells were obtained by dissecting femurs and tibias from euthanised mice and removing attached tissue. Bone marrow was isolated into 90 mm culture dishes by cutting off the ends of bones, and flushing them with medium. Bone marrow was disrupted to a single cell suspension, by pipetting. Bone marrow cell suspensions were plated at $1x10^6$ cells per ml in complete DMEM supplemented with 10 ng/ml recombinant mouse GM-CSF. On day 3, non-adherent cells comprised mostly by granulocytes were carefully removed, with the addition of fresh medium supplemented with GM-CSF. On day 5 to 7, loosely adherent DC clusters were removed, disrupted to single cells and used as an enriched source of immature BM-DCs in various assays.

For BMDM cultures, bone marrow cells were isolated and cultures in complete DMEM supplemented with M-CSF (30% of L929 fibroblast-conditioned medium as M-CSF source). On day 3 and day 5, spent culture medium was replenished with

appropriately supplemented fresh culture medium. Adherent BMDMs were obtained for use in assays at 5 to 7 days of culture.

### *2.4 Lymphocyte co-culture assay:* (Belkaid et al., 2002)

BMDCs were plated at a density of $2x10^5$ cells per well in 2 ml of complete medium and were either allowed infection with $5 x10^6$ Lm per well in 1 ml of DMEM for 4-6 h or left uninfected. After 4-6 h of infection, the wells were gently washed thrice with ice-cold PBS to remove non-phagocytosed parasites. Draining popliteal lymph nodes were isolated from various groups of infected mice and total lymph node T cells, $CD69^-$ T cells or $CD62L^{low}$ effector CD4 T cells were purified for various experiments by magnetic cell sorting (MACS, Miltenyi Biotec, Germany). Sort purity was checked flow cytometrically and confirmed to be >90% pure. Purified cells were resuspended in 2 ml complete medium and added onto the BMDCs at DC: T cell ratio of 3:1, and cells were spun down onto the adherent DCs at 800 rpm for 3 min. At 16 h of co-culture, cells were isolated and CD69 upregulation was assessed. Intracellular IFN-γ estimation was done on cells at 24 h and/or 48 h in culture. Culture supernatants were collected at 24 and/or 48 h after T cell addition, and supernatant cytokine quantification was performed.

### *2.5 Parasite clearance assay:*

For parasite clearance assays in vitro, macrophages were adhered onto autoclaved 22 mm glass cover-slips in 6-well plates. Cells were plated into wells containing the cover-slips at $5x10^6$ cells per well in 2 ml of complete medium, and allowed to adhere for 1 h at 37°C. The wells were then washed with 2 ml PBS, and $25x10^6$

fluorescent dye (Syto 13)-labelled stationary-phase Lm were overlaid onto the cells in 2ml of DMEM and allowed to infect the macrophages at 37°C for 4 h. The wells were then gently washed thrice with ice cold PBS to remove non-phagocytosed parasites. This was considered as time 0 h of infection, and at various times following that, the coverslips were gently picked out of culture and fixed with a 2% paraformaldehyde (PFA) solution and glycerol-mounted onto glass slides for quantification by fluorescence microscopy (IX81, Olympus, Japan)**(Fig. IIA)**.

Macrophage infection was quantified by counting the frequency of infected cells as well as the parasite number per infected cell. At least 300 cells were counted for each sample and expressed as mean ± standard error (SE) of triplicates.

Parasites were labeled with 5 μM of the nuclear staining cell permeable dye Syto 13 using standard protocols as recommended by the manufacturer (Molecular Probes).The ability of Lm to proliferate after syto13 labeling was tested and found to be unchanged. Also the dividing parasites retained the fluorescence label, even up to 4 days in culture **(Fig. IIA)**.

## *2.6 Maleylation*

### *2.6.1 Protein maleylation:*

The protein to be maleylated was dissolved in borate buffer (5 mM, pH 8.5) at 20 mg/mL, followed by addition of powdered maleic anhydride (Sigma-Aldrich) with constant stirring. The amount of maleic anhydride added was 2.5 times (w/w) than the amount of protein used. pH was maintained between 8.5 and 9.0 with 1N NaOH through out maleic anhydride addition. At the end of the reaction, pH was finally

adjusted to 7.5 and excess maleic anhydride was removed by dialysis against PBS at $4^0$C (Abraham et al., 1995).

### *2.6.2 Protein estimation by BCA method:*

Protein concentrations were estimated by the Bicinchonic acid (BCA) method. Various dilutions of protein and a known protein standard in a volume of 20 µL were taken, to which 200 µL of reagent C (see below) was added and incubated at $37^0$C for 20-30 min. Absorbance was measured at 570 nm on microplate reader (Bio-tek instruments, USA). Protein concentration of experimental samples was calculated by extrapolating from the standard curve thus obtained.

| **Reagent A** | | **Reagent B** | |
|---|---|---|---|
| BCA | 1% | $CuSO_4.5H_2O$ | 4% |
| $Na_2CO_3.H_2O$ | 2% | | |
| NaOH | 0.4% | | |
| $NaHCO_3$ | 0.95% | | |
| $C_4H_4O_6Na_2.2H_2O$ | 0.16% | | |

pH was adjusted to 11.25

**Reagent C** was obtained by mixing reagent A and B in a ratio of 50:1.

### *2.6.3 Estimation of extent of maleylation:*

The extent of maleylation was estimated using the trinitrobenzenesulfonic acid (TNBS) assay for the loss of free ε- amino groups. TNBS reacts with the free amino groups under mild alkaline conditions to give rise to trinitrophenyl (TNP) derivatives. Briefly, 4% sodium bicarbonate, 0.1% TNBS and 1mg/ml of maleylated protein were mixed in equal volumes (100 µl) each and incubated at $40^0$C for 2 hrs, at the end of which 100 µl of 10% sodium dodecyl sulphate was added. The reaction was terminated by adding 1N HCl (50 µl), and absorbance was measured at 335 nm on microplate reader (Bio-tek instruments). The difference in absorbance between native and maleylated proteins indicated the extent of maleylation. Maleyl proteins that showed at least 95% maleylation were used for experiments.

### *2.7 Statistical analysis:*

For in vivo experiments, data are shown as mean ± SE for three mice per group and are representative of three to seven independent experiments. For in vitro cultures, data are shown as mean ± SE for triplicate samples and is representative of three independent experiments. Wherever mentioned, data have been compared by two-tailed student's t test, where $p<0.05$ was considered significant.

## *3. Purification of T cells*

Pooled total lymph node cells form uninfected mice or lesion draining popliteal lymph node cells from Lm infected mice were stained with biotin conjugated

monoclonal antibodies (mAb) as required, for 45 min on ice. Cells were washed with MACS buffer (DMEM supplemented with 1% FCS) and incubated on ice for 30 min with immuno-magnetic streptavidin beads (Miltenyi Biotec) diluted in MACS buffer. The magnetically labeled cell suspension was washed and loaded onto a MACS positive separation column. Positively purified column bound cells were used for further assays. For purification by depletion, cells in the flow through fraction were collected for assays.

Positive purification for various cell subsets was achieved by labeling with biotin conjugated antibody reactive to the subset of interest; mAbs were used against Thy1.2 (total T cells), CD4, and TCRVβ3, TCRVβ6 or TCRVβ14. Antibody cocktail containing mAbs against CD62L, CD69 and CD44 was used for purification of $CD62L^{low}$ effector T cells by depletion. Biotinylated anti-CD8 was also added to the above cocktail for purification of CD4 effector T cells.

## *4. Enzyme linked immunosorbent assay (ELISA)*

### *4.1 Cytokine ELISA:*

Amount of cytokine secreted in culture supernatants was assayed by sandwich ELISA according to manufacturer's instruction. 96 well flat bottom polystyrene ELISA plates (Nunc, Denmark) were coated with capture antibody, anti mouse IFN-γ/IL-10, diluted in carbonate-bicarbonate buffer (pH 9.2) and incubated at 4°C for 14-16 h. The plates were blocked with blocking buffer (10% FCS in PBS containing 0.1% Tween-20, PBS-T) for 2 h at room temperature to prevent non-specific binding of proteins to the plate. After blocking, various dilutions of culture

supernatants (from stimulated cultures) or standard were added and incubated for 1 h at room temperature. The bound cytokines were detected by adding biotinylated anti-mouse IFN-γ/IL-10 and streptavidin-horseradish peroxidase (HRP) conjugate, diluted to appropriate concentration in PBS containing 10% FCS. Plates were incubated for 1 h at room temperature. After each incubation, plates were washed thrice with PBS-T. To develop the ELISA, citrate-phosphate substrate buffer (pH 5.0) containing $H_2O_2$ (30% w/w; 1 µl/ml of the substrate buffer) and OPD (0.5 mg/ml of substrate buffer) was added to each well. $H_2O_2$ is the substrate for HRP enzyme while OPD is a chromogenic substrate and reacts with radicals released during primary reaction of HRP and $H_2O_2$ to yield color. 2N $H_2SO_4$ (Qualigens) was added to terminate the reaction. Absorbance was measured at a wavelength of 490 nm using a microplate reader and compared with the IFN-γ/IL-10 standard.

### *4.2 Immunoglobulin ELISA:*

For detection of IgM or IgG in serum, 96-well ELISA plates were coated with 2 µg/ml goat anti-mouse Ig (Bangalore Genei, India). The plates were blocked with PBS containing 1% lactogen and loaded with diluted sera. The bound antibody was detected with anti-mouse IgM-HRP or IgG-HRP (Southern Biotech, USA) followed by 0.1 M citrate-phosphate buffer containing 1 µl/ml $H_2O_2$ (Merck, India) and 0.5 mg/ml OPD (Sigma). Absorbance was measured at a wavelength of 490 nm using a microplate reader.

## *5. Myeloid cell effector function assays*

### *5.1 Induction and estimation of nitrite:*

Adherent peritoneal macrophages were plated into 96 well plates at $5x10^5$ cells per well and stimulated with titrating doses of bacterial LPS. After 48 h of incubation, accumulated nitrite in the culture supernatant was estimated using the Griess reaction. Briefly, 50 µl of Griess reagent (1% sulfanilamide and 0.1% napthyl-ethylenediamine at a ratio of 1:1 dissolved in 2.5% orthophosphoric acid) was added to 50 µl of culture supernatant and incubated at room temperature for 5 min. Absorbance of the wells was then read at 550 nm, and nitrite levels were calculated in micro-moles (µM) based on a standard curve read from titrating amounts of sodium nitrite standard solution.

### *5.2 Reactive oxygen species induction and estimation:*

Dichlorofluorescein diacetate (DCF-DA), a membrane permeable derivative of 2'7'-dichlorofluorescein (DCFH) was used at a concentration of 5 µM to label cells. On entering the cells DCF-DA is converted to DCFH, by cleavage of the diacetate group, both being non-fluorescent. $5x10^5$ cell per well of adherent macrophages or eosinophils were loaded with DCF-DA dissolved in PBS for 45 min at 37°C. Cells were then washed and stimulated with titrating amounts of LPS added in PBS supplemented with 1% FCS. On induction of oxidative burst, DCFH is converted to highly fluorescent 2'7'-dichlorofluorescein (DCF), mainly mediated by the released $H_2O_2$. The resultant fluorescence, both intracellular and extracellular, is measured

using a fluorimeter (BMG Labtechnologies, Australia) at an excitation of 485 nm and read emission of 530 nm.

### *5.3 Phagocytosis assay:*

Macrophages phagocytosis of fluorescently labeled opsonized *E-coli* (FITC-*E coli*) was performed according to the manufacturer's instruction using the Phagotest kit (ORPEGEN Pharma, Germany). Briefly, heparinized blood samples were obtained by retro-orbital venepuncture from anesthetized mice, incubated on ice for 10 min. Opsonized FITC-*E coli* were then added and samples were incubated at 37°C for 10 min in a pre-heated water bath to allow phagocytosis, similarly treated control sample was incubated at 4°C for 10 min. Extracellular fluorescence was then quenched, erythrocytes lysed and the blood cells were fixed. Following this cells were stained with propidium iodide (PI) for 10 min on ice. Cells were then washed, suspended in PBS and analyzed by flow cytometry.

### *5.4 Carrageenan induced footpad inflammation:*

Carrageenan-induced inflammation was induced by a subcutaneous injection of 0.5% iota-carrageenan (Sigma) dissolved in normal saline into one hind footpad and normal saline alone into the contralateral footpad (50 µl/footpad) (Otterness and Moore, 1988). Footpad thickness was measured prior to injections (T0) and later at intervals of 3 h using a dial-gauge micrometer (Mitutoyo), and expressed as the increase in footpad thickness over T0 at various time points after injection.

## *6. Staining for flow-cytometry*

### *6.1 Surface staining:*

For surface staining, 0.3 x $10^6$ to 1x $10^6$ cells were incubated with 50 µL of primary staining reagent appropriately diluted in staining buffer (PBS containing 0.5% BSA and 0.1% sodium azide), on ice for 45 min in 96 well round bottom polystyrene plates (Nunc). Control samples were incubated with appropriate isotype matched control antibody. The cells were washed thrice with cold staining buffer, followed by incubation for 45 min with 50 µl of appropriate secondary reagent diluted in staining buffer at working concentration. Finally, cells were washed two to three times with ice cold staining buffer and re-suspended in PBS to be analysed on BD LSR (Becton and Dickinson, USA) or Elite, ESP flow cytometer (Beckman Coulter, USA). The data were analysed using Flow Jo software (Treestar, USA).

### *6.2 Intracellular cytokine staining:*

In order to stain for antigen specific CD4 T cells expressing IFN-γ, cells were stimulated with Lm infected BMDCs or BMDCs in presence of mOVA in culture for 24 h to 48 h. The uninfected or unstimulated cultures respectively, served as control. Monensin (10 µM) was added during last 2 h of stimulation to all the cultures. At the end of 2 h, cells were harvested and washed with ice cold staining buffer (PBS, 0.5% BSA, 10 µM monensin), followed by an incubation at $4^0$C for 45 min with the appropriate antibody for surface staining (CD4/CD44) that had been diluted to working concentration in the above staining buffer. Cells were washed with PBS containing 10 µM monensin, fixed with 2% paraformaldehyde and stained with anti-

mouse IFN-γ antibody conjugated to FITC/PE diluted in 50 µl permeabilization buffer containing 0.03% saponin and 50 mg/ml ovalbumin (Vig et al., 2004). After incubation at $4^0$C for 45 min, cells were washed and re-suspended in PBS to be analyzed on a flow cytometer.

### *6.3 Staining of blood cells:*

Peripheral blood was obtained by retro-orbital venepuncture, anti-coagulated with 2 IU/ml of heparin sodium salt solution. Whole blood was then layered onto 3% dextran, in a 1:1 ratio and RBCs were allowed to sediment at room temperature for 30-45 min. Buffy coat cells containing leukocytes were carefully collected and mixed with PBS at a ratio of 1:4. Cells were then pelleted by centrifugation at 1400 rpm for 5 min at 25°C.

## *7. Mice*

### *7.1 Mouse strains used:*

All mice were used at 4-8 weeks of age. The mouse strains used and the respective genotypes were CBA/J (H-2k), CBA/CaJ (H-2k), CBA/N (H-2k) C57BL/6 (H-2b), bg/bg (H-2b), IL-5 Tg (H-2k), iNOS$^{-/-}$ (H-2b), BALB/c (H-2d), B10.LP*nu/nu* (H-2b), TCRβ$^{-/-}$ (H-2b). c-Rel$^{-/-}$ (H-2b) mice were a kind gift from Dr. Ranjan Sen (NIA, NIH, USA).All other strains were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were bred in the small animal facility of the National Institute of

Immunology (New Delhi, India). All experiments were done with the approval of the Institutional Animal Ethics Committee.

### *7.2 Generation of XID/nude mice - Breeding strategy and screening:*

Adenosine deaminase deficient athymic nude mice show deficient αβ T cell development, which is transmitted as an autosomal recessive trait. The XID defect is transmitted as an X-linked recessive trait. Therefore, male B10.PL nude mice and female CBA/N mice were utilized as founder mice. The F1 generation thereof was uniformly heterozygous for both the defects. The F2 generation obtained from random mating of these F1 mice, were then screened for the nude phenotype which is manifested as absence of coat hair in the mice and were further genotyped by genomic-PCR for the XID mutation.

**Fig. IA** shows the expected pedigree chart for this breeding strategy. In this, 6.25% of male and female F2 progeny were expected to be of the XID/*nu* genotype as indicated by filled blocks in the pedigree. For genotyping the XID mutation, genomic PCR was performed to amplify a 558 bp of *Btk* exon-2 **(Fig. IB)**. The C to T transition in an XID mutation results in the loss of a Hha I restriction site, thus allowing for identification of WT and XID alleles by digesting the 558 bp PCR product. The WT allele gave three fragments of 222, 291 and 45 bp, whereas the XID mutation resulted in two fragments of 222 and 336 bp (Rohrer and Conley, 1999).

This genotyping protocol however could not accurately distinguish between female F2 progeny which were heterozygous or homozygous for the XID mutation. Therefore, genotyped male XID/*nu* progeny were back-crossed with female CBA/N mice and the progeny generation (G1), of which all mice now inherited the XID

mutation, were intercrossed by random mating to obtain the G2 generation, of which all progeny carried the XID mutation and 25 %, with 12.5% male and 12.5% female, progeny were XID/*nu*. Simultaneously, male nude mice from the F2 generation, genotyped to be negative for the XID mutation were back-crossed with female CBA/CaJ mice, the WT control for the CBA/N mouse. G2 progeny as indicated from this back-cross were used as age matched WT/*nu* control mice with the XID/*nu* mice in all further experiments.

Genotyping Primers:

5' GAC TGT GGA AGA AGG AGC 3'

5' GGC ATA GAG TGA GTT CTT AC 3'

### *7.3 Generation of XID/TCR $\beta^{-/-}$ mice - Breeding strategy and screening:*

CBA/N female mice were bred with male TCR $\beta^{-/-}$ mice. The F1 generation was intercrossed and the resulting F2 generation mice were genotyped by genomic PCR screening for the XID/TCR $\beta^{-/-}$ progeny. XID genotyping was done as described in the previous section. On genotyping for the TCR β allele according to the genotyping protocol recommended by the Jackson Laboratory, inheritance of a wild-type TCR β allele resulted in amplification of a 540 bp fragment, TCR $\beta^{+/-}$ (heterozygous) mice showed fragments of 540 bp and 280 bp, and the TCR $\beta^{-/-}$ mice showed only a 280 bp fragment **(Fig. IIB)**.

Genotyping Primers: For Jackson Laboratory mouse strain no. 002118

5' CTT GGG TGG AGA GGC TAT TC 3'

5' AGG TGA GAT GAC AGG AGA TC 3'

5' TGT CTG AAG GGC AAT GAC TG 3'

5' GCT GAT CCG TGG CAT CTA TT 3'

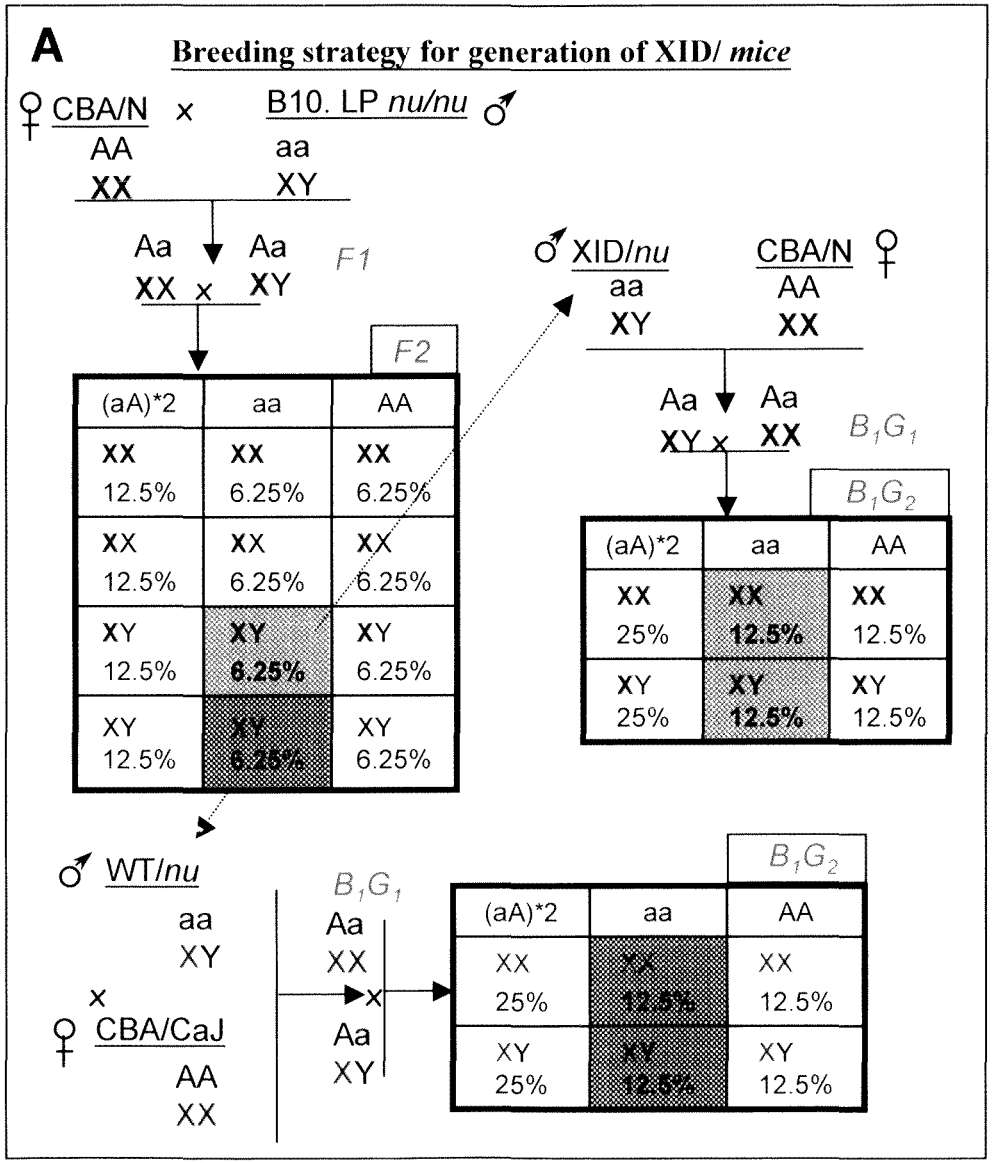
## *8. Histology:*

Footpad tissue was dissected out and fixed in Bouin's solution. After paraffin embedding, 5 μm sections were then prepared and hematoxylin- and eosin-stained using standard protocols. Prepared samples were then mounted on glass slides. All images were acquired on an inverted light microscope (Olympus, IX81) and analyzed using DP-10 software (Olympus).

## *9. Total and differential leukocyte count:*

To perform differential leukocyte counts (DLC), thin blood smears were made on a clean glass slide and stained with Leishman's stain. Cells were identified by morphology seen under oil immersion at a magnification of 100X. A total of 400 cells were counted and the frequency of various leukocyte subsets was expressed as a percentage.

For estimation of total leukocyte counts, peripheral blood was diluted 1:20 with leukocyte dilution fluid (2% acetic acid in water) to lyse RBCs and the cells were counted on a hemocytometer.



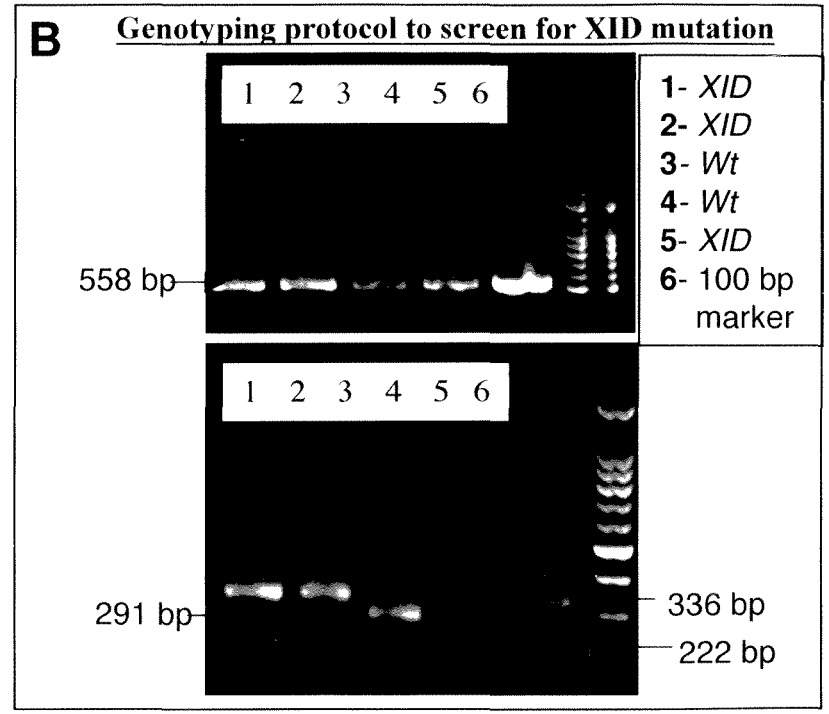




**Figure I: Breeding strategy and screening protocol of XID/*nu* mice**

**(A)** Female CBA/N mice were bred with male B10.LP *nu/nu* mice to generate F1 and F2 mice. Genotyped male XID/*nu* or WT/*nu* mice were back-crossed with female CBA/N or CBA/CaJ mice respectively. First generation of back-cross progeny ($B_1G_1$) were intercrossed and the litter ($B_1G_2$) were genotyped and used for experiments. **(B)** 558 bp region of exon-2 of *Btk* was PCR amplified from genomic DNA, digested with Hha-I and the resultant fragments were resolved by electrophoresis on a 2% agarose gel.

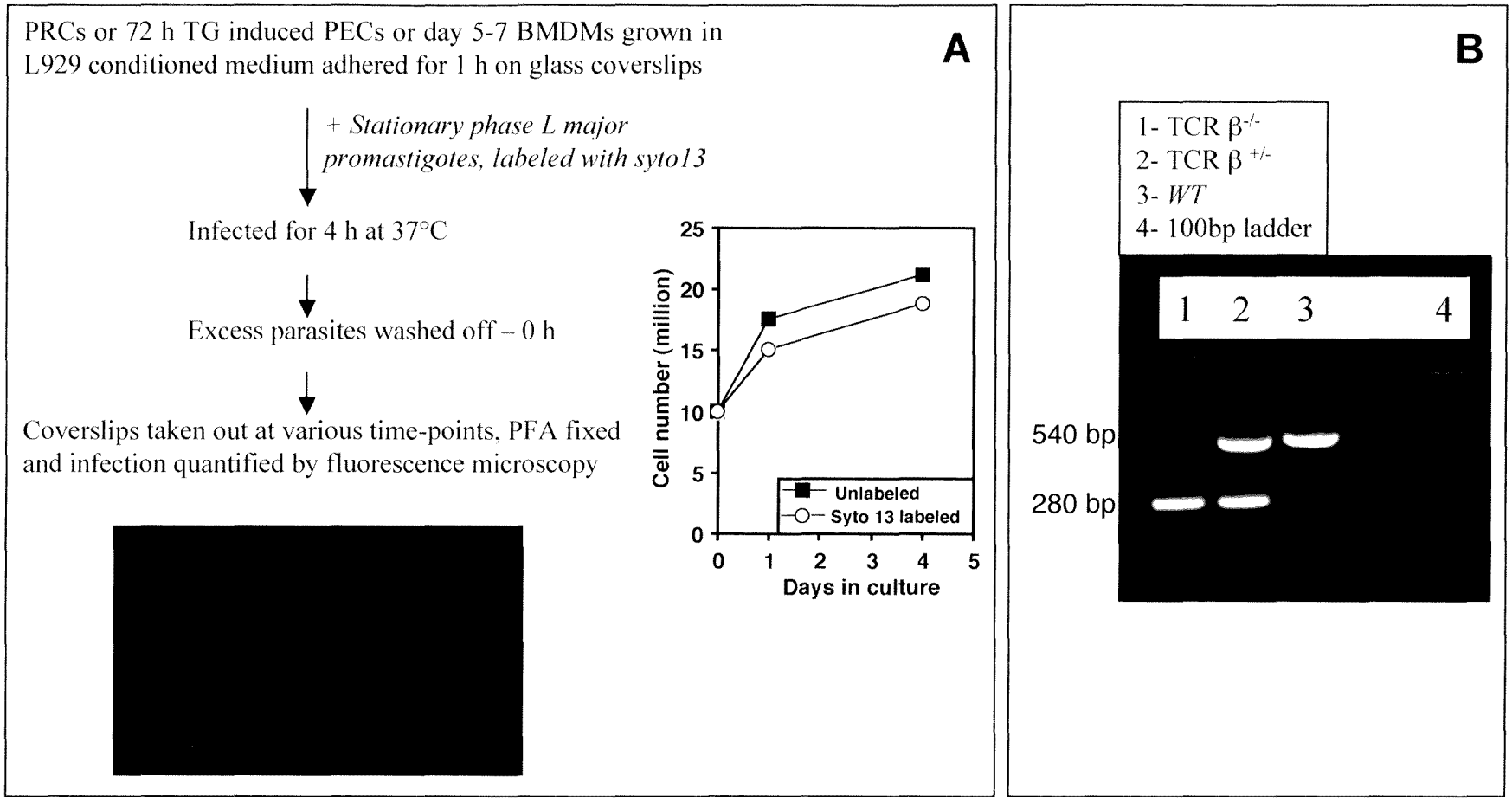



**Figure II: Parasite clearance assay and genotyping of TCR $\beta^{-/-}$**

**(A)** Protocol for parasite clearance assay. Image shows BMDMs infected with Syto 13 labeled parasites and the graph depicts parasite recoveries at indicated time-points in culture from a starting population of 10 x $10^6$ unlabeled or Syto 13 labeled parasites. **(B)** Mouse genomic DNA was PCR amplified using specific primers recommended by the Jackson Laboratory, and the resultant PCR products were resolved on a 2% agarose gel.

# Results

# Results

## *Role of signaling intermediate Btk in regulating cellular interactions in inflammation*

Bruton's tyrosine kinase (BTK), a member of the TEC family of non receptor tyrosine kinases, functions as a major intermediate in signal transduction pathways downstream of a variety of cell surface receptors to modulate both cellular developmental and functional properties. BTK has been shown by various extensive studies to be a key molecule involved in B cell development and function, both in humans and mice. Previous work has focused on dissecting the role of BTK in myeloid cells using the CBA/N (X-linked immunodeficiency; XID) mouse, which carries an arginine to cysteine (R28C) mutation at the 28$^{th}$ amino acid position in the pleckstrin homology domain of BTK, thereby abrogating its membrane recruitment and signaling functions (Mohamed et al., 1999). Through these studies a role has been demonstrated for BTK in mediating inflammatory functions and development of myeloid cells including macrophages and neutrophils (Horwood et al., 2003; Kawakami et al., 1998; Kawakami et al., 2006; Mangla et al., 2004; Schmidt et al., 2004). It was therefore appropriate to ask if BTK plays a similar role in the eosinophil lineage.

## *Role of Btk in regulating eosinophil effector functions*

### *Eosinophils constitute a high proportion of granulocytes in IL-5 Tg mice*

IL-5 is a major cytokine driving eosinophil recruitment and function. In the IL-5 transgenic (Tg) mouse, where IL-5 expression is controlled by an inducible

metallothionein promoter, eosinophils have been reported to constitute the majority of the polymorphonuclear (PMN) cells in peripheral circulation (Tominaga et al., 1991). This was confirmed by total and differential leukocyte counts on peripheral blood of IL-5 Tg and C57BL/6 wild-type (WT) mice **(Fig. 1A)**. The differential leukocyte counts revealed a high frequency of eosinophils in the peripheral blood smear of the IL-5 Tg mice, compensated by a lower frequency of neutrophils as compared to the WT. Total circulating leukocytes per cu mm of blood were also substantially higher in the IL-5 Tg mice **(Fig. 1B)**. These results were also confirmed flow cytometrically by staining dextran separated peripheral blood leukocytes for mouse eosinophil specific surface sialic acid glycoprotein, Siglec-F. Siglec-F bright, Gr-1 dull leukocytes were identified and scored as eosinophils **(Fig. 1C)**. In the first 16 h after peritoneal instillation of brewer's thioglycollate broth (TG), PMNs are the major cell type to be recruited. In the IL-5 Tg mice, 16 h TG induced peritoneal exudate cells (PECs), in keeping with the peripheral blood PMN profile, were largely composed of eosinophils with a frequency of up to 50-70% of recruited cells as compared to less than 1% eosinophils in WT mice.

### *Compromised eosinophil effector functions in XID/IL-5 Tg mice*

In order to generate a rich source for XID eosinophils, IL-5 Tg male mice were crossed with female CBA/N mice. The male F1 progeny all of which inherited the XID mutation and expressed the IL-5 transgene were used as XID/IL-5 Tg and the heterozygous female littermates as WT/IL-5 Tg mice. Dextran separated peripheral blood leukocytes from the XID/IL-5 Tg and WT/IL-5 Tg were stained with Siglec-F and analyzed for eosinophil frequency by flow cytometry **(Fig. 2A)**. On analysis of the frequencies of Gr-1+Siglec-F-

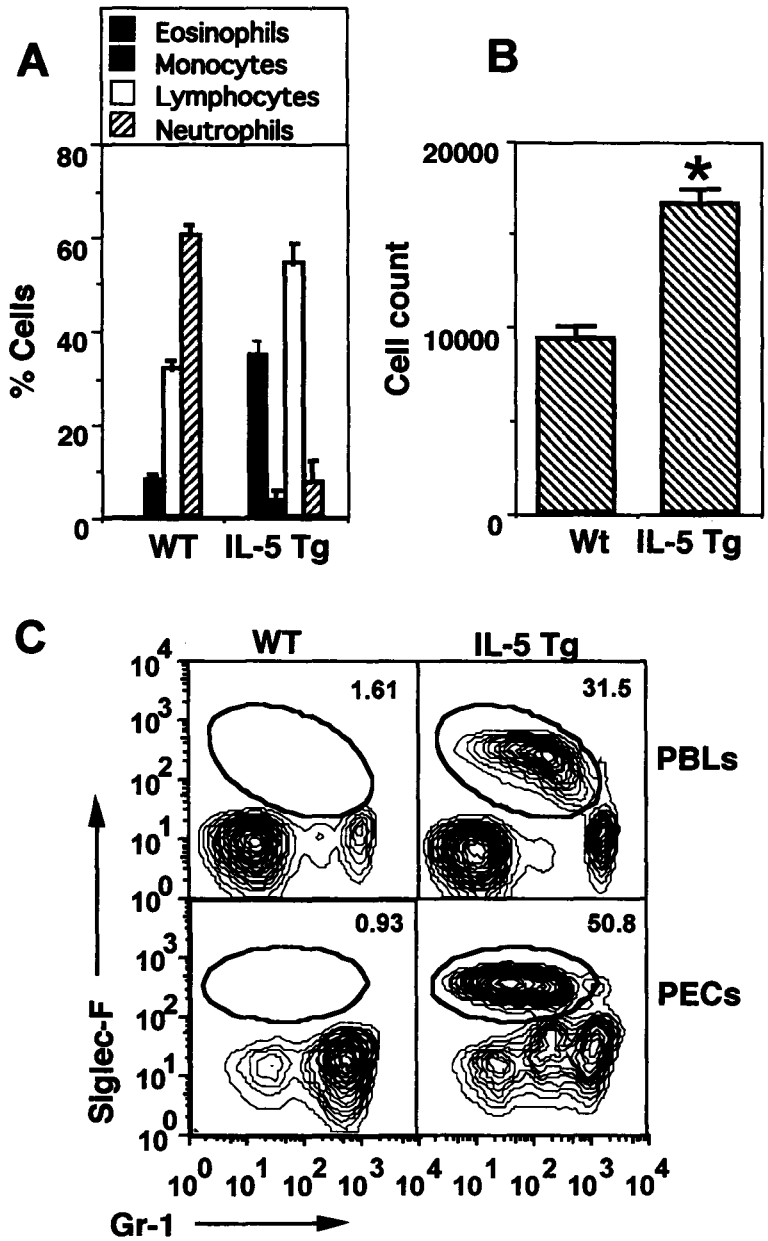



***Figure 1: Eosinophils constitute a high frequency of circulating granulocytes in the IL-5 Tg mice***

Peripheral blood samples were obtained from WT (C57BL/6) and IL-5 Tg mice and differential leukocyte count **(A)** and total leukocyte counts per cubic microlitre of blood **(B)** were estimated. Peripheral blood leukocytes (PBLs) and Peritoneal exudate cells (PECs) **(C)** were stained for Siglec-F and Gr-1 and analyzed by flow cytometry to estimate frequency of eosinophils, identified as Siglec-$F^{bright}$Gr-$1^{dull}$ cells. Data have been compared using two-tailed student's t-test.(*p=0.002)

neutrophils and Gr-1-Siglec-F+ eosinophils, the XID/IL-5 Tg mice showed a slightly higher eosinophil frequency **(Fig. 2B)**. When considered in the context of the mild defect in PMN development in XID mice (Mangla et al., 2004), a possible interpretation could be that the XID mutation has a more deleterious effect on PMN generation than it has on the eosinophil lineage. These results will need further confirmation to ascertain if this indeed is the case.

The ability of eosinophils to migrate in response to peritoneal instillation TG was examined next. Eosinophils were found to constitute a majority of the recruited granulocytes, 16 h after TG (Brewer's thioglycollate broth) instillation. However, the total number of recruited eosinophils was lower in the XID/IL-5 Tg mice as compared to the WT/IL-5 Tg **(Fig. 2C)**.

The major myeloid cell effector molecules generated in an inflammatory response are reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI). XID macrophages and neutrophils have been previously shown to be poorer at RNI and ROI generation in response to stimulation with bacterial lipopolysaccharide (LPS). No RNI generation could be elicited from eosinophils on stimulation with LPS. However, ROI generation could be demonstrated with LPS stimulation on 16 h TG elicited peritoneal eosinophils. TG elicited eosinophils were resuspended at $5x10^5$ cells/ml, loaded with dye dichlorofluorescein-diacetate (DCF-DA) and stimulated with titrating doses of LPS. ROI induction was then measured at various times of stimulation using a fluorimeter. XID eosinophils showed poorer induction of ROI when stimulated with either 30μg **(Fig. 2D)** or 10μg **(Fig. 2E)** of LPS at all the time points tested. These results indicated a role for

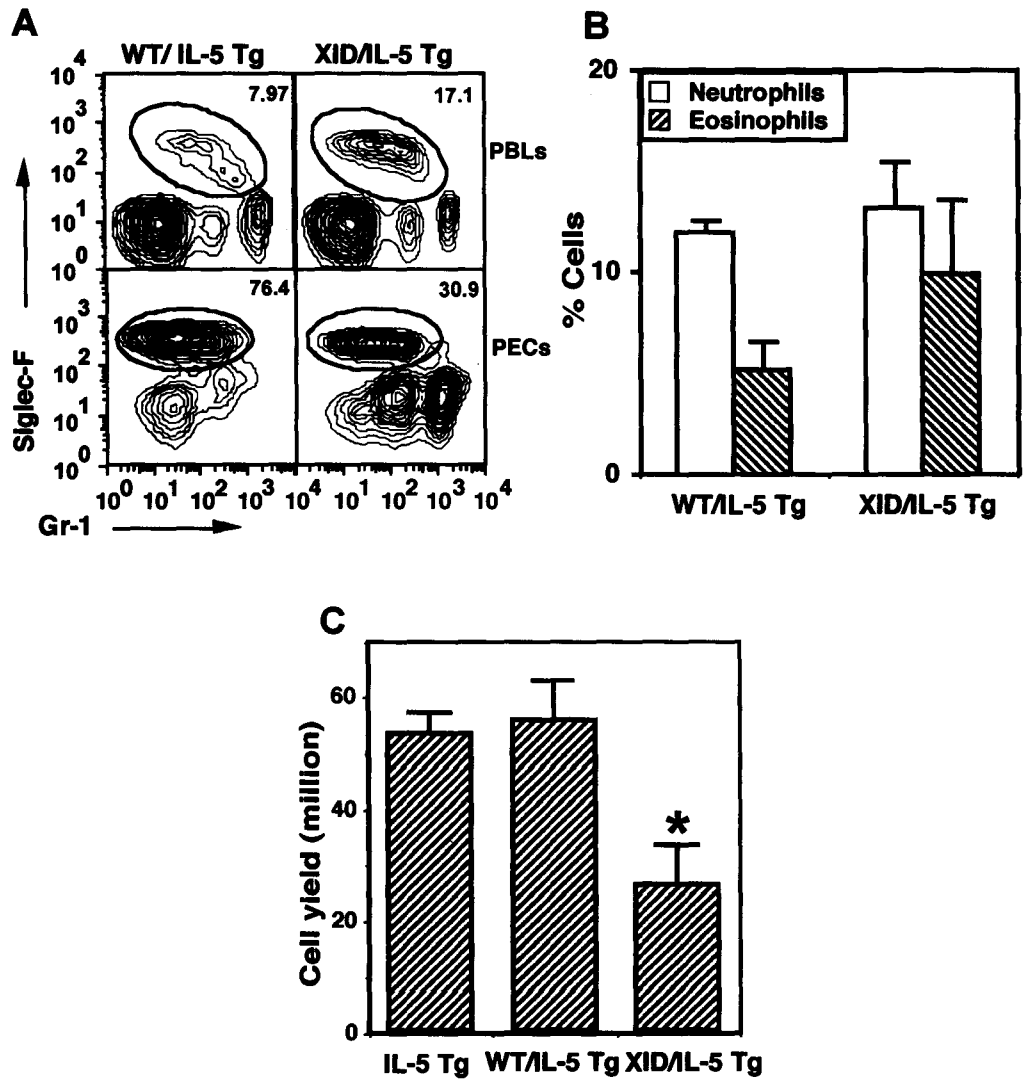



***Figure 2: XID eosinophils show poorer recruitment and ROI induction***

PBLs or PECs from WT/IL-5 Tg or XID/IL-5 Tg mice were stained with Siglec-F and Gr-1, analyzed by flow cytometry **(A)** and frequency of eosinophils (Siglec-$F^+$) ad neutrophils (Gr-$1^+$) estimated on peripheral blood leukocytes and represented as mean $\pm$ SE (n=3)**(B).** Total number of PECs recovered for all the strains were also calculated and are represented as mean $\pm$ SE (n=4) **(C).** Data have been compared using two-tailed student's t-test.(*p=0.027)

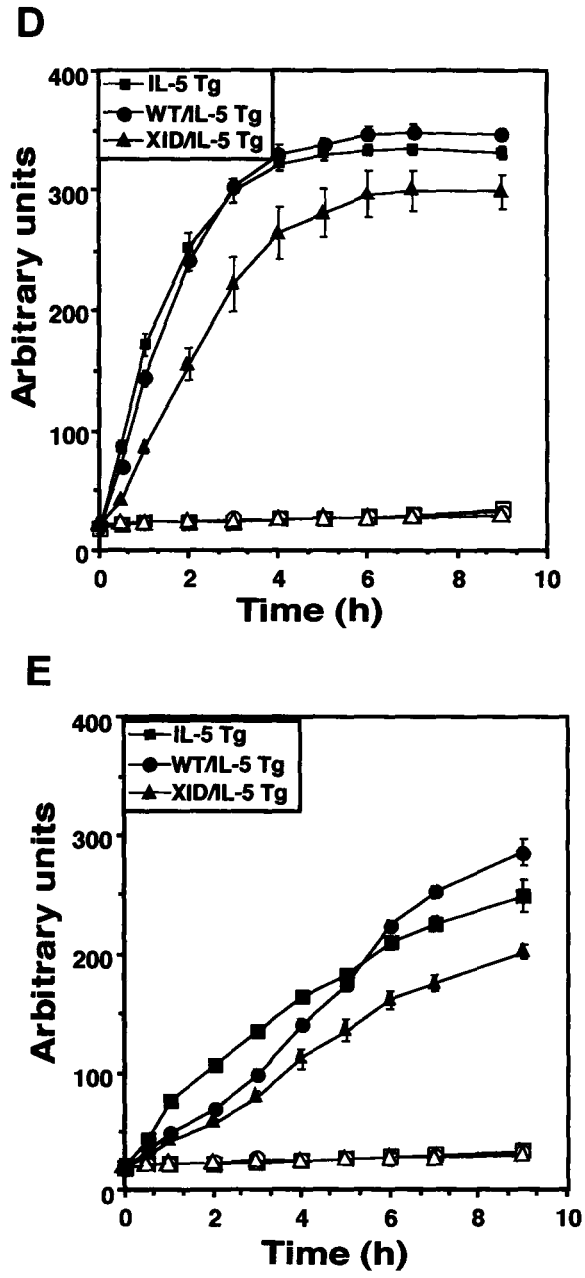



***Figure 2 (contd.): XID eosinophils show poorer recruitment and ROI induction***

16 h TG induced eosinophils were loaded with DCF-DA dye and stimulated with 50 μg **(D)** or 30 μg **(E)** LPS to induce ROI generation. ROI generation was detected over subsequent times as indicated. Filled symbols represent cells stimulated with LPS and open symbols represent unstimulated controls.

BTK in the regulating eosinophils effector responses, as has earlier been reported in other cells of the myeloid lineage.

## *Role of c-Rel in regulating myeloid cell functions*

Previous work has demonstrated poor induction of the Rel family of NF-kB transcription factors. In LPS stimulated XID mouse macrophages (Mukhopadhyay et al., 2002). c-Rel deficient mouse macrophages have also been reported to show compromised induction of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) as well as effector cytokine IL-12, an important inducer of IFN-γ (Sanjabi et al., 2000). These findings indicated a possible role for c-Rel in regulation of myeloid effector functions. The c-Rel knockout (c-Rel$^{-/-}$) mouse model was chosen as the model system for this study.

## *Unaltered leukocyte frequency and number in peripheral blood of c-Rel$^{-/-}$ mice*

Peripheral blood smears of c-Rel$^{-/-}$ and WT (C57BL/6) mice were stained with Leishman's stain and differential leukocyte count was performed. Blood smear of c-Rel$^{-/-}$ mice showed slightly higher frequency of lymphocytes balanced by a lower granulocyte frequency as compared to the WT. However, the total leukocyte count was within the normal range in both the strains **(Fig. 3A)**.

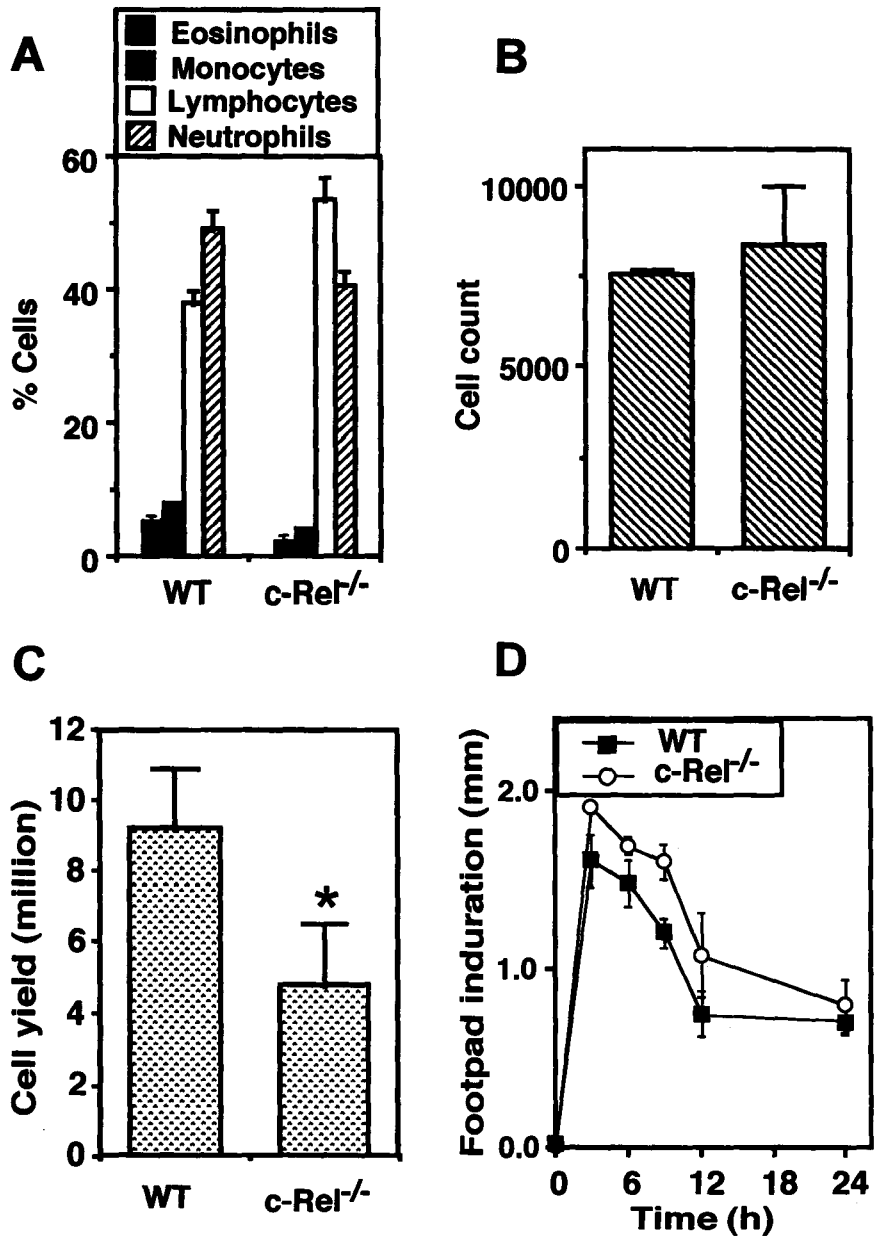



***Figure 3: Peripheral blood leukocyte frequency and recruitment to sites of inflammation are unaffected in c-Rel$^{-/-}$ mice***

Peripheral blood total **(A)** and differential **(B)** leukocyte counts of WT (C57BL/6) and c-Rel$^{-/-}$ were estimated. Cell yields of 72 h PECs recruited in response to TG in BL/6 and c-Rel$^{-/-}$ are expressed as mean $\pm$ SE (n=9) **(C)**. Footpad induration induced by injection of 0.5% iota carrageenan, expressed as millimeter increase in footpad thickness in comparison to contralateral footpad injected with normal saline **(D)**. Values are represented as mean $\pm$ SE (n=5 mice per group). Data have been compared using two-tailed student's t-test.(*p=0.051).

## *Myeloid cell recruitment to sites of inflammation in c-Rel$^{-/-}$ mice*

WT and c-Rel$^{-/-}$ mice were primed with TG given intraperitoneally (i.p). 72 h later peritoneal exudate cells which mainly comprised of macrophages were elicited and cell yields estimated. It was observed that similar numbers of macrophages were recruited to the peritoneum in the c-Rel$^{-/-}$ as compared to the WT **(Fig. 3B)**.

To analyze granulocyte functioning *in vivo*, carrageenan was injected subcutaneously (s.c) into the footpad of WT and c-Rel$^{-/-}$ mice to induce an acute inflammation mediated by infiltrating granulocytes. The resulting increase in footpad induration was observed over the next 24 h as a measure of efficiency of granulocyte recruitment. Contralateral footpad injected with 0.9 % saline served as controls. c-Rel$^{-/-}$ attained similar peak footpad swelling attained by 3 h and showed similar kinetics of recovery thereafter as compared to WT mice **(Fig. 3C)**.

## *Myeloid cell effector functions in c-Rel$^{-/-}$ mice*

The ability of leukocytes, both monocytes and granulocytes, in heparinized whole blood from WT and c-Rel$^{-/-}$ mice to phagocytoze fluorescence labeled E-coli (FITC-*E coli*) was estimated by a flow cytometry based phagocytosis assay. Heparinized whole blood from WT and c-Rel$^{-/-}$ mice was incubated with fluorescence labeled heat killed E-coli (FITC-*E coli*) at 37°C for 10 min. A similarly processed sample, incubated on ice at 4°C for 10 min, acted as the control. Flow cytometric analysis on PI gated cells was done to assess frequency of cells that have phagocytozed FITC$^{+}$ *E-coli*. Mean fluorescence intensity of FITC$^{+}$ cells gave an estimate of the number of bacteria per cell. The frequency of cells

that had taken up the labeled bacteria as well as per cell bacterial load was similar between the c-Rel$^{-/-}$ and WT leukocytes **(Fig. 4A)**.

TG elicited peritoneal macrophages were stimulated with titrating doses of LPS. After 48 h of stimulation, RNIs detected as accumulated nitrite in the culture supernatants were estimated by Griess reaction. Both c-Rel$^{-/-}$ and WT cells showed comparable levels of nitrite output at all the titrating amounts of LPS tested **(Fig. 4B)**.

Reactive oxygen species generation was next examined. For this, peritoneal macrophages were loaded with dye DCFH-DA and stimulated with two different doses of LPS (30μg and 50μg) and ROI induction was measured at subsequent time points. Both c-Rel$^{-/-}$ and WT macrophages showed equivalent ROI induction at the lower dose of LPS (30μg) stimulus at all the time points tested **(Fig. 5A)**. However, at the higher dose of LPS (50μg) stimulus, c-Rel$^{-/-}$ showed slightly lower levels of ROI induction at early time points, but eventually attained WT levels by 8 to 10 h of stimulation **(Fig. 5B)**.

It was possible that ROI induction at higher doses requires transcriptional and /or translational events to take place in order to generate the higher levels of oxygen intermediates and that these events may not be crucial at lower doses of LPS stimulation. This could explain the apparent discrepancy of an observed deficit in ROI induction in c-Rel$^{-/-}$ macrophages at the higher LPS stimulus (50μg).

To examine this, ROI induction was studied in WT macrophages stimulated with 50μg LPS in the presence of inhibitors of protein synthesis and transcription, Cycloheximide

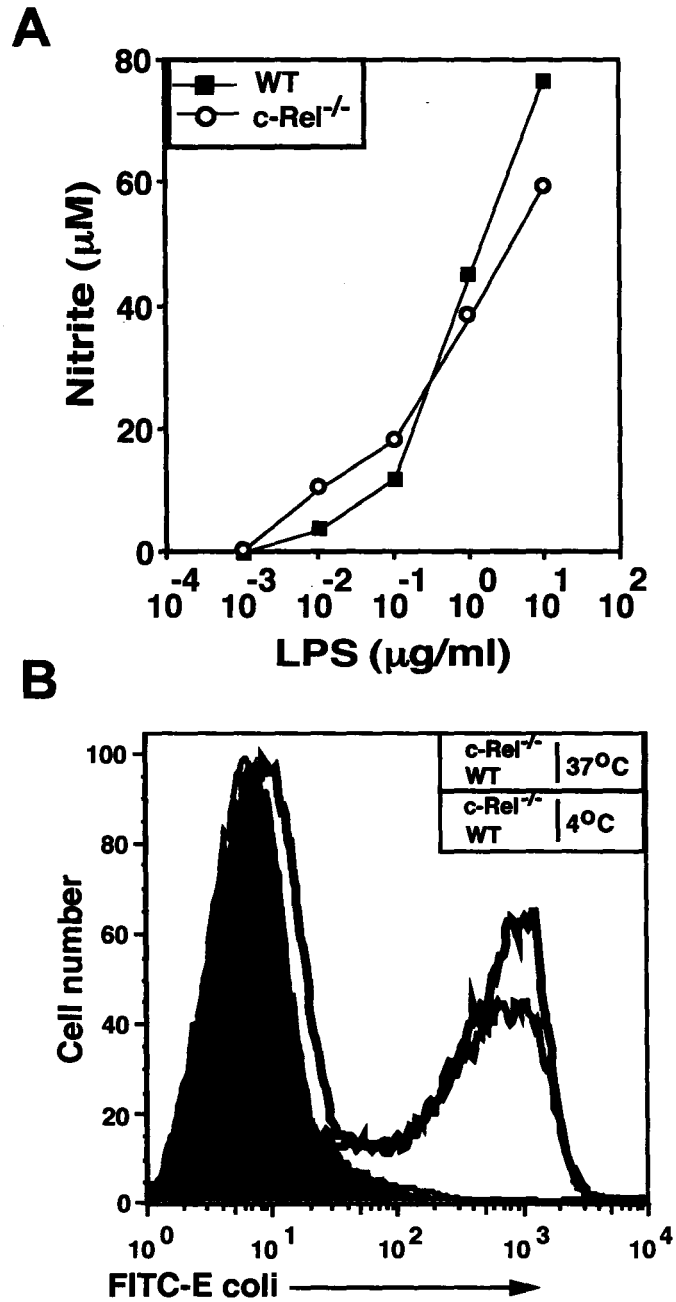



*Figure 4: Macrophage effector function in c-Rel $^{-/-}$ mice are unaltered*

72 h TG induced peritoneal macrophages were stimulated with titrating amounts of LPS, at 48 h of culture supernatant nitrite accumulation was estimated by Griess test **(A)**. Whole blood leukocytes from WT (C57BL/6) (red thick line) or c-Rel$^{-/-}$ (blue thick line) were allowed to phagocytose fluorescencetly labelled E-coli at 37°C and bacterial uptake was estimated on PI positive cells (according to manufacturer's protocol) by flow cytometry **(B)**. WT (shaded histogram) or c-Rel$^{-/-}$ (black thin line) cells incubated with bacteria at 4°C acted as controls.

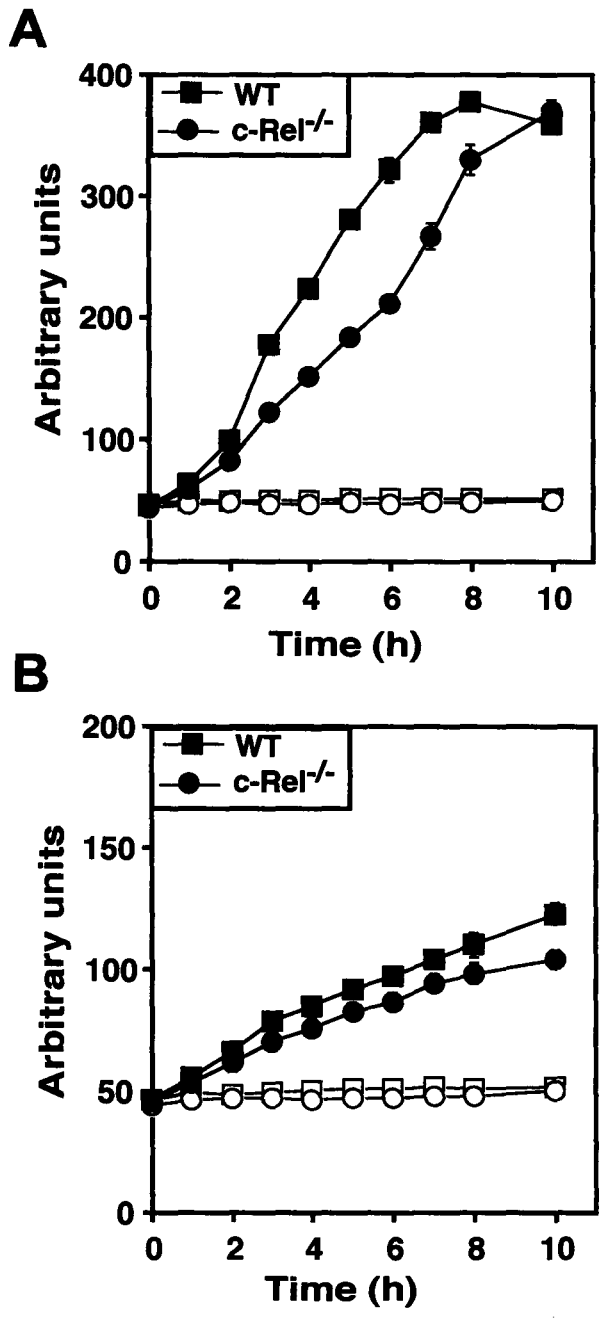



***Figure 5: c-Rel$^{-/-}$ macrophages show no compromise in ROI induction***

Macrophages from c-Rel$^{-/-}$ and WT (C57BL/6) mice were loaded with DCF-DA dye and ROI generation was induced with either 50μg **(A)** or 30μg **(B)** of LPS. Filled symbols represent LPS stimulated cells and open symbols represent unstimulated controls.

and Actinomycin D respectively. No compromise in ROI induction was seen in the presence of either Cycloheximide or Actinomycin D **(Fig. 6)**. Thus, confirming the absence of any role for fresh transcriptional or translational events in ROI induction in macrophages on LPS stimulation.

Many of the NF-kB family of transcription factors are known to have functionally redundant roles (Ghosh et al., 1998). Therefore, in the absence of c-Rel, the other Rel family members might compensate for its functions resulting in no apparent deficit in the development or effector functions of macrophages.

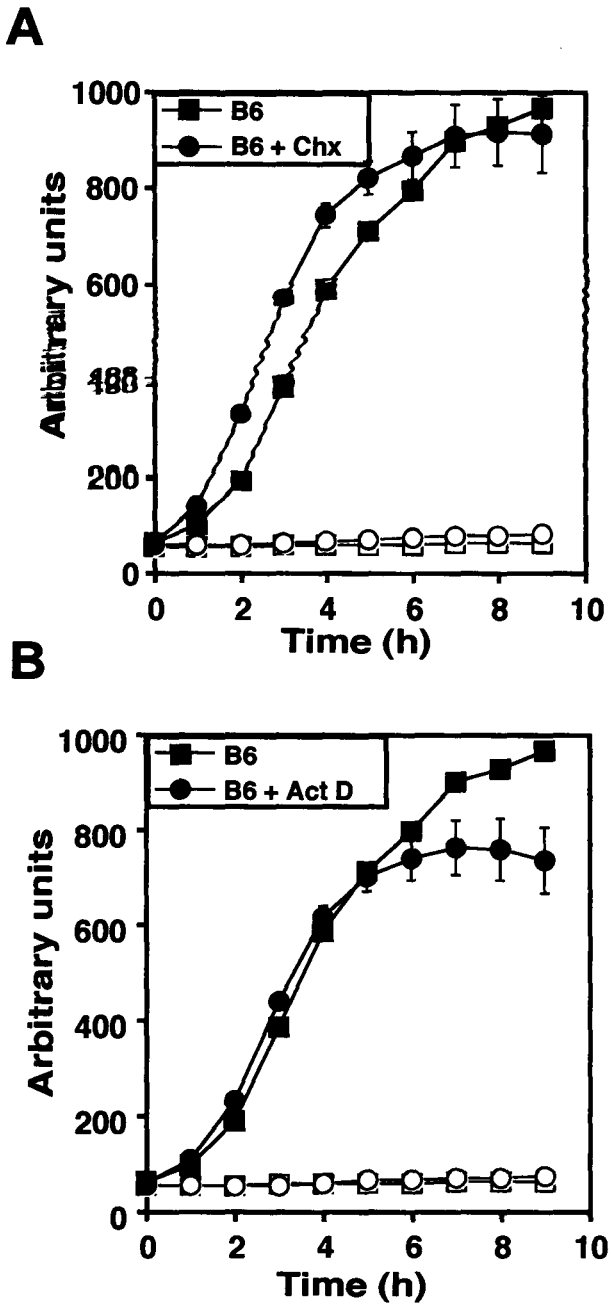



***Figure 6: Induction of transcription or translation are not essential for ROI generation***

C57BL/6 macrophages pre-treated with 100 μg/ml Cycloheximide (Chx) **(A)** or 1 μg/ml of Actinomycin D (Act D) **(B)** for 1 h were loaded with DCF-DA dye and stimulated with 50 μg of LPS in the presence of the inhibitor and ROI generation was estimated over time as indicated. Data are shown as mean ± SE of triplicate a culture. Open symbols represent cells which received no LPS stimulation.

## *Cellular cross-talk in the role of BTK in B cell development*

The human disease caused by non functional BTK, X-linked agammaglobulinemia (XLA) is characterized by a B cell developmental block at the pro- to pre-B cell transition, resulting in a severe deficiency of mature B cells and circulating immunoglobulin of all isotypes (Conley, 1985). The XID defect in mice, either in the XID mutant CBA/N or the BTK deficient *Btk*$^{-/-}$, results in a less severe phenotype. XID mice have lower number of peripheral B cells at 50-60% of normal frequency, severely reduced IgD$^{+}$ mature B cells in lymphoid organs and lower levels of circulating IgM and IgG3, with normal levels of the other immunoglobulin isotypes (Hardy et al., 1983; Hardy et al., 1984; Kerner et al., 1995). Anecdotal reports show that deficiency of T cells (Wortis et al., 1982) or of CD40 (Khan et al., 1997) in the XID mice leads to a worsening of the B cell immunodeficiency approaching in severity that seen in humans, thereby, indicating a possible role for T cell mediated signaling to B cell development.

To examine this further, the CBA/N mice were initially bred with athymic *nude (nu)* mice to generate the XID/*nude* mice. Age matched WT/*nu* were used as controls.

### *Reduced serum immunoglobulin levels in the XID/nu mice*

Total circulating serum immunoglobulins of IgM and IgG isotypes in the XID/*nu*, CBA/N and the corresponding WT mice, WT/*nu* and CBA/CaJ respectively, were estimated by enzyme linked immunosorbent assay (ELISA). Both CBA/N and XID/*nu* mice showed reduced levels of IgM **(Fig. 7A)** with normal levels of IgG **(Fig. 7B)** as

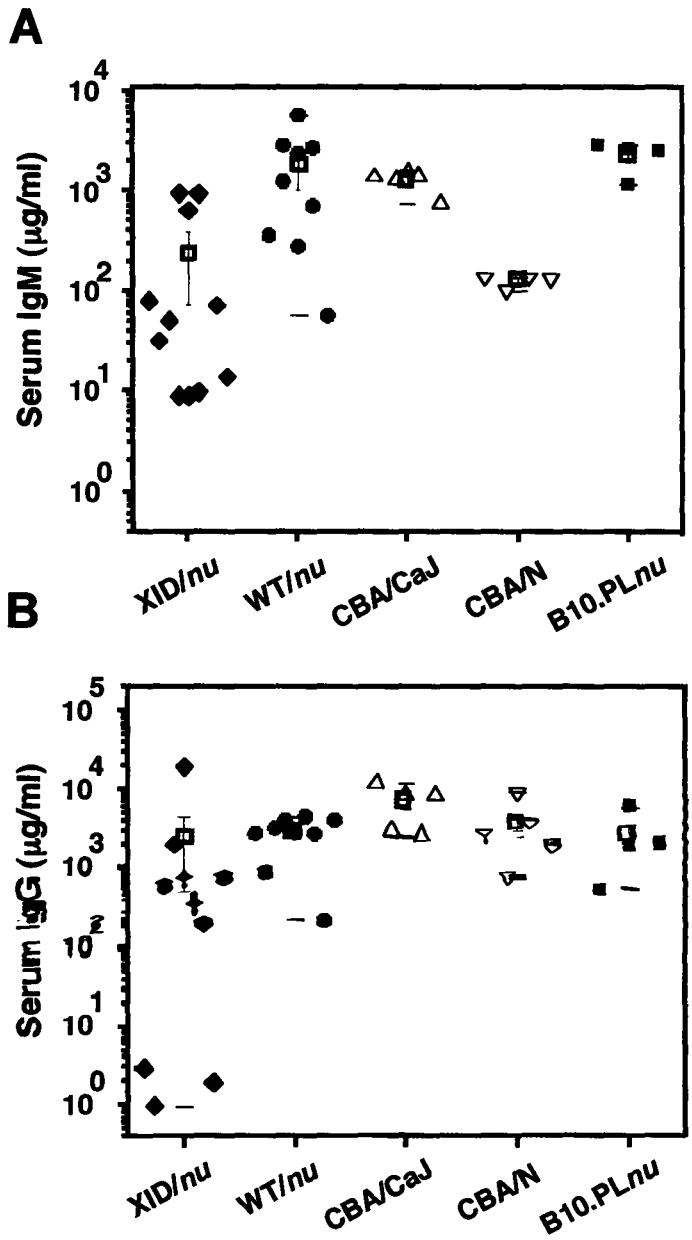



***Figure 7: Reduced serum IgM and variable IgG levels in XID/nu mice***

Serum IgM **(A)** or IgG **(B)** levels were estimated in 4-8 week old mice of the various genotypes as indicated. Symbols correspond to individual mice and boxes represent mean $\pm$ SE values for the indicated genotype.

compared to the wild-type CBA/CaJ or WT/*nu*. In some individual XID/*nu* mice there was a marked reduction in serum IgM associated with very low levels of serum IgG. This heterogeneity in the levels of serum immunoglobulin in the XID/*nu* mice could be due to the presence of mature T cells in the periphery, subsequent to escaping the *nude* T cell developmental block which has been reported to be leaky in its expression. To examine this, peripheral lymphoid organ cell profiles of representative mice showing reduced IgM levels ($IgM^{low}IgG^{high}$) or reduced levels of both IgM and IgG ($IgM^{low}IgG^{low}$) were analyzed.

## *B cell frequency in bone marrow and peripheral lymphoid organs of XID/nu*

Frequency of $B220^+$ splenic B cells was lower in the CBA/N and $IgM^{low}IgG^{high}$ XID/*nu* mice when compared to the WT mice. The $IgM^{low}IgG^{low}$ XID/*nu* mice, on the other hand showed a more severe depletion of the splenic B cell compartment, manifested by an exceedingly low splenic B cell frequency **(Fig. 8A)**.

The $IgM^{low}IgG^{high}$ XID/*nu* also showed reduced numbers of mature circulating B cells of the $IgD^+$ subset coupled with a higher number of immature $IgM^+$ B cells. This was very similar to the subset distribution of the B cell compartment observed in the CBA/N mice, indicative of block in the splenic transition of $IgM^+$ immature B cells into $IgD^+$ mature B cells. On the other hand, the $IgM^{low}IgG^{low}$ XID/*nu* mice showed a more pronounced reduction in the $IgD^+$ as well as the $IgM^+$ subset of B cells **(Fig. 8B)**.

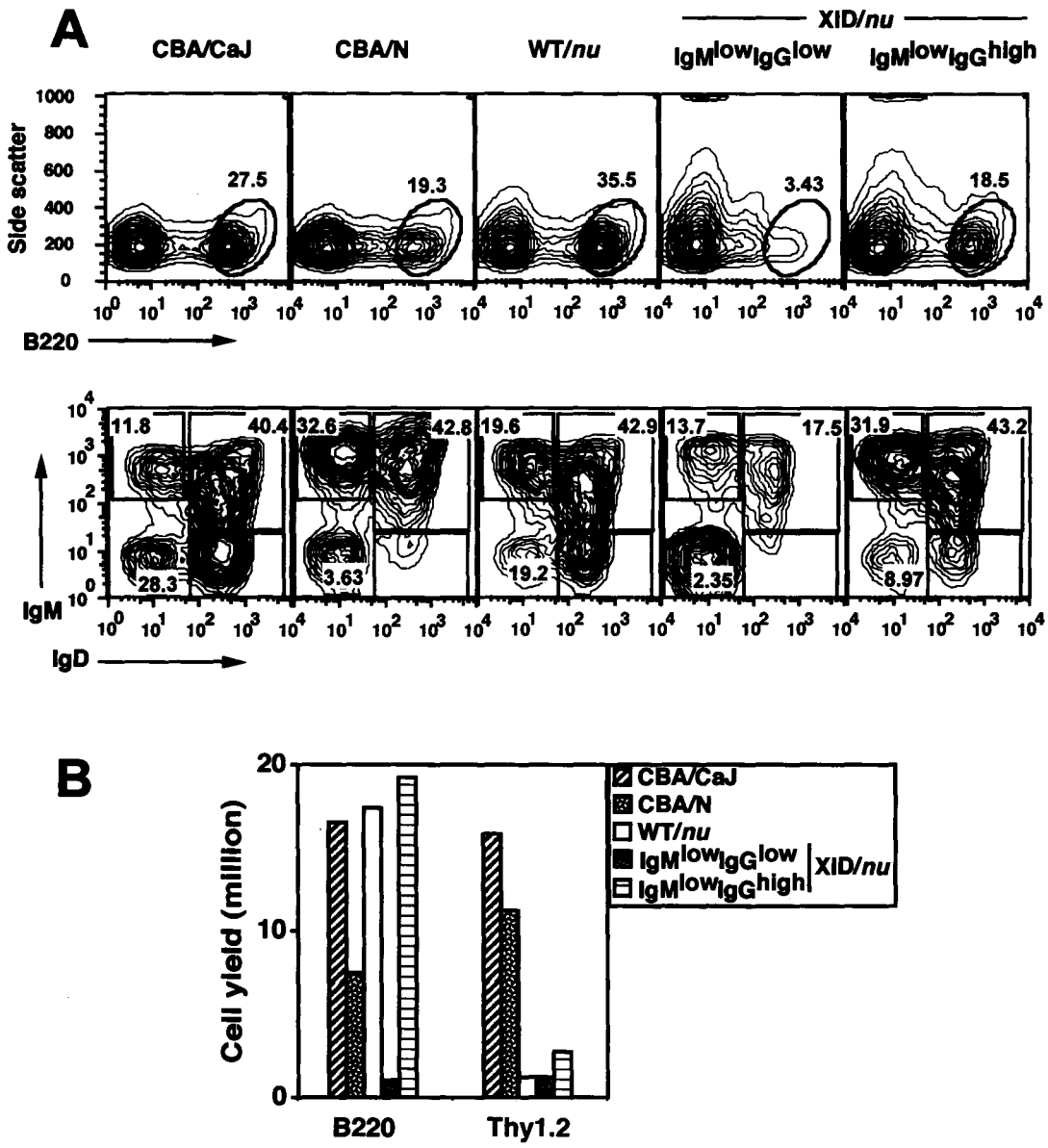



***Figure 8: IgM$^{low}$IgG$^{low}$ XID/nu mice show severely compromised splenic B cell maturation and low T cell frequency***

Spleen cells from mice of indicated genotypes were stained with B220/IgD/IgM and frequency of B cells estimated **(A)**. B220$^+$ B cells were further analyzed to reveal immature (B220$^+$IgM$^+$) and mature (B220$^+$IgD$^+$) B cells **(B)**. Absolute number per spleen of B220$^+$ B cells and Thy1.2$^+$ T cells was also calculated in all the mice tested **(C)**

Splenic B cell yields from the $IgM^{low}IgG^{low}$ XID/*nu* were significantly lower than in the $IgM^{low}IgG^{high}$ XID/*nu* or WT/*nu.* On analyzing splenic T cell yields, higher numbers of splenic T cells were recovered from the $IgM^{low}IgG^{high}$ XID/*nu* mice as compared to the $IgM^{low}IgG^{low}$ XID/*nu* mice **(Fig. 8C)**.

Thus, XID mice bred onto an *athymic nude* genetic background show evidence of a severe reduction in mature B cell frequency as well as total circulating immunoglobulin as compared to T cell sufficient XID mice. Further, this B cell defect appeared to be rescued with even a marginal increase in the number of circulating T cells.

Since the *nude* defect is an epithelial cell defect manifested in all stromal epithelial cells, it was imperative to identify the phenotype as T cell intrinsic. Also, the *nude* defect does not result in a complete T cell developmental block, so a more effective approach was needed to attain complete T cell ablation. This was achieved by generating XID/TCR $\beta^{-/-}$ mice, where the T cell receptor (TCR) β chain is absent leading to a complete deficiency of αβ T cells.

CBA/N female mice were bred with male TCR $\beta^{-/-}$ mice. The F1 generation was intercrossed and the resulting F2 generation mice were genotyped by genomic PCR (Rohrer and Conley, 1999) screening for the XID mutation as well as the TCR β chain deletion and were used in further experiments.

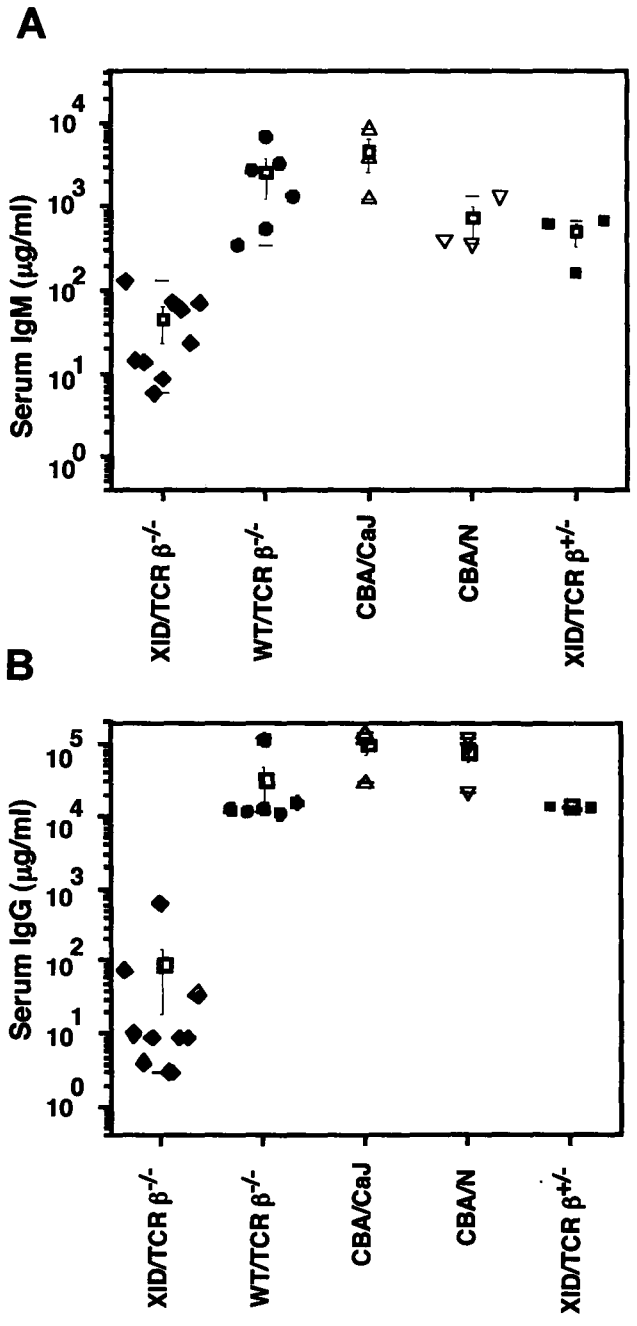



***Figure 9: Severely reduced serum IgM and IgG levels in XID/TCR β$^{-/-}$ mice***

Serum IgM **(A)** or IgG **(B)** levels were estimated in 4-8 week old mice of the various genotypes as indicated. Symbols correspond to individual mice and boxes represent mean ± SE for the indicated genotype.

### *Severely reduced serum immunoglobulin levels in the XID/TCR β$^{-/-}$ mice*

Total circulating serum IgG and IgM levels were estimated in XID/ TCR β$^{-/-}$ progeny and the corresponding WT mice. The XID/TCR β$^{-/-}$ mice showed 100-fold lower levels of serum IgM **(Fig. 9B)** as compared to the CBA/CaJ or TCR β$^{-/-}$ mice, and these serum levels were at least 10-fold lower than in the CBA/N or littermate XID/TCR β$^{+/-}$ mice. More remarkably, 400-fold lower serum IgG levels was observed in the XID/TCR β$^{-/-}$ mice **(Fig. 9C)** compared to CBA/CaJ, CBA/N, TCR β$^{-/-}$ or XID/TCR β$^{+/-}$ littermates, all of which displayed equivalent levels of IgG.

Though the serum immunoglobulin profiles showed a qualitatively similar pattern as shown by the XID/*nu* mice, there was a quantitatively greater reduction as well as lesser heterogeneity in serum immunoglobulin levels in all the XID/TCR β$^{-/-}$ mice tested.

### *B cell frequency in bone marrow and peripheral lymphoid organs of XID/TCR β$^{-/-}$ mice*

Peripheral lymphoid organs from representative XID/TCR β$^{-/-}$ and WT mice were next examined. Both spleen and lymph nodes from the XID/TCR β$^{-/-}$ mouse were very small in size and the total splenic yield was $5x10^5$ cells and lymph node cell yield was less than $1x10^5$ cells.

Bone marrow cell yields were also lower than that in the wild-type or the CBA/N. Frequency of B220$^+$ B cells were extremely low in the XID/TCR β$^{-/-}$ and most of the B220$^+$ B cells were pro/pre B cells (CD43$^+$ B220$^+$) with an almost complete absence of

the immature B cell subset ($CD43^-B220^+$) **(Fig. 10A)**. The XID/TCR $\beta^{-/-}$ also showed a very low frequency of surface $IgM^+$ B cells, which represent the immature B cell subset poised to exit the bone marrow **(Fig. 10B)**.

XID mice, in the absence of circulating T cells, show a more severe agammaglobulinemia, with low peripheral B cell frequency and an almost complete arrest of B cell development at the pro/pre to immature B cell transition, closely resembling the human XLA. Thus, presence of T cells seems to play a pivotal role in effecting a complete rescue of B cell developmental arrest in the bone marrow but fail to reverse the peripheral B cell maturation arrest in the spleen.

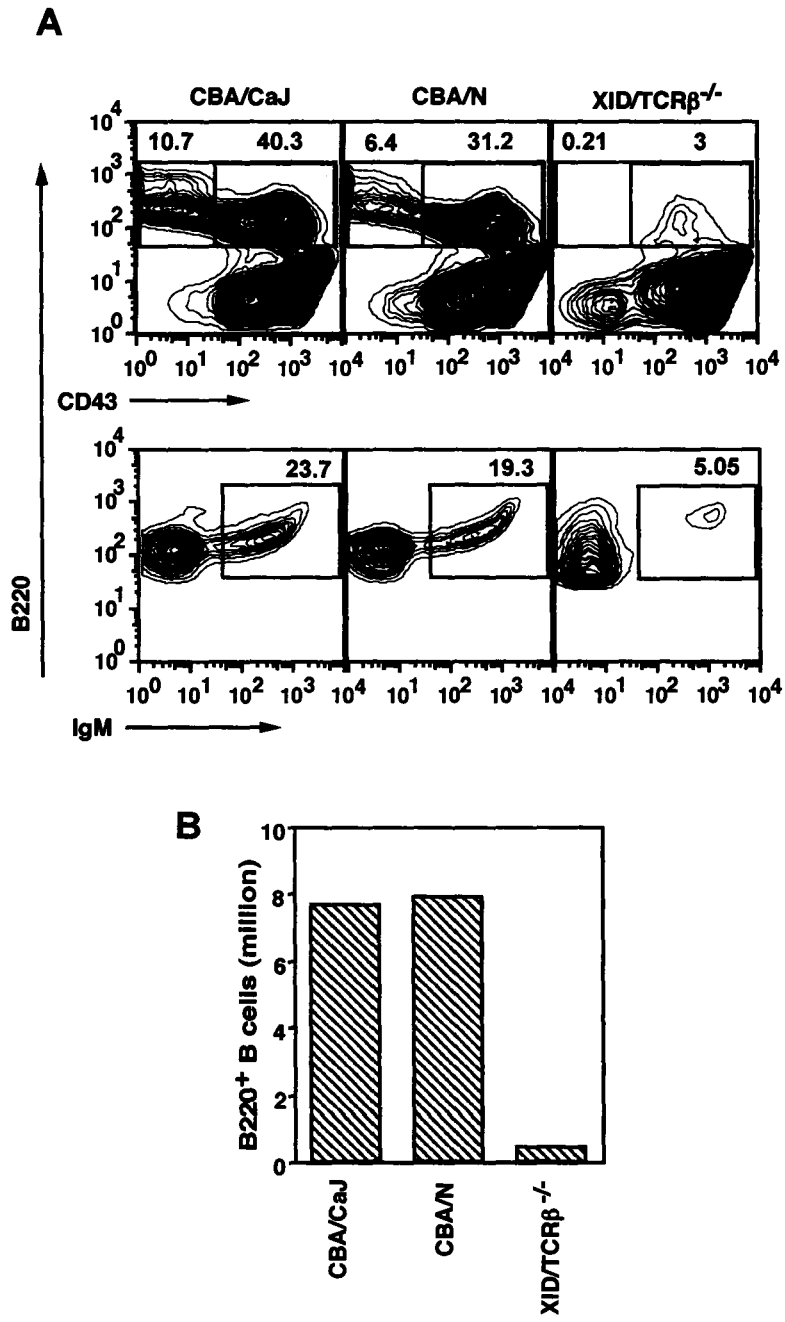



***Figure 10: XID/TCRβ $^{-/-}$ mice show severely compromised bone marrow B cell maturation***

Bone marrow cells from CBA/CaJ, CBA/N and XID/TCRβ$^{-/-}$ were stained for B220/IgM/CD43 and analyzed to reveal pro/pre (B220$^{-/low}$CD43$^{+}$) and immature (B220$^{+}$CD43$^{-}$) B cell subsets **(A-upper panel)**. Frequency of IgM$^{+}$ immature B cells on B220$^{+}$ B cells is also shown **(A-lower panel)**. Total number of B220$^{+}$ B cells per femur and tibia was also calculated **(B)**.

## *Role of lysosomal transporter LYST, in regulating myeloid cell functions*

Following phagocytosis of pathogens by myeloid cells, they are targeted through a series of vesicles of the endo-lysosomal system, where pathogen destruction and antigen processing for further presentation to lymphoid lineage cells such as B and T cells occur simultaneously. Mutation in the lysosomal transport protein, LYST, is known to lead to an accumulation of giant dysfunctional lysosomes, associated with delayed antigen presentation and decreased cytolytic granule release, thereby resulting in an immunodeficiency syndrome, the Chediak-Higashi syndrome, associated with susceptibility to recurrent bacterial infections (Ward et al., 2000). The murine model for the *Lyst* mutation, the bg/bg mouse strain, was studied to elucidate the role for such a mutation in modulating the myeloid cell inflammatory responses.

## *Myeloid cells of bg/bg mice show no defect in recruitment to sites of inflammation*

Macrophage recruitment in response to peritoneal instillation of TG was found to be similar between wild-type C57BL/6 (WT) and the bg/bg mice **(Fig. 11A)**. Similarly, bg/bg mice showed no defect in PMN (polymorphonuclear) cell-driven acute inflammation induced by subcutaneous injection of carrageenan. The kinetics as well as peak footpad swelling achieved was similar in bg/bg and WT mice **(Fig. 11B)**. Next, the ability of whole blood leukocytes, both monocytes and granulocytes, from WT and bg/bg

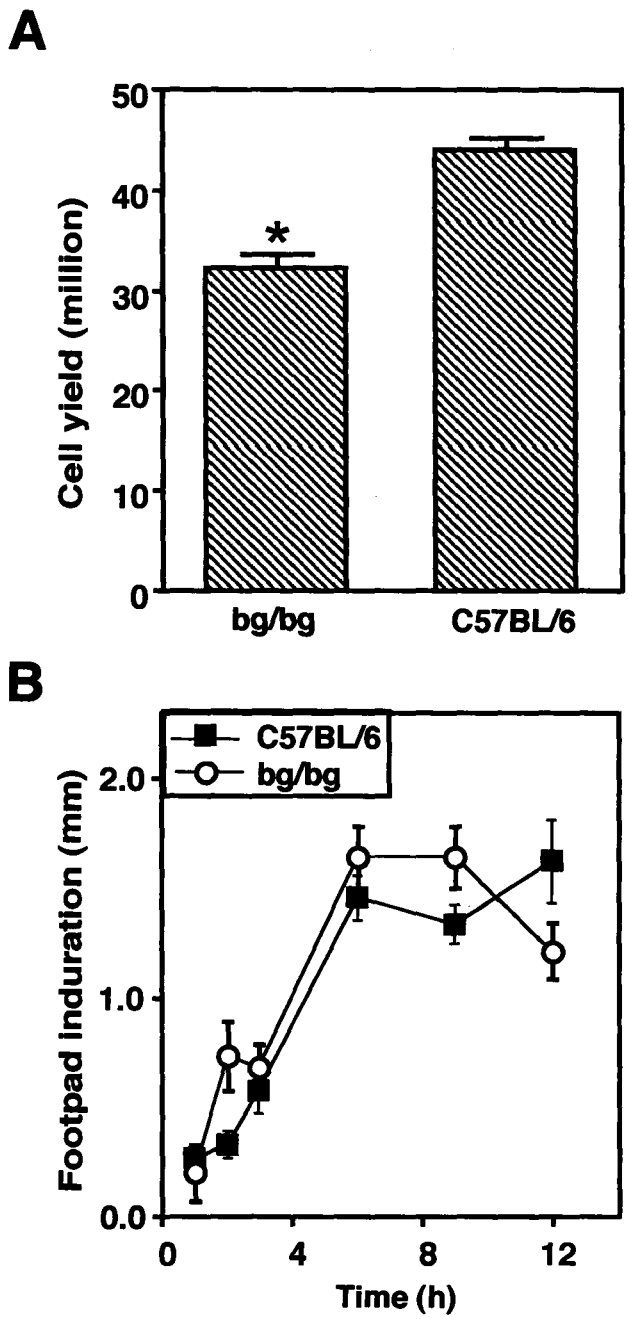



***Figure 11: bg/bg mice show no defect in myeloid cell recruitment***

Peritoneal recruitment of macrophages in response to TG expressed as mean±SE (n=5) **(A)**. Footpad induration induced by injection carrageenan, expressed as millimeter increase in footpad thickness in comparison to contralateral footpad injected with normal saline **(B)**. Data has been compared using two-tailed student's t-test (*p=0.052).

mice to phagocytose fluorescence labeled E.coli (FITC-*E coli*) was estimated by a flow cytometry based phagocytosis assay. The frequency of cells internalizing the labeled bacteria as well as per cell bacterial load was similar between the bg/bg and WT leukocytes **(Fig. 12)**. Therefore bg/bg myeloid cells exhibited no deficiency in phagocytic ability or recruitment of myeloid cells.

### *bg/bg mice show increased susceptibility to cutaneous leishmaniasis*

The protozoan parasite *Leishmania major* (Lm) induces a clinically chronic cutaneous infection with the parasite establishing the infection by colonizing the lysosomes of host macrophages. Paradoxically, the clearance of these parasites by effector molecules like RNIs is also affected in the lysosomes. In view of the known lysosomal function defect of bg/bg mice, the functional consequences of the same on the disease progression in a leishmanial infection was analyzed.

C57BL/6, BALB/c and bg/bg mice were infected in the footpad with $5x10^6$ stationary phase Lm promastigotes. The course of infection was followed by measuring the consequent increase in footpad thickness over subsequent weeks. BALB/c mice are known to be genetically susceptible to leishmanial infection and accordingly, showed accelerated lesion formation. C57BL/6 mice, which are known to be genetically resistant to Lm infection, rapidly cleared the lesions. On the other hand despite being on the same resistant genetic background as the C57BL/6, the bg/bg mice showed a relatively high susceptibility to Lm infection with progressive lesion formation **(Fig. 13)**.

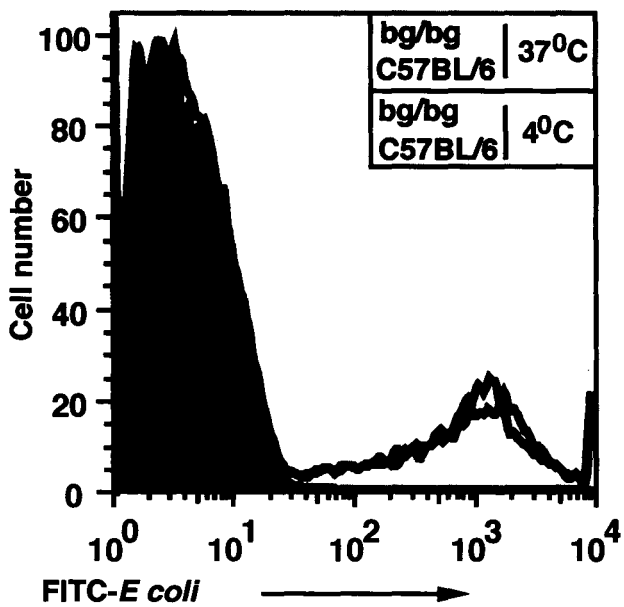



***Figure 12: Macrophage effector functions in bg/bg mice were normal***

Whole blood leukocytes from C57BL/6 (red thick line) or bg/bg (blue thick line) were allowed to phagocytose fluorescently labeled E-coli at 37°C and bacterial uptake was estimated on PI positive cells by flow cytometry. C57BL/6 (shaded histogram) or bg/bg (black thin line) cells incubated with bacteria at 4°C acted as controls.

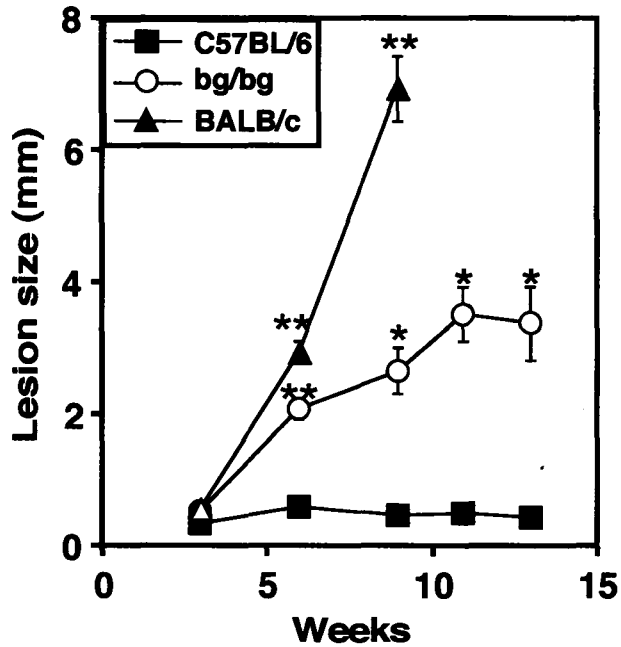



*Figure 13: bg/bg mice display greater susceptibility to cutaneous leishmaniasis*

Mice were infected in the footpad with $5x10^6$ stationary phase Leishmania major (Lm) promastigotes, the course of infection was followed over subsequent weeks and expressed as increase in thickness of infected footpad compared to contralateral uninfected footpad. Data represent mean lesion size (millimeters) $\pm$SE of 8 mice per group. Data have been compared using two-tailed student's t-test (*$p<0.005$,**$p<0.001$).

On histopathological examination of hematoxylin- and eosin-(H&E) stained footpad sections at 12 weeks of infection, bg/bg mice showed massive inflammatory cell infiltration with destruction of tissue architecture, with no evidence of skin ulcerations **(Fig. 14B)**. Similarly processed BALB/c footpads showed extensive skin ulceration associated with massive inflammatory cell infiltration resulting in extensive tissue destruction **(Fig. 14C)**, whereas C57BL/6 lesions were characterized by moderate perivascular inflammatory infiltrates in the absence of any evident tissue damage **(Fig. 14A)**. Parasitized macrophages were observed among the infiltrating lymphocytes in lesions from both bg/bg **(Fig. 14E)** and BALB/c **(Fig. 14F)** mice, but were absent in the C57BL/6 mice, which expectedly showed no evidence of the presence of any parasitized macrophages at this time-point of infection **(Fig. 14D)**.

### *bg/bg macrophages show compromised Lm clearance*

The ability of macrophages from the bg/bg mice to clear Lm infection was next examined in an *in vitro* parasite clearance assay. Adherent TG elicited peritoneal macrophages from bg/bg, C57BL/6 and iNOS$^{-/-}$ (inducible nitric oxide synthase-deficient) mice were infected with Lm and parasite clearance was analyzed over time. iNOS$^{-/-}$ macrophages have been reported to show compromised Lm clearance due to an inability to induce RNIs, which are key effector molecules essential for effective parasite elimination. Both frequency of parasitized macrophages (parasitaemia) and the number of parasites per infected macrophage (parasite burden) were estimated. Cells from all the three strains showed equivalent parasitaemia and similar parasite burdens at the outset, however the iNOS$^{-/-}$ and bg/bg macrophages displayed compromised parasite clearance

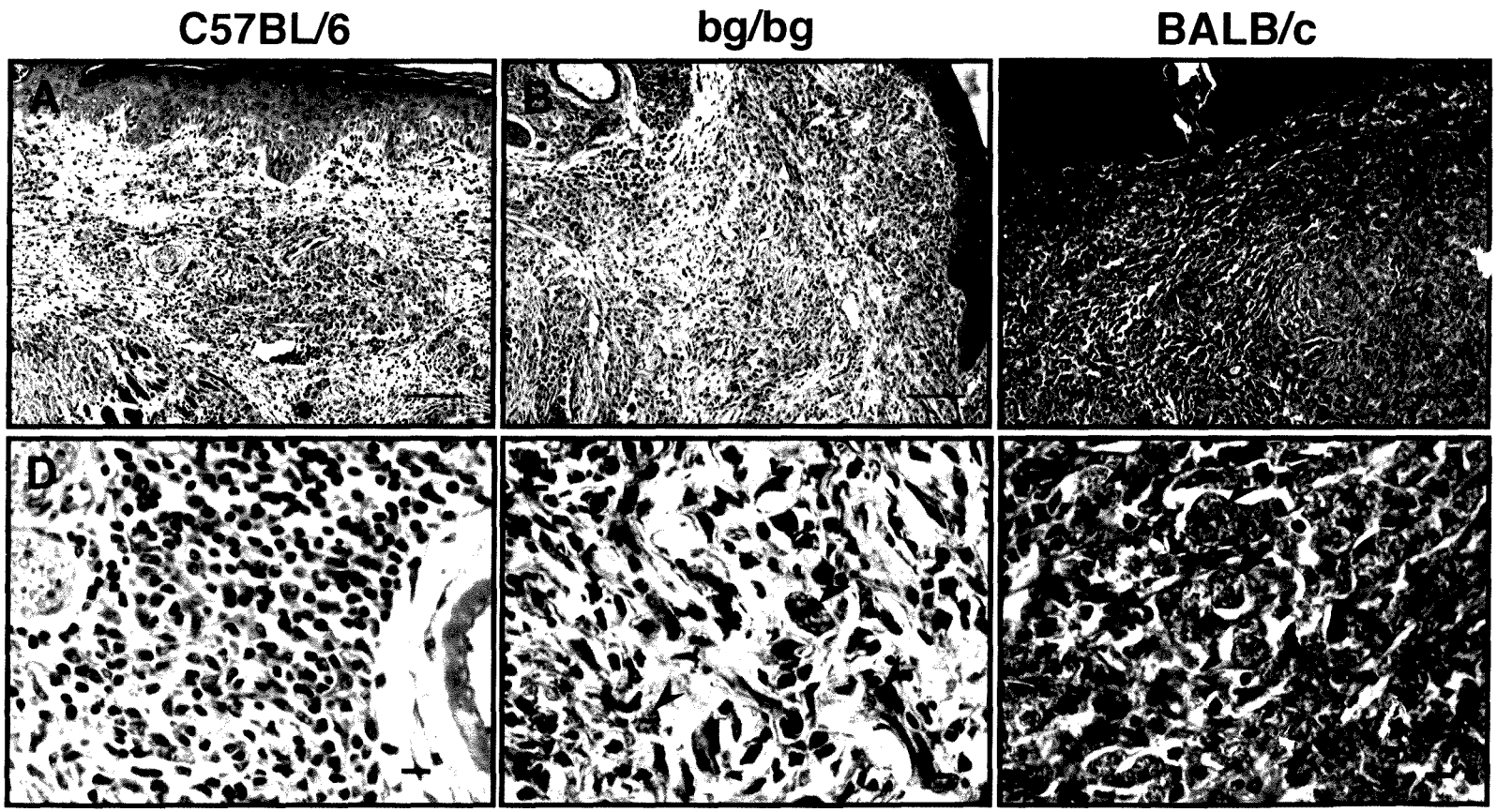



***Figure 14:*** H&E-stained footpad sections from BL/6 **(A)**, bg/bg **(B)** and BALB/c **(C)** mice were examined at 12 weeks of infection. Images **A,B & C** were taken at a magnification of 20X. Scale bar represents 100 μm. Parasitized macrophages were visualized under oil immersion at a magnification of 100X **(D,E,F)**. Scale bar represents 10 μm. Arrowheads indicate parasite laden macrophages.

at all the subsequent time points tested. Both parasitaemia as well as parasite burden remained significantly higher in bg/bg and iNOS$^{-/-}$ macrophages even as late as 48 h of infection **(Fig. 15)**.

Similarly, when bone marrow derived macrophages (BMDM) were infected with Lm, similar frequencies of cells were infected to begin with, but the bg/bg and iNOS$^{-/-}$ macrophages showed higher parasitaemia as well as parasite burden at all subsequent time points tested **(Fig. 16)**.

Ability to clear an Lm infection in mice correlates with their ability to generate an IFN-γ preponderant Th1 immune response, and IFN-γ mediates clearance by inducing iNOS and the subsequent production of RNIs in parasitized macrophages. Therefore the ability of bg/bg macrophages to respond to IFN-γ was next tested.

### *Enhanced Lm clearance in IFN-γ primed bg/bg macrophages*

To analyze for IFN-γ responsiveness, BMDMs primed with IFN-γ for 16 h were subsequently infected with Lm and nitrite generation was estimated. IFN-γ primed Lm infected BMDMs from both C57BL/6 and bg/bg mice showed a similar level of enhancement in nitrite generation compared to unprimed infected BMDMs, with no increase in nitrite production by the iNOS$^{-/-}$ BMDMs **(Fig. 17A)**. Despite the enhanced nitrite levels generated, the compromise parasite clearance displayed by bg/bg macrophages was only partially rescued in IFN-γ primed bg/bg BMDMs. Also the improvement in clearance was slower in the bg/bg, being evident only after 48 h as

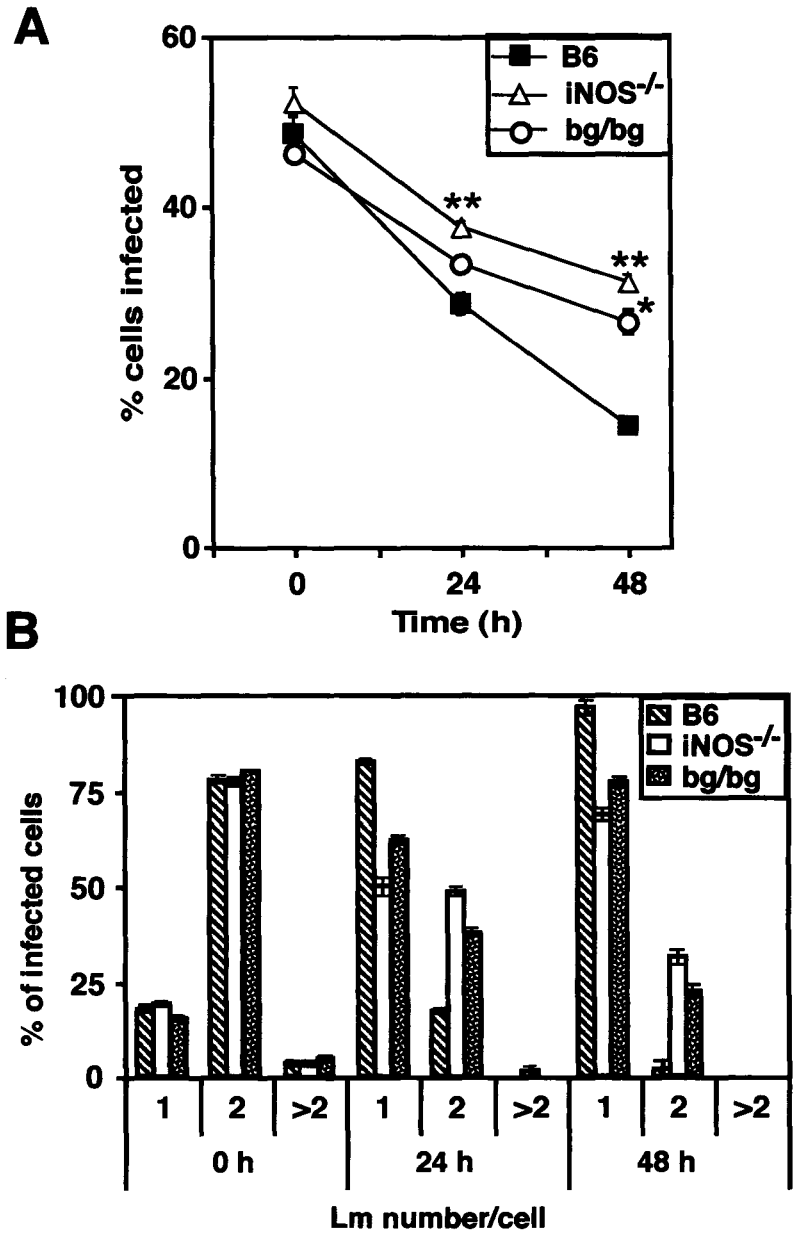



***Figure 15: bg/bg peritoneal macrophages display poor Lm parasite clearance***

Macrophages from C57BL/6, iNOS$^{-/-}$ and bg/bg mice were infected *in vitro* with fluorescently labeled Lm at an MOI of 50:1. Parasite clearance was estimated as % of macrophages infected (parasitaemia) **(A)** and number of parasites per infected macrophage (parasite burden) **(B)** at subsequent time points. Data represent mean±SE of triplicates of 300 cells each. Data were compared to C57BL/6 by two-tailed student's t-test (*$p<0.01$,**$p<0.005$).

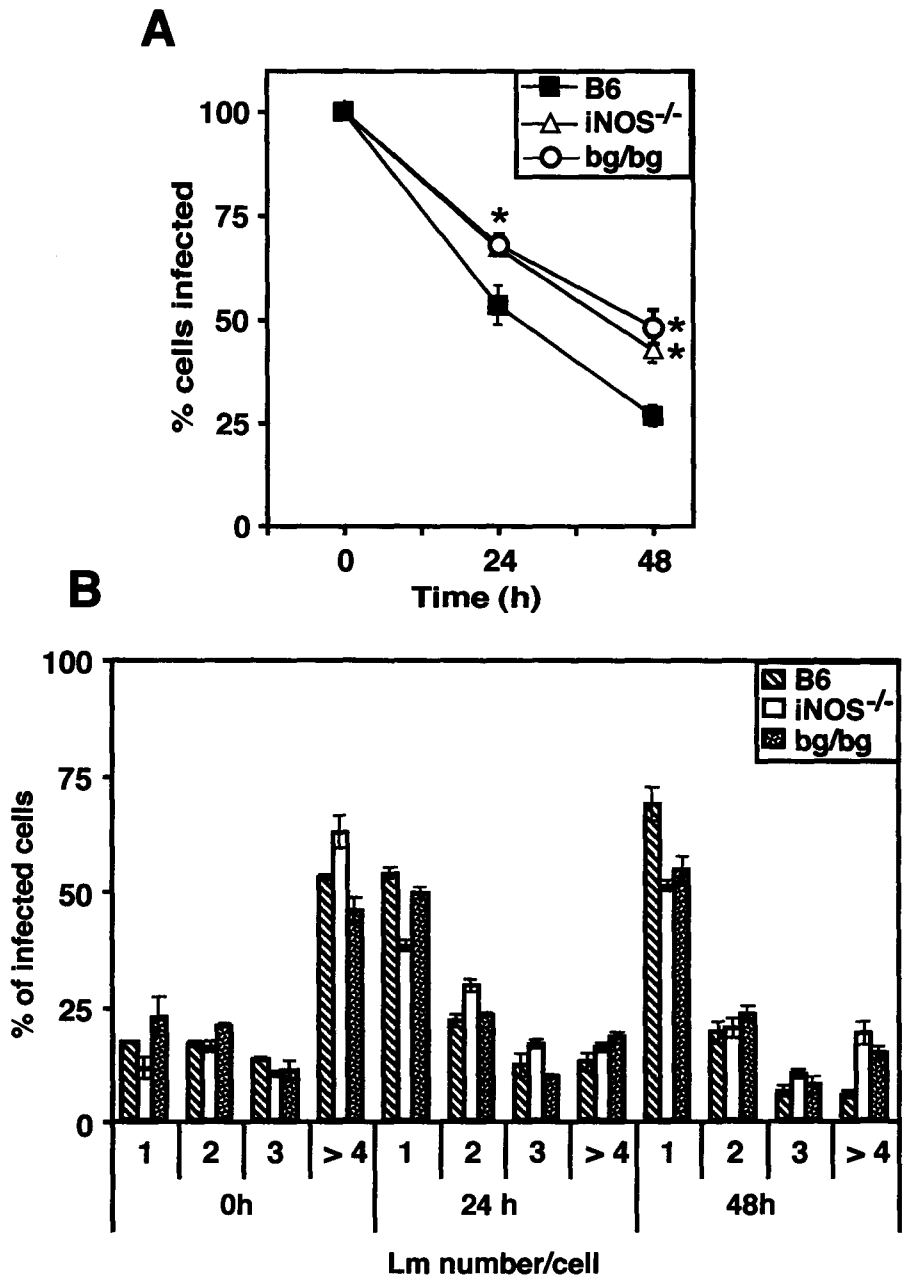



***Figure 16: bg/bg bone marrow derived macrophages (BMDMs) show poor Lm clearance***

BMDMs derived from C57BL/6, iNOS$^{-/-}$ and bg/bg mice were infected *in vitro* with fluorescently labeled Lm at MOI of 50:1. Parasite clearance was estimated as % of macrophages infected (parasitaemia) **(A)** and number of parasites per infected macrophage (parasite burden) **(B)** at subsequent time points. Data represent mean±SE of triplicates of 300 cells each.Data were compared to C57BL/6 by two-tailed student's t-test (*$p<0.05$).

compared to C57BL/6 BMDMs which displayed enhanced clearance even by 24 h of infection **(Fig. 17B)**.

Since IFN-γ could induce Lm clearance in bg/bg mice albeit slowly, it was essential to analyze the Lm specific T cell response, especially the Th1/Th2 balance, in the bg/bg. First, Lm specific T cell frequency was estimated in lesion draining lymph nodes from C57BL/6, BALB/c and bg/bg mice. For this, CD69-expressing cells were removed by magnetic sorting, and the remaining CD4 T cells were re-stimulated *in vitro* in lymphocyte co-culture with Lm infected or uninfected syngenic C57BL/6 or BALB/c bone marrow-derived dendritic cells (BMDCs) functioning as antigen-presenting cells (APCs). After 16 h of re-stimulation, Lm-specific CD4 T cells were identified by their ability to express the early T cell activation marker CD69 in response to stimulation. Similar frequencies of CD69 upregulating CD4 T cells were found in lesion draining lymph node from C57BL/6, bg/bg and BALB/c mice **(Fig. 18A, B)**. Culture supernatant Th1/Th2 cytokine profiling revealed similarly high levels of IFN-γ in supernatants form bg/bg and C57BL/6 cultures, while BALB/c cells made very low levels of the cytokine **(Fig. 18C)**. However, large amounts of secreted IL-4 were detected in BALB/c cultures as compared to both the C57BL/6 and bg/bg cultures, both of which made very little IL-4 **(Fig. 18D)**.

These data indicate that the inability of bg/bg mice to control a leishmanial infection does not result from a shift an inability to mount an IFN-γ dominant Th1 response, but is due to a compromise in elimination of parasites by bg/bg macrophages.

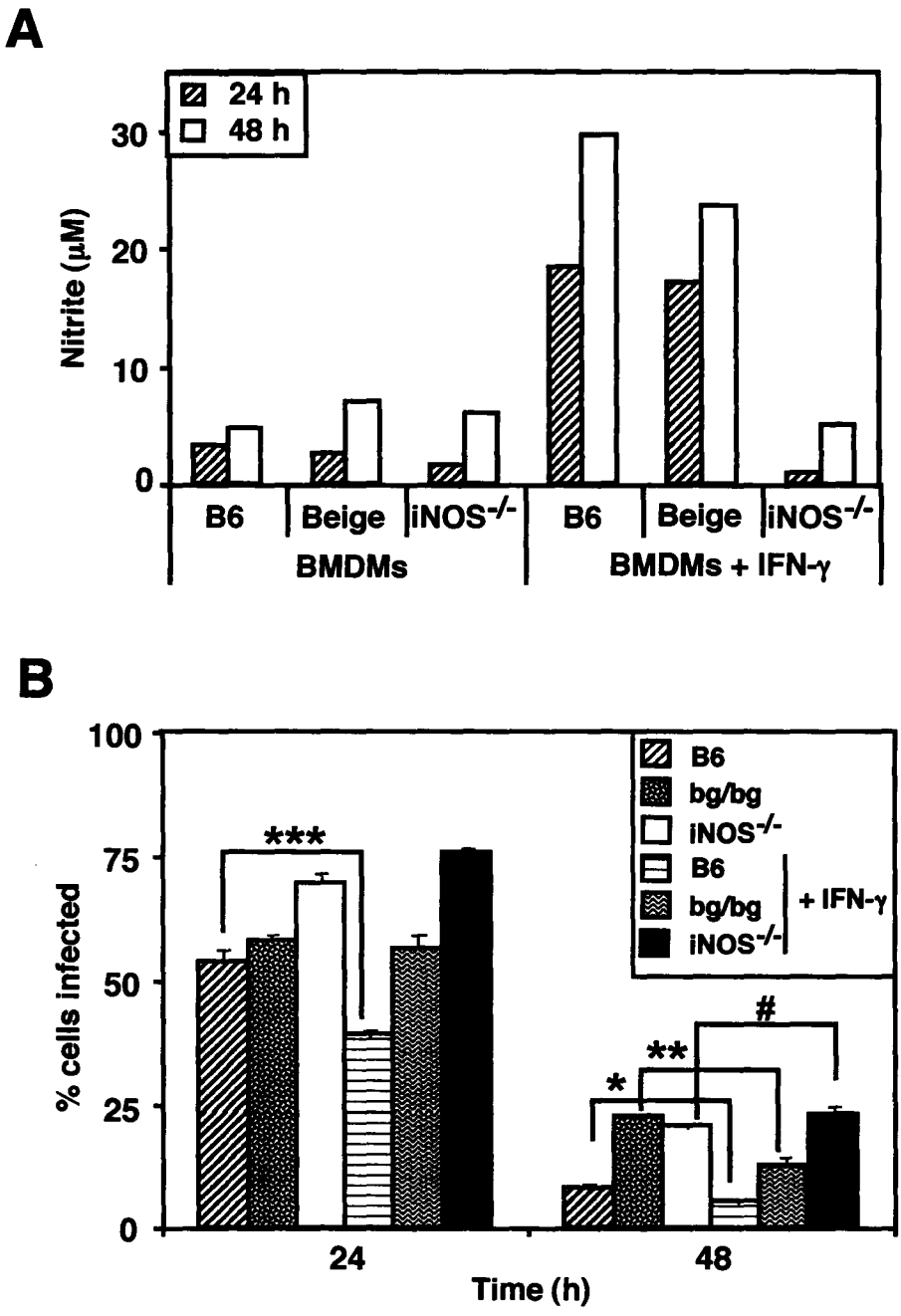



***Figure 17: IFN-γ primed bg/bg BMDMs display late and incomplete improvement of clearance***

Unprimed BMDMs or BMDMs primed overnight with 30 units/ml IFN-γ were infected with labeled Lm at MOI of 50:1. Culture supernatant nitrite levels **(A)** and frequency of Lm infected cells expressed as percentage normalized to 0 h values, (taken as 100%) **(B)** were estimated. Data were compared between IFN-γ primed and unprimed BMDMs by two-tailed student's t-test.
(*p=0.059,**p=0.017,***p=0.020,#p=0.25)

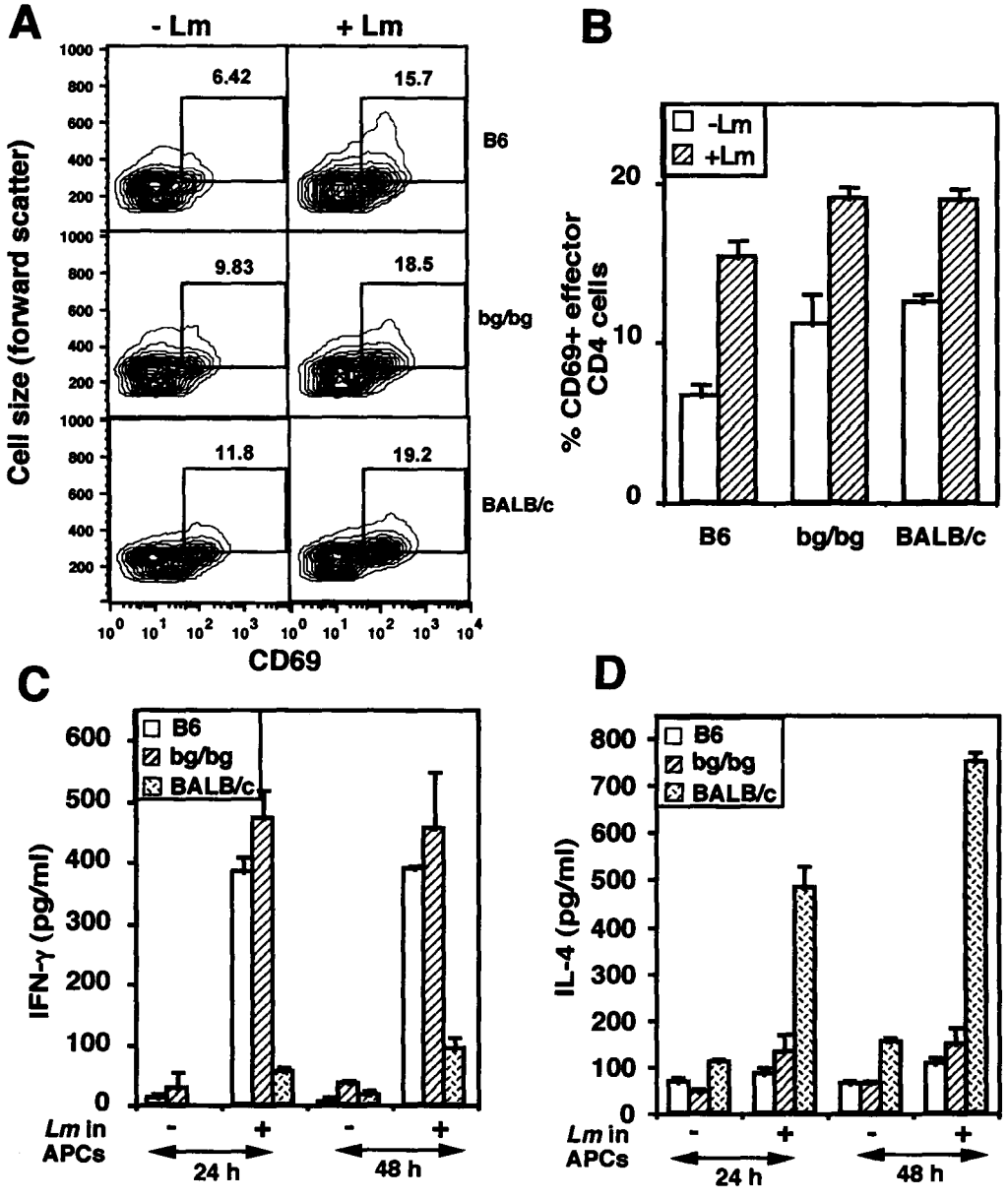



***Figure 18: Comparable Lm-specific T cell frequency and IFN-γ response in bg/bg and BL/6 mice***

CD69/CD62L depleted effector T cells from lesion draining lymph nodes of Lm infected B6, bg/bg and BALB/c mice were co-cultured with Lm infected of uninfected B6 (for B6 & bg/bg) or BALB/c derived BMDCs. At 16 h of co-culture, cells were stained for CD4/CD69 and Lm-specific CD4 T cell identified as $CD69^+CD4$ cells. Cells were initially gated on $CD4^+$ cells and analyzed for CD69 upregulation by flow cytometry **(A)** and frequency of $CD69^+CD4$ effectors was estimated **(B)**. Supernatant IFN-γ **(C)** and IL-4 **(D)** was estimated by ELISA.

These results highlight that even a delayed outcome of cellular interactions, in this case between the primed Th1 T cells and the macrophages, can change the eventual outcome of Lm infection, potentially leading to significant increments in disease susceptibility.

## *Modulation of disease susceptibility by germline retroviral insertions -v-SAg-7*

Genome-integrated retroviruses have been identified to occupy a substantial fraction of both mouse and human genomes. These proviral insertions are best known for the ability of some of them to code for superantigen proteins (SAgs) that associate with major histocompatibility complex class II (MHCII) molecules and stimulate T cells bearing particular T cell receptor V-beta (TCRVβ) segments leading to activation and/or deletion of these T cells (Janeway, 1991). Both, human endogenous retroviruses (HERVs) and mouse endogenous murine tumor viruses (*mtv*s) have been reported to mediate susceptibility to infections, tumors and autoimmune disease processes (Bhadra et al., 2006; Casanova and Abel, 2004; Clausen, 2003; Gorgette et al., 2002; Schirrmacher et al., 1998). Though the biological basis of these associations has not been elucidated as yet, they appear not to be restricted to the T cell deleting functions of the encoded SAgs.

To analyze the role of such evidently environmental modulators on determining disease outcomes, the course of and immunity to Lm infection in two closely related apparently Lm-resistant inbred mouse strains the CBA/J and CBA/CaJ, was studied. The genetically closely related CBA/J and CBA/CaJ mice differ in the proviral integrants they carry. CBA/J genome contains *mtv-6* and *mtv-7* integration in addition to *mtv-8 and mtv-9,* present in both CBA/J and CBA/CaJ (Tomonari et al., 1993).

### *CBA/J mice show chronic low-grade cutaneous leishmanial infection*

On footpad infection with *L major*, CBA/J mice exhibited greater lesion severity, even in the early weeks of infection. This was followed by a sustained chronic infection with no decrease in the lesion severity. In comparison, the CBA/CaJ mice had small lesions to begin with that steadily reduced further and culminated in complete recovery by 10 weeks post-inoculation **(Fig. 19A)**. Simultaneously infected C57BL/6 mice showed resistance to Lm infection, while BALB/c showed an expectedly severe and progressive infection necessitating euthanasia of the mice at 6-8 weeks post-inoculation **(Fig. 19B)**. H&E stained footpad sections from CBA/J, CBA/CaJ, BL/6 and BALB/c mice were examined between 12-14 weeks of infection. CBA/J mice showed moderate inflammatory cell infiltration and evidence of granuloma formation with preserved skin and tissue architecture **(Fig. 20B)**; CBA/CaJ footpad sections displayed no evidence of inflammation with normal tissue architecture **(Fig. 20A)**. BALB/c footpads showed extensive skin ulceration with massive inflammatory cell infiltration and loss of tissue architecture **(Fig. 20D)**, whereas C57BL/6 lesions showed moderate perivascular inflammatory infiltrates **(Fig. 20C)**.

### *Chronicity of Lm infection is associated with inheritance of mtv-7*

The *mtv-6* and *mtv-7* integrants have been reported to follow a simple mendelian form of inheritance pattern and undergo independent assortment (Frankel et al., 1991). They also represent the minor histocompatibility loci *mls-3* and *mls-1* (Peters et al., 1986), and code for the viral superantigens (v-SAg) v-SAg-6 and v-SAg-7 respectively (Frankel et al., 1991; Howell et al., 1995). These superantigens bind to distinct T cells bearing

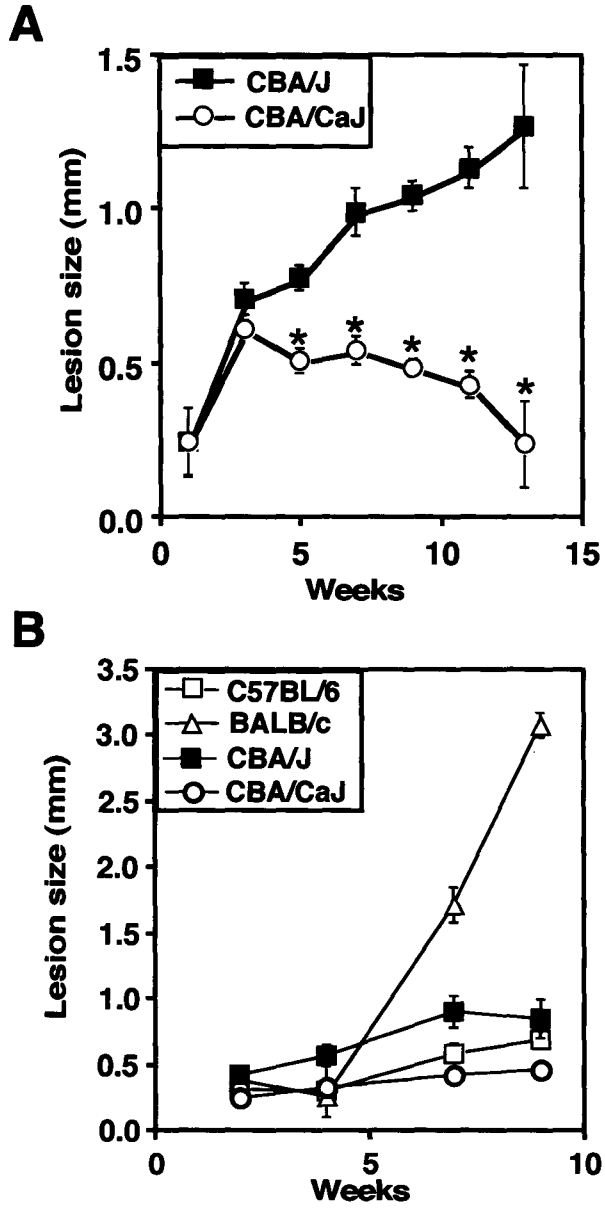



***Figure 19: CBA/J mice show chronic progressive leishmanial infection***

Mice were infected in the footpad with $5x10^6$ stationary phase Lm, the course of infection was followed by measuring the increase in footpad thickness and expressed as increase in thickness of the infected footpad compared to the contralateral uninfected footpad **(A)**.Comparative lesion profiles in CBA/J, CBA/CaJ, C57BL/6 and BALB/c **(B)**. Values indicate mean lesion size (millimeters) $\pm$ SE of 8 animals each. Data were compared using two-tailed student's t-test (*$p<0.005$)

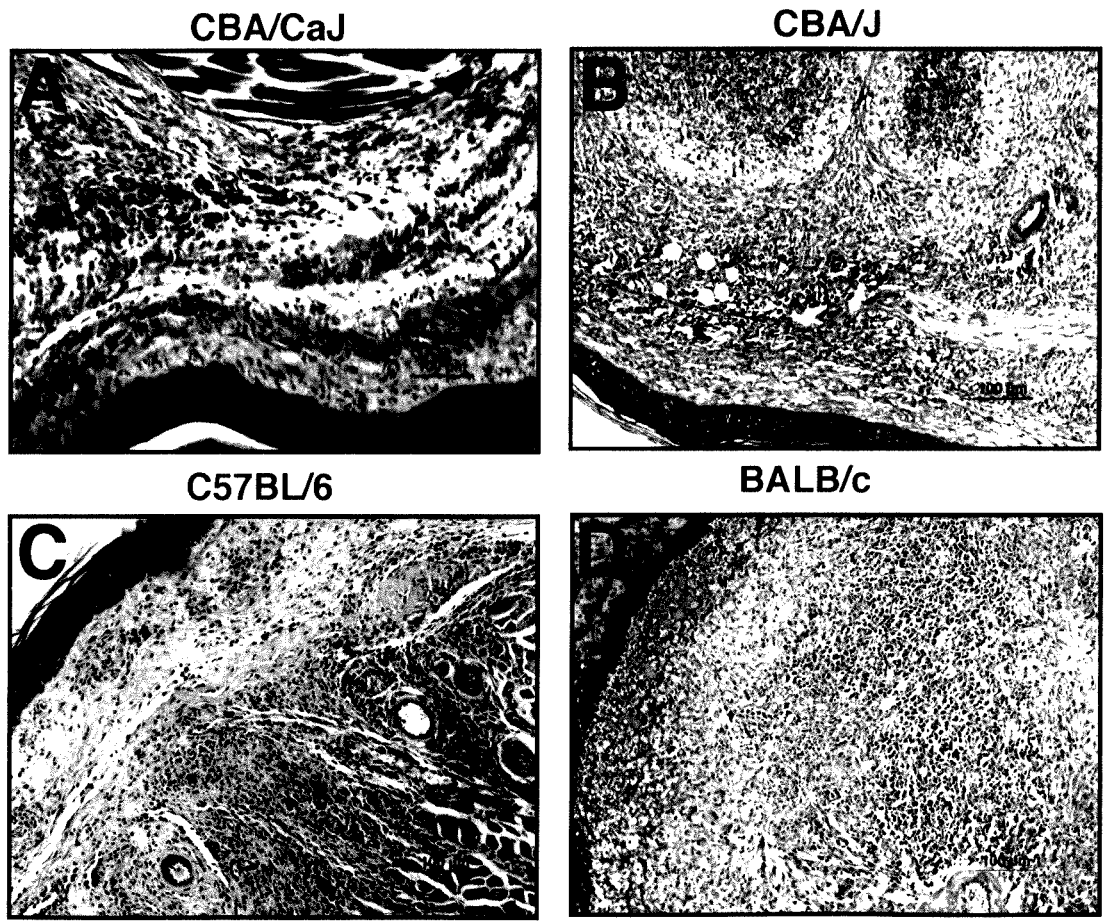



*Figure 20:* H&E-stained footpad sections from CBA/CaJ **(A)**, CBA/J **(B)**, BL/6 **(C)** and BALB/c **(D)** mice were examined between 12-14 weeks of infection. All images were taken at a magnification of 20X. Scale bar represents 100 μm.

specific T cell receptor-V-beta (TCRVβ) segments and delete them during thymic development. TCRVβ6-bearing T cells are deleted by v-SAg-7 but not by v-SAg-6, and vice versa for TCRVβ3 (Tomonari et al., 1993). This property of the v-SAgs was utilized to examine the potential contribution of these proviral integrants to the relative Lm susceptibility of CBA/J mice by intercrossing the two strains. Inheritance of *mtv-6* and *mtv-7* was tracked by testing for the deletion of TCRVβ3- and TCRVβ6-bearing T cells respectively.

On footpad infection with Lm, (CBA/J x CBA/CaJ) F1 mice showed similar susceptibility to that shown by the parental CBA/J strain **(Fig. 21A)**. F1 mice showed deletion of both TCRVβ3- and TCRVβ6-bearing T cells, while F2 mice generated by interbreeding F1 mice showed all four expected mendelian patterns of inheritance of *mtv-6* and *mtv-7* **(Fig. 21B)**. Among the F2 generation, mice inheriting both *mtv-6* and *mtv-7* or *mtv-7* alone were relatively susceptible to Lm infection, like the parental CBA/J strain **(Fig. 22A)**. However, F2 mice inheriting either *mtv-6* alone, or those inheriting neither mtv-6 nor *mtv-7*, were relatively resistant to Lm infection, like the parental CBA/CaJ strain **(Fig. 22B)**.

## *Lm-specific T cell frequencies are equivalent in CBA/J and CBA/CaJ mice*

Since *mtv-7* appeared to correlate with relative susceptibility to Lm infection in CBA/J mice, the possibility that the magnitude of the T cell response in CBA/J mice was reduced due to loss of the v-SAg-7-reactive TCR subset from the immune repertoire was

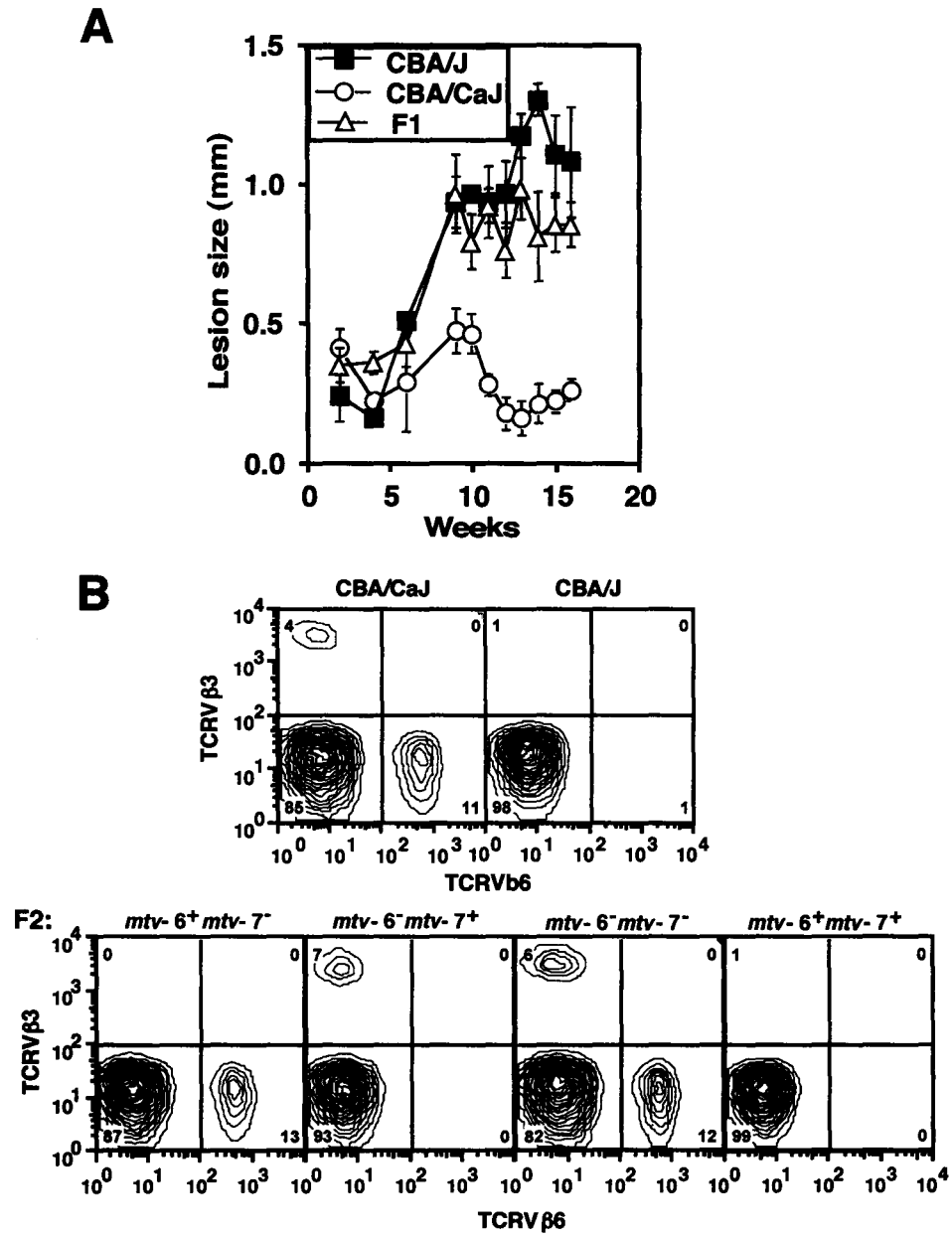



*Figure 21: (CBA/J x CBA/CaJ) F1 mice show chronic Lm infection*

CBA/J, CBA/CaJ and (CBA/J x CBA/CaJ) F1 mice were infected in the footpad with Lm and lesion size progression was estimated **(A)**. Data have been compared using two-tailed student's t-test (*$p<0.05$) (n=8 mice per group). (CBA/J x CBA/CaJ) F2 generation mice were screened for the inheritance of *mtv-6* and *mtv-7* integrations by staining peripheral blood mononuclear cells (PBMCs), and analyzing for Vβ3 and Vβ6 subsets on CD4 positive cells by flow cytometry **(B)**.

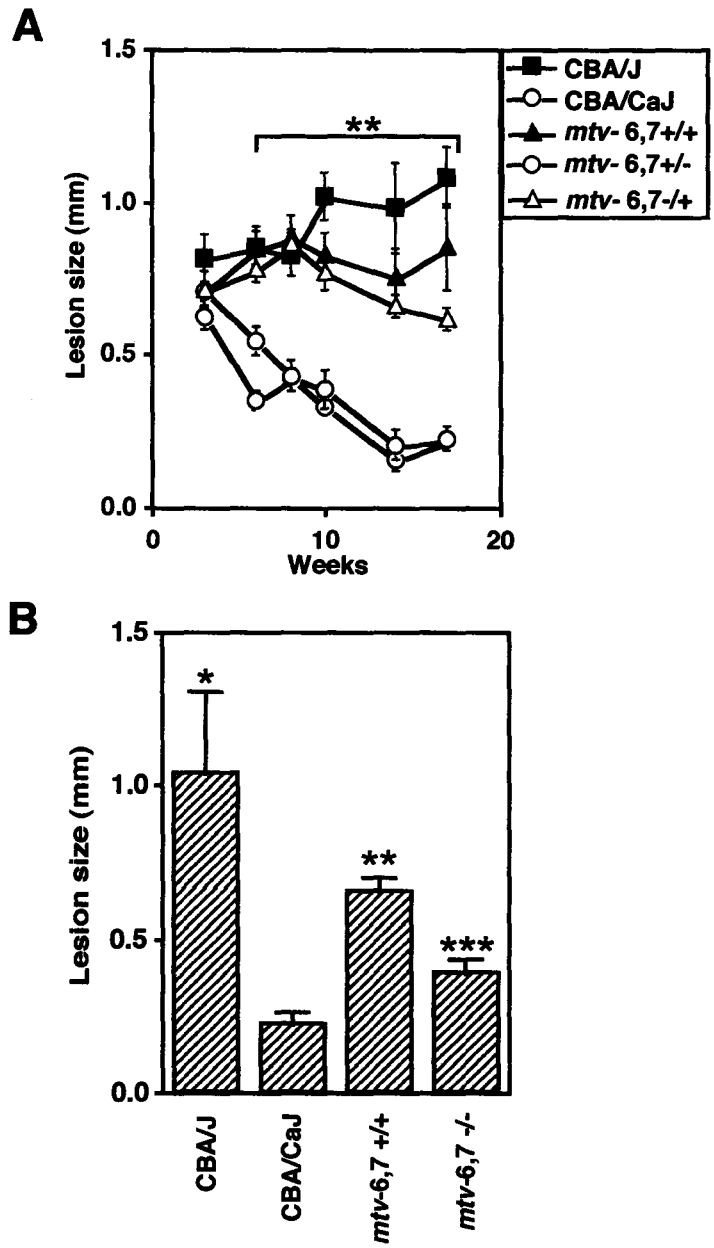



***Figure 22: mtv-7 inheritance confers susceptibility to chronic Lm infection***

(CBA/J x CBA/CaJ) F2 mice grouped on the *mtv* inheritance pattern, were infected with Lm and lesion progression monitored over subsequent weeks of infection **(A). (B)** Represents footpad lesion size at 15 weeks of infection for the indicated *mtv* integrant mice. All data are represented as mean lesion size (millimeters) $\pm$ SE (n=8 or more mice per group). All groups were compared with CBA/CaJ (B) or CBA/CaJ and *mtv-6*+ F2 mice, using two-tailed student's t-test (*$p<0.05$, **$p<0.005$, ***$p=0.06$).

examined. At early time points post-inoculation, lesion draining popliteal lymph node cell recoveries was similar between CBA/J and CBA/CaJ mice. Frequency and total cell numbers of both CD4 **(Fig. 23A)** and CD8 T cells **(Fig. 23B)** were also similar between CBA/J and CBA/CaJ. Next, the frequencies of effector CD4 T cells of the $CD44^{high}CD62L^{low}$ phenotype were examined in both the CD4 **(Fig. 23A)** as well as CD8 **(Fig. 23B)** T cell subsets. Equivalent expansion of the CD4 effector T cell subset was seen between CBA/J and CBA/CaJ. However, the CD8 T cells did not contribute significantly to the early expansion of the effector T cell pool, and displayed no differences among the two strains.

The frequencies of Lm-specific CD4 T cells in the draining lymph nodes of infected CBA/J or CBA/CaJ mice were estimated, early during infection. For this, CD69 depleted CD4 T cells from Lm-infected or uninfected CBA/J and CBA/CaJ mice were re-stimulated *in vitro* in a lymphocyte co-culture assay. Lm infected or uninfected BMDCs derived from CBA/CaJ mice were used as antigen-presenting cells (APCs), since they would not trigger v-SAg-6- and v-SAg-7-reactive T cells in an Lm-nonspecific fashion. At early time points during infection, the Lm-specific CD4 T cell frequencies in draining lymph nodes of CBA/ J and CBA/CaJ mice were found to be similar **(Fig. 24)**.

Next, Lm-specific cell frequencies in the $CD44^{high}CD62L^{low}$ effector populations in draining lymph nodes was examined. For this, naive $CD62L^{high}$ T cells were removed by magnetic sorting, and the remaining CD4 T cells were confirmed by flow cytometry to be $CD44^{high}$. These cells were re-stimulated *in vitro* and Lm-specific CD4 T cells were

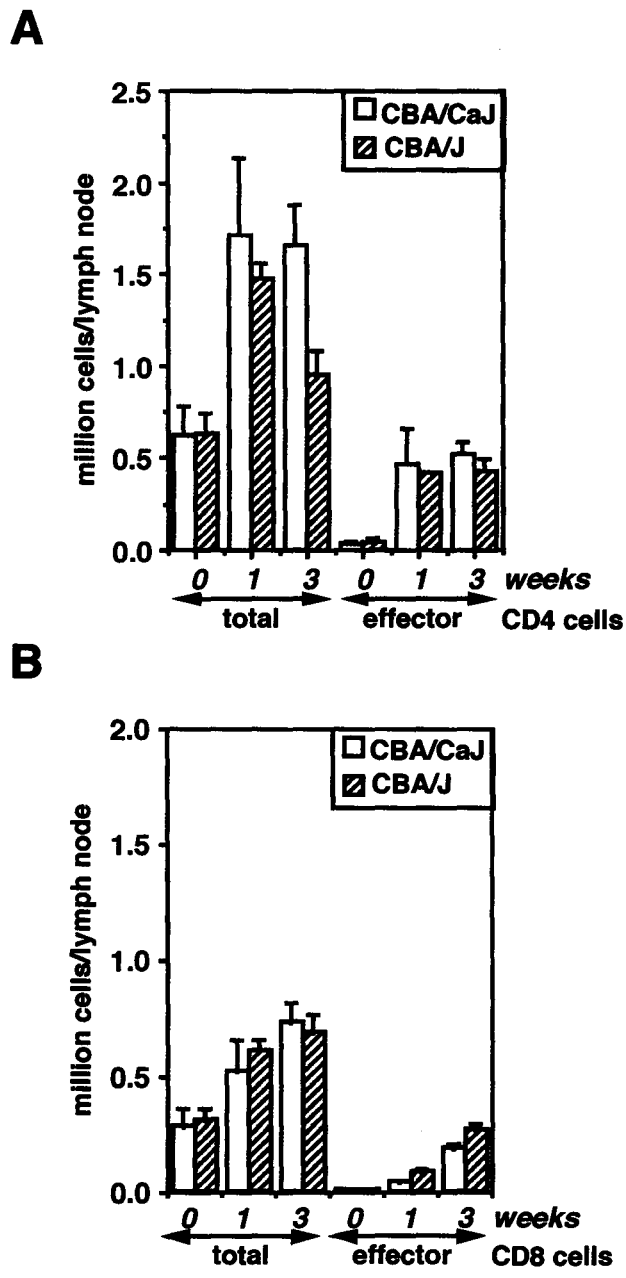



***Figure 23: Similar magnitude of Lm induced T cell response in CBA/J and CBA/CaJ***

Total or "effector" (CD4/8 CD62L$^{low}$CD44$^{high}$) CD4 **(A)** or CD8 T **(B)** cell recoveries were calculated from total cells recovered per lesion draining popliteal lymph node, based on the frequency of the specific cell population obtained by flow cytometric analysis. Cell recoveries from uninfected mice are shown as 0 week values. 3 mice per group were used for each time point analyzed. Data are represented as mean cell recovery per lymph node (million) $\pm$ SE.

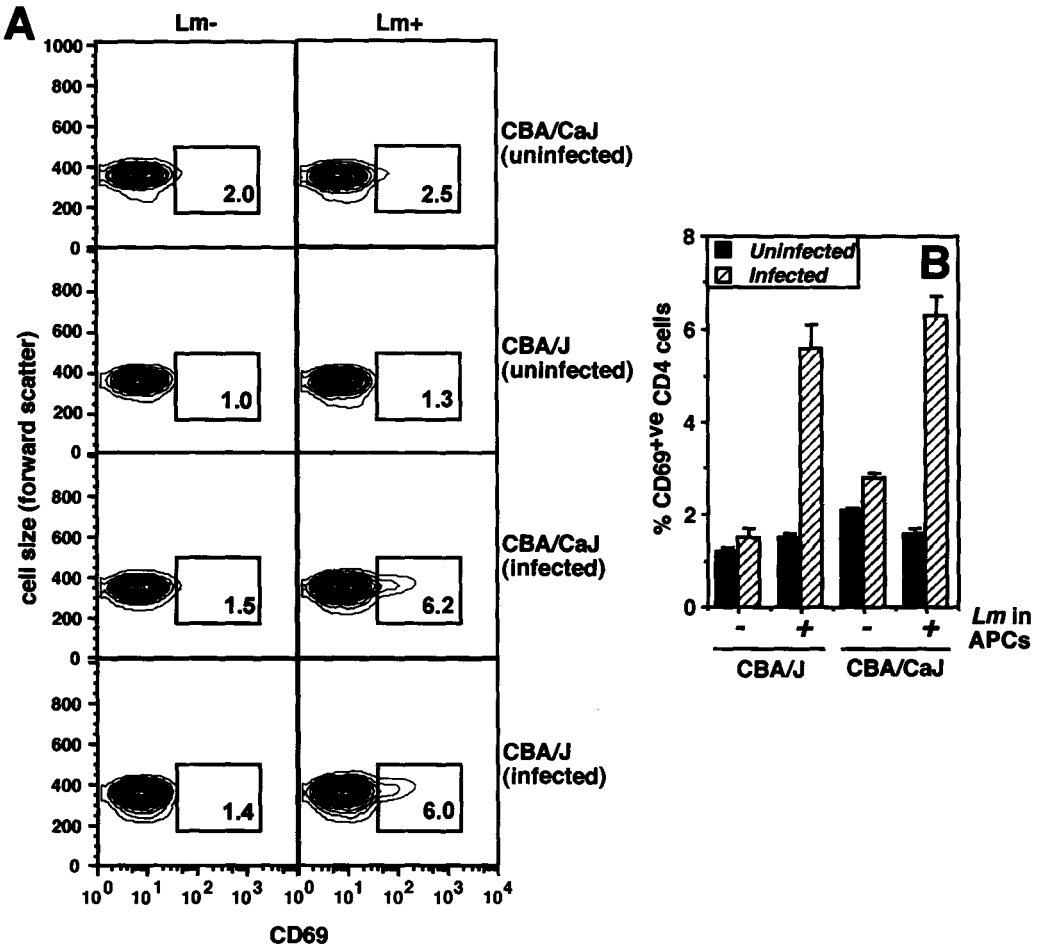



***Figure 24: Similar frequency of Lm specific CD4 T cells in CBD/J and CBA/CaJ***

CD69 depleted CD4 T cells from infected or uninfected CBA/J or CBA/CaJ mice were co-cultured with uninfected or Lm infected CBA/CaJ derived BMDCs. 16 h later, cells were surface stained for CD4/CD69 and Lm-specific CD4 T cells were identified flow cytometrically by specific upregulation of CD69 in response to Lm infected BMDCs, compared to uninfected BMDCs. Cells were initially gated on CD4 cells, the represented frequency identifies CD69 upregulation on cells of increased size **(A)**. Data represent percentage frequency of CD69 positive CD4 T cells in culture $\pm$ SE **(B)**.

identified. Higher frequencies of Lm-specific CD4 T cells were found in the effector CD4 T cell populations, however no differences were apparent between Lm-infected CBA/J and CBA/CaJ mice **(Fig. 25)**.

## *Lm-specific CBA/J T cells make lower levels of IFN-γ than CBA/CaJ T cells*

Supernatants from lymphocyte co-cultures with draining lymph node CD4 T cells of Lm-infected CBA/J or CBA/CaJ mice were analyzed for cytokine levels after 24 and 48 h of culture. Consistently, CBA/CaJ CD4 T cells made more IFN-γ than CBA/J T cells **(Fig. 26A)**. Similarly set up lymphocyte co-cultures with purified $CD62L^{low}$ effector CD4 T cells from infected CBA/J and CBA/CaJ mice, yielded higher total levels of IFN-γ in the supernatant, but CBA/J CD4 effectors still made persistently lower levels of IFN-γ than the CBA/CaJ CD4 effector cells **(Fig. 26B)**. When IL-10 was estimated in these cultures, comparable levels of the cytokine were secreted by effector CD4 T cells from both strains **(Fig. 26C)**. None of the classical Th2 cytokines like IL-4, IL-5 or IL-13 were detectable in any of these cultures.

Next, using intracellular IFN-γ staining, the frequencies of CD4 T cells making IFN- γ were determined. Among the draining lymph nodes CD4 T cells from 3 weeks after Lm infection, the frequencies IFN-γ making CD4 T cells were lower in CBA/J than in CBA/CaJ mice at 40 h of co-culture **(Fig. 27)**.

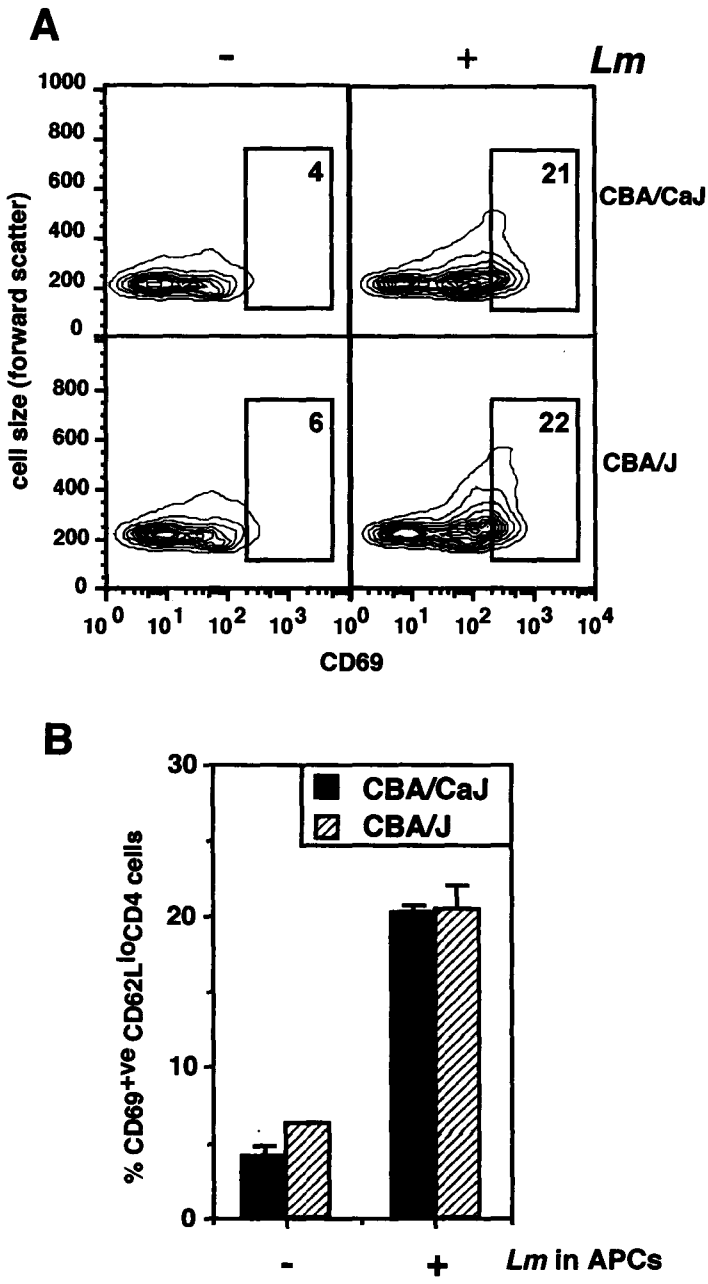



***Figure 25: Similar frequency of Lm-induced effector CD4 T cells in CBA/J and CBA/CaJ***

Purified CD69 depleted "effector" CD4 T cells from infected CBA/J or CBA/CaJ mice were co-cultured with Lm infected or uninfected CBA/CaJ BMDCs at T cells:DC ratio of 3:1. After 16 h of culture, CD69 upregulation was assessed by flow cytometry **(A).** Frequency of $CD69^+$ effector CD4 T cells, expressed as a percentage of effector CD4 T cell population in culture $\pm$ SE **(B).**

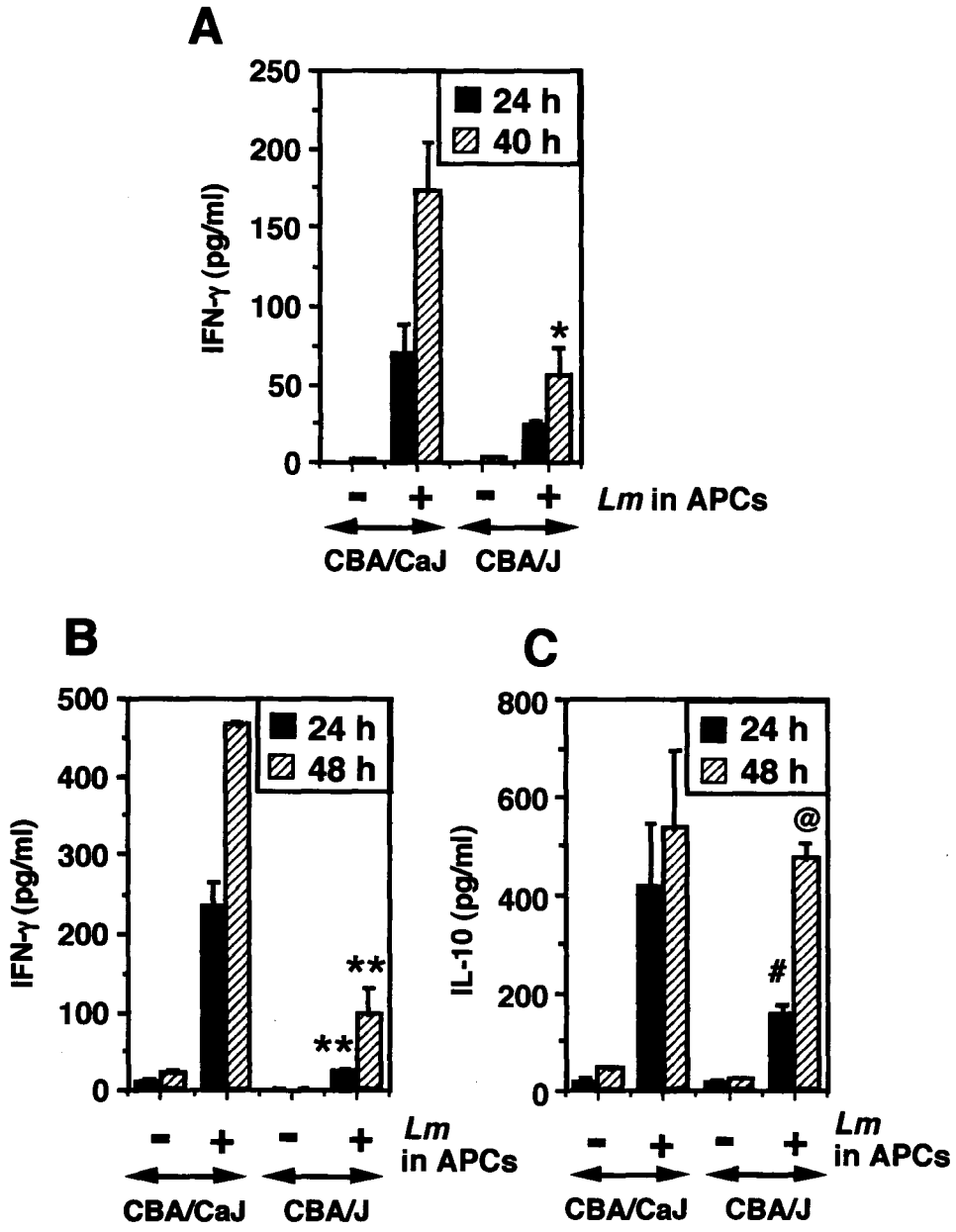



***Figure 26: CBA/J T cells make lower amounts of IFN-γ; similar levels of IL-10 compared to CBA/CaJ***

Purified total and "effector" CD4 T cells infected CBA/J or CBA/CaJ were co-cultured with Lm infected or uninfected CBA/CaJ BMDCs at a T cell: BMDC ratio of 3:1. At indicated time points, supernatant IFN-γ levels were estimated for the CD4 T cell **(A)** and CD4 effector T cell co-culture; in addition supernatant IL-10 levels were also estimated in the latter **(C)**. Data represent mean $\pm$ SE of triplicate cultures. CBA/J and CBA/CaJ were compared using two-tailed student's t-test (*$p<0.05$, **$p<0.005$, #$p=0.13$, @$p=0.72$)

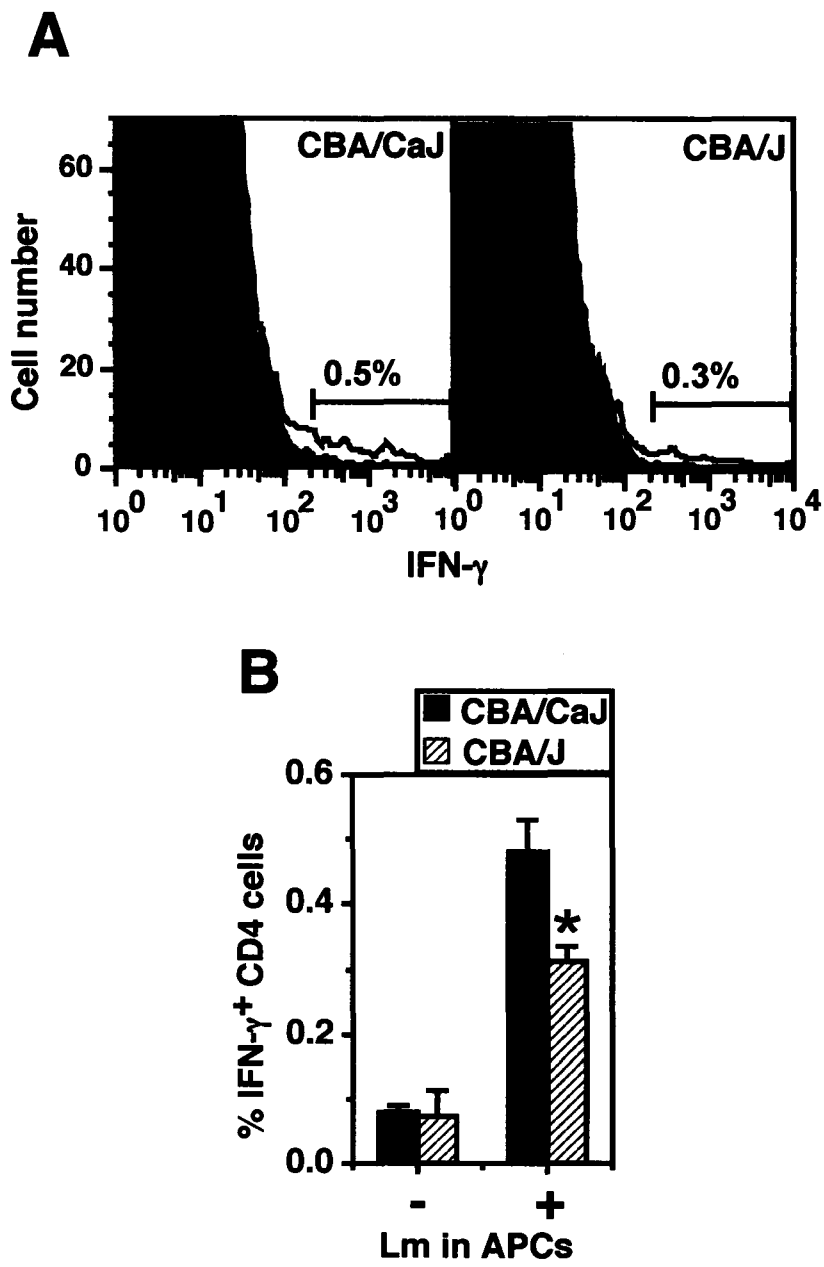



***Figure 27: Frequency of IFN-γ making CD4 cells was higher in CBA/CaJ***

Purified CD4 T cells from Lm infected CBA/J or CBA/CaJ mice were co-cultured with Lm infected or uninfected CBA/CaJ BMDCs at a ratio of 3:1. At 40 h of culture, CD4 T cells were harvested from culture and stained for cell surface CD4 and intracellular IFN-γ **(A)** and frequency of IFN-γ positive CD4 cells was estimated **(B)**. Data were compared by two-tailed student's t-test (*$p<0.05$)

The frequency of IFN-γ synthesis induced by co-culture with Lm-infected BMDCs in $CD62L^{low}$ effector CD4 T cells from infected mice was also estimated. At 24 h of stimulation in culture, CBA/J mice had fewer IFN-γ-making effector CD4 T cells than CBA/CaJ mice **(Fig. 28A)**. However, the frequencies of cells upregulating CD69 expression at the same time point were similar between cultures from the two strains **(Fig. 28B)**. These differences persisted at 48 h in culture as well **(Fig. 28C&D)**. These differences were accompanied by a concomitantly low level of IFN-γ secreted into the culture supernatants of CBA/J effector CD4 cells at both the time points **(Fig. 28E)**.

The Lm-specific CD4 T cell frequencies in draining lymph node $CD62L^{low}$ effector CD4 cells from Lm-infected (CBA/J x CBA/CaJ) F2 mice, were also examined. The frequencies of effector CD4 cells upregulating CD69 upon stimulation with Lm-infected BMDCs were similar between F2 mice inheriting *mtv-6* alone, *mtv-7* alone, or both *mtv-6* and *mtv-7* **(Fig. 29A)**, indicating comparable magnitudes of Lm-specific T cell responses. Further, despite the comparable Lm-specific T cell frequencies, IFN-γ levels were lower in cultures of T cells from F2 mice inheriting *mtv-7* alone or both *mtv-6* and *mtv-7*, than in mice inheriting *mtv-6* alone **(Fig. 29B)**. Therefore, indicating an association of *mtv-7* inheritance with lowered Lm-specific IFN-γ responses, without any associated shift towards a Th2 response.

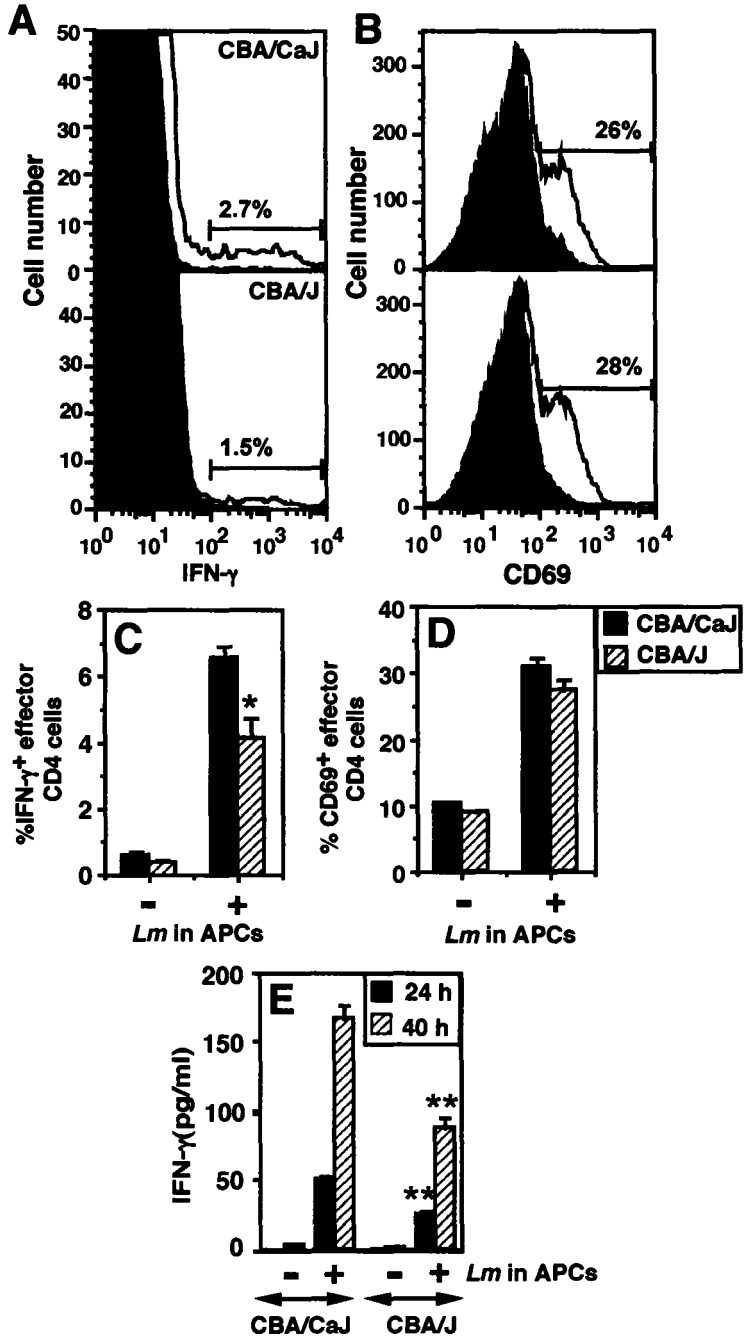



*Figure 28: Higher frequency of IFN-γ positive CD4 T cells in CBA/CaJ*

Purified effector CD T cells from infected CBA/J or CBA/CaJ mice co-cultured with CBA/CaJ BMDCs at a ratio of 3:1 were stained for CD4/CD69 and intracellular IFN-γ at 24 h (A,B) and 48 h (C,D) of culture. Supernatant IFN-γ was also estimated **(E)**. Data were compared by two-tailed student's t-test (*$p<0.05$, **$p<0.005$)

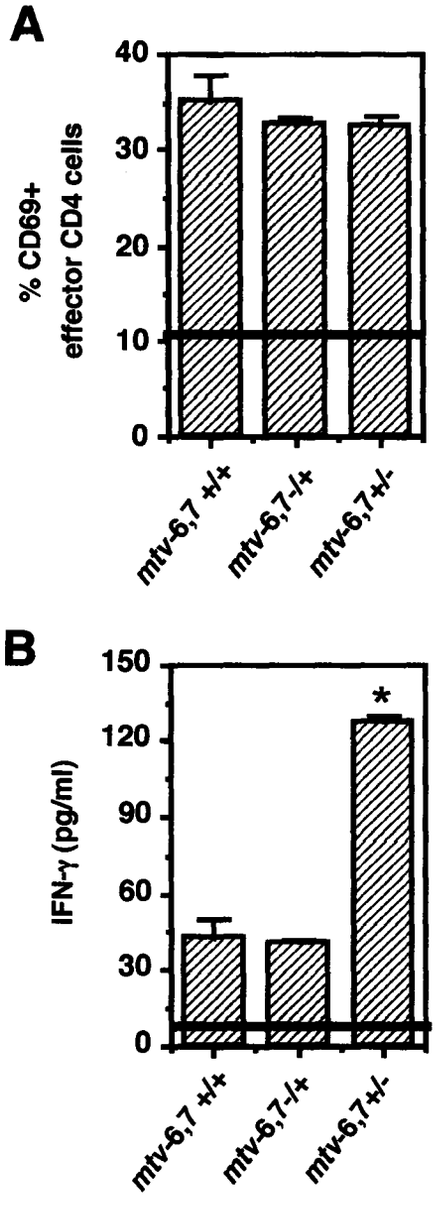



***Figure 29: (CBA/J x CBA/CaJ) F2 mice inheriting mtv-7 show poor Lm-specific IFN-γ response***

CD69 depleted effector CD4 T cells from *mtv-6*, *mtv-7* and *mtv-6&7* integrant F2 mice were co-cultured with Lm infected or uninfected CBA/CaJ BMDCs at a ratio of 1:1; frequency of CD69 positive CD4 T cells **(A)** was assessed by flow cytometry and supernatant IFN-γ was quantified by ELISA **(B)**. Horizontal bar represents the supernatant IFN-γ levels in uninfected BMDC co-culture. Data were compared using two-tailed student's t-test (*$p<0.005$)

### *Low IFN-γ levels made by CBA/J T cells are not due to absence of v-SAg-7-reactive TCRVβ subsets*

Since the magnitude of the T cell response was similar between CBA/J and CBA/CaJ, it was possible that lymph node CD4 T cells from the CBA/CaJ bearing v-SAg-7-reactive TCRVβ subsets contributed disproportionately more to the Lm-specific response, either in frequency of responder cells and/or in the IFN-γ levels generated. To address this, the Lm-specific CD4 T cell frequencies in $CD62L^{low}$ effector CD4 T cells bearing various TCRVβ segments from CBA/J and CBA/CaJ mice were examined in a co-culture assay. TCRVβ3 and TCRVβ6 were tested as representative TCRVβ subsets deleted by v-SAg-6 and v-SAg-7 respectively, and TCRVβ4, TCRVβ10 or TCRVβ14 as subsets unaffected by these v-SAgs in both strains. Lm-specific CD4 T cell frequencies were similar in all TCRVβ subsets in CBA/CaJ mice, and were comparable to the v-SAg-unaffected subsets in CBA/J mice **(Fig. 30A)**. Since the frequency of Lm-committed effector CD4 cells was similar between all the TCRVβ subsets irrespective of v-SAg reactivity, possibility of TCRVβ subset specific variation in the IFN-γ commitment was next examined. Lymphocyte co-culture was set up using purified T cells of various TCRVβ subsets from infected CBA/J and CBA/CaJ. TCRVβ3, TCRVβ6 and TCRVβ14 as representative subsets that recognize v-SAg-6, v-SAg-7, or neither respectively, were purified. Unfractionated T cells were also used in the assay. IFN-γ levels estimated in culture supernatants at 24 h of culture. T cells of all TCRVβ subsets in a given strain made equivalent levels of IFN-γ. However, T cell subsets from CBA/J mice made less IFN-γ than that made by similar TCRVβ subsets from CBA/CaJ **(Fig. 30B)**. Thus, there was a generalized reduction of IFN-γ output from Lm-specific CBA/J T cells than from

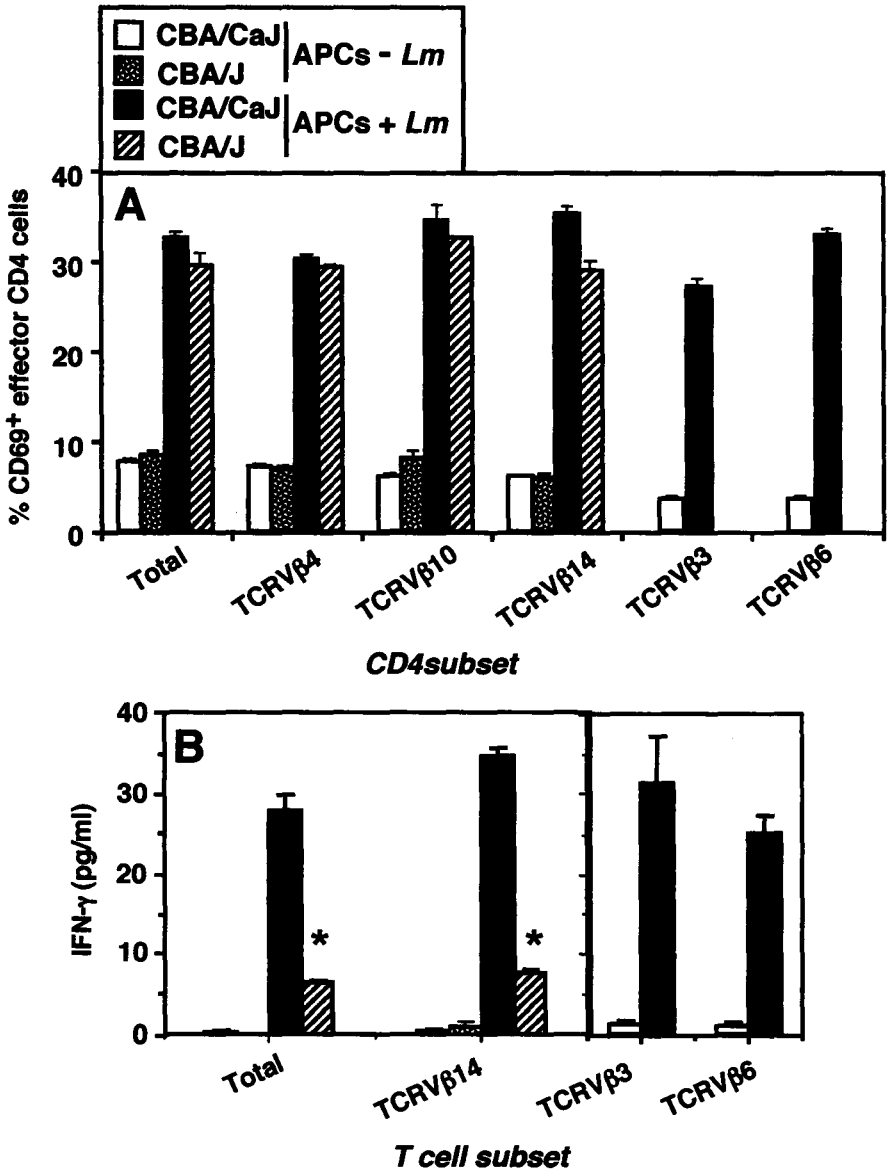



***Figure 30: Lack of vSAg-7 reactive T cell subsets was not responsible for compromised Lm-specific IFN-γ response in CBA/J***

After 16 hrs of co-culture of CD69 depleted effector CD4 T cells, CD69 upregulation was analyzed on vSAg-6&7 reactive CD4 T cell subsets (Vβ3&Vβ6) from CBA/CaJ and non-reactive CD4 T cell subsets from both CBA/CaJ and CBA/J (Vβ4, Vβ10&Vβ14)(**A**). Purified TCRVβ T cells of vSAg-6&7 reactive and non-reactive subsets were co-cultured for 24 h and supernatant IFN-γ was estimated (**B**). Data were compared by two-tailed student's t-test (*$p<0.005$).

CBA/CaJ T cells, rather than any effect of deletion of v-SAg-7-reactive TCRVβ subsets from the CBA/J repertoire.

### *Lm-directed CBA/J and CBA/CaJ macrophage functions are similar*

Since macrophage effector responses play a crucial role in effecting Lm clearance, it was next examined whether the difference in Lm susceptibility could be ascribable to differences in Lm-killing capacity between CBA/J and CBA/CaJ macrophages. Adherent macrophages from peritoneal resident cells (PRCs) or TG elicited peritoneal macrophages (PECs), or M-CSF-grown bone marrow-derived macrophages (BMDMs) were infected with fluorescence labeled Lm and clearance of infection was scored over time by fluorescence microscopy. PRCs **(Fig. 31A)** and PECs **(Fig. 31B)** showed similar peak levels of infection at 50% of cells showing infection at 0 h at an MOI of 50:1, followed by complete clearance by 48 h. Both parasitaemia as well as parasite burden were similar at all time points between CBA/J and CBA/CaJ **(Fig. 31D)**. iNOS$^{-/-}$ macrophages (both PRCs and PECs) showed delayed clearance compared to wild-type-BL/6 or CBA/J and CBA/CaJ **(Fig. 31E)**.

BMDMs, at a similar MOI of 50:1, showed higher peak infection at 0 h and somewhat slower Lm elimination **(Fig. 31C)**. However, no differences were apparent in both the parasite burden as well as frequency of Lm infected cells between the two strains at all time points.

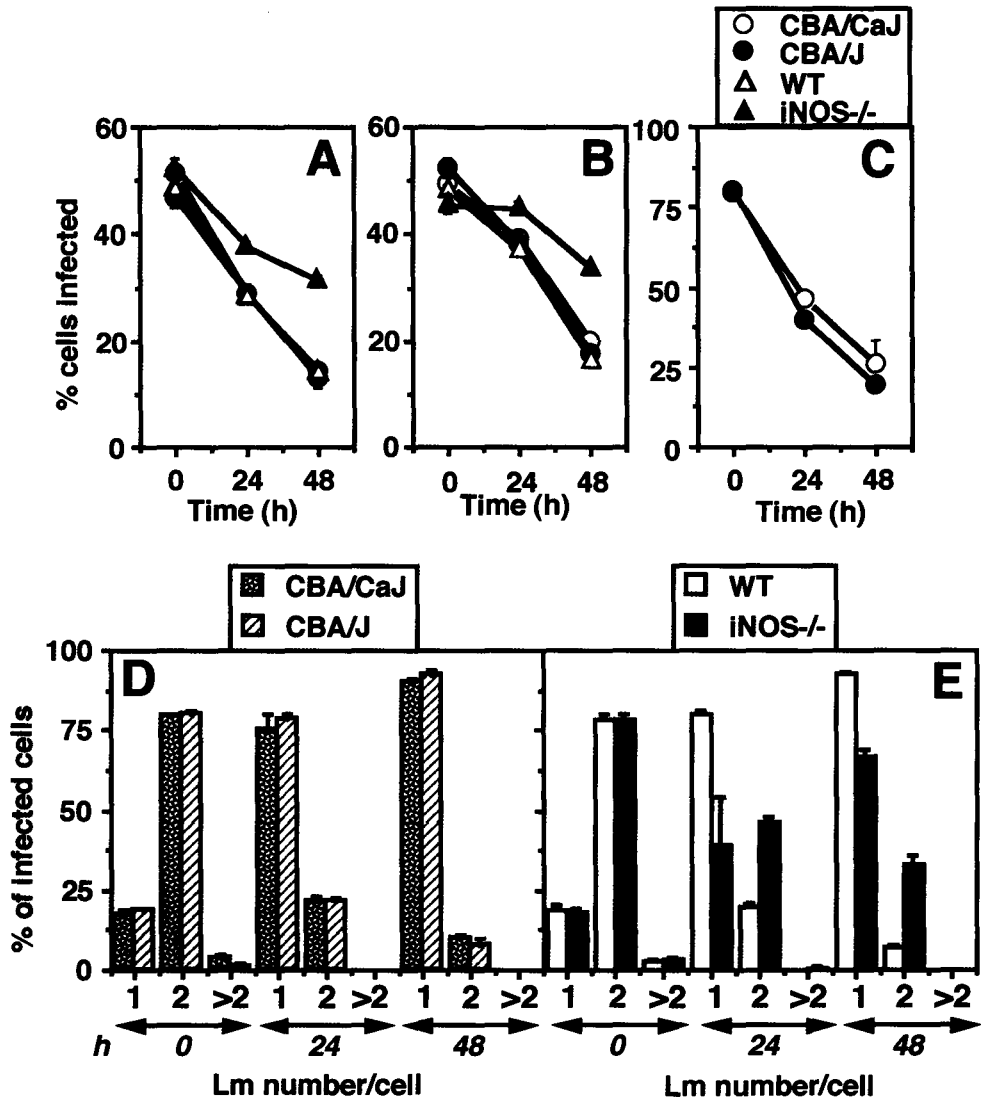



***Figure 31: CBA/J and CBA/CaJ macrophages show comparable Lm clearance ability***

Adherent PRCs **(A)**, PECs **(B)** or BMDMs **(C)** were infected with 25 x$10^6$ fluorescently labeled stationary phase Lm at a parasite:macrophage ratio of 50:1 and parasite clearance was quantified by fluorescence microscopy. **(A,B,C)** represent frequency of infected cells at various time points, **(D)** shows quantification of parasite burden per infected cell on infected PRCs. At least 900 cells were counted for each group in sets of 300 cells each and expressed as mean $\pm$ SE.

Next, the ability of Lm infected CBA/CaJ and CBA/J DCs to generate effector molecules and pro-inflammatory cytokines, was tested since Lm infection has been reported to result in inhibition of both these responses (Carrera et al., 1996; Proudfoot et al., 1996; Proudfoot et al., 1995). BMDCs from both CBA/J and CBA/CaJ were infected with Lm, and IFN-γ or LPS was added to the cultures, or left untreated. In one group, IFN-γ was added 24 h after infection to mimic a more physiological scenario of T cell help an infected DC. Lower levels of NO were induced in Lm infected DCs, with the IFN-γ treated DCs secreting more NO than the LPS treated DCs, and the group receiving an IFN-γ stimulus after 24 h of infection showing the most inhibition, with an NO level close to background uninfected DCs **(Fig. 32A)**. Induction of pro-inflammatory cytokine IL-12 also revealed a similar pattern of inhibition on Lm infection **(Fig. 32B)**. TNF-α levels in the supernatant were most robustly induced on LPS than IFN-γ stimulation **(Fig. 32C)**, this was consistent with the role of LPS as a potent inducer of TNF-α (Tsan et al., 2001). However, no differences were observed between CBA/J and CBA/CaJ BMDCs in NO, IL-12 or TNF-α induction.

### *APC identity in recall does not alter the level of IFN-γ production*

Since the source of re-stimulating APCs used in the co-culture assays was CBA/CaJ BMDCs, it was also possible to hypothesize that v-SAg-7-expressing DCs might be defective in eliciting an Lm-specific recall response. Therefore, IFN-γ responses induced on re-stimulation with CBA/J and CBA/CaJ DCs and any difference in the elicited responses was examined. Since only v-SAg-6/7-non-reactive TCRVβ subsets could be used for this purpose, purified TCRVβ14 T cells from Lm-infected CBA/J and CBA/CaJ

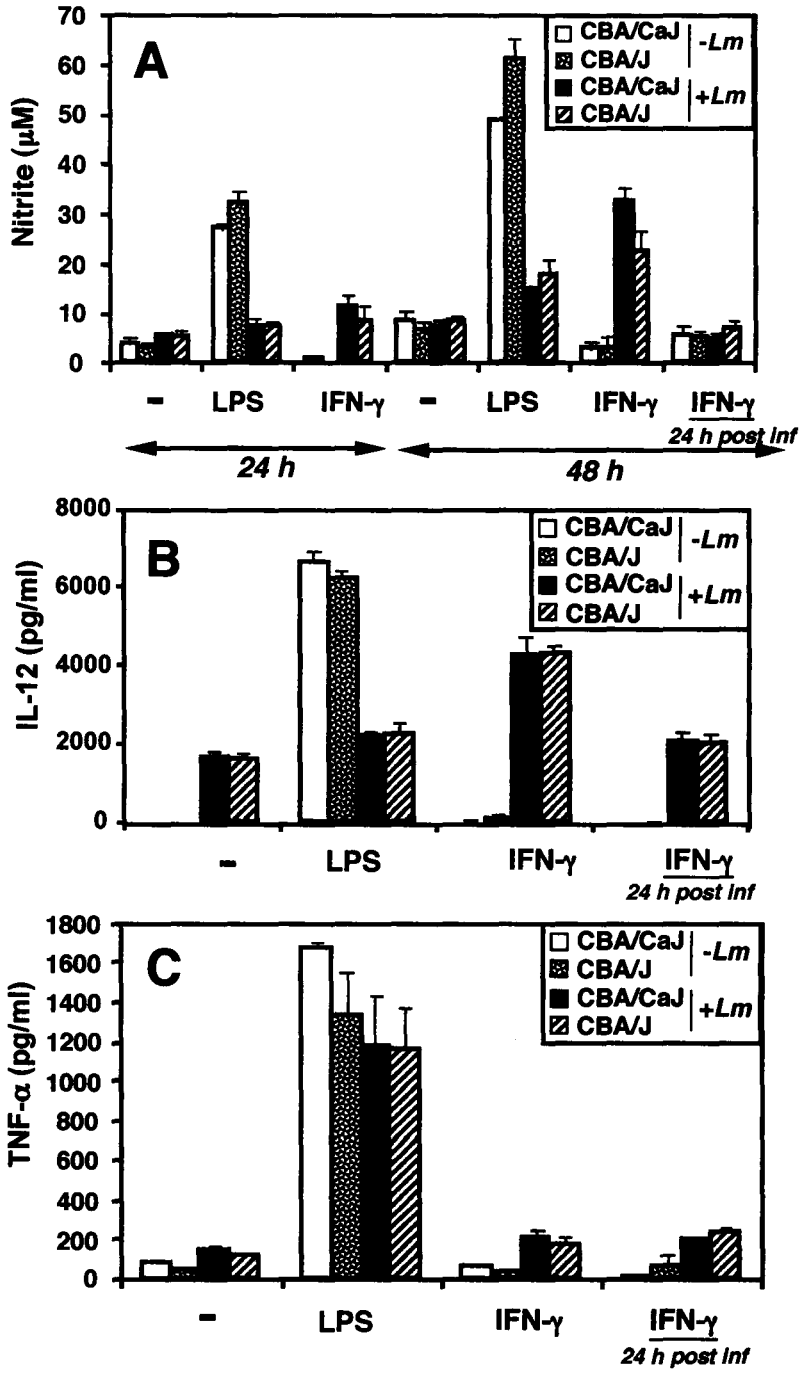



***Figure 32: Lm infected CBA/CaJ and CBA/J BMDC show cytokine and nitrite responses***

CBA/CaJ and CBA/J BMDCs were infected with Lm at a parasite:macrophage ratio of 25:1, in some groups, LPS (30 μg/ml) or IFN-γ (30 IU/ml) was added at the time of infection, or at 24 h of infection as indicated. Supernatant nitrite levels were estimated by Griess test at 24 h and 48 h of culture **(A)**. Levels of secreted IL-12 (B) and TNF-α (C) were estimated after 48 h of culture. Data represent mean ± SE values of triplicate cultures.

mice were co-cultured with either CBA/J or CBA/CaJ Lm-infected DCs. IFN-γ levels were then estimated in the culture supernatants at 40 h of re-stimulation. It was observed that, regardless of the source of the re-stimulating DCs, CBA/J TCRVβ14 T cells consistently made lower levels of IFN-γ then CBA/CaJ T cells **(Fig. 33)**. Similarly, CBA/CaJ TCRVβ14 T cells made higher IFN-γ levels than CBA/J T cells **(Fig. 33)**.

### *Altered T cell priming by CBA/J BMDCs is not immunogen independent*

To explore whether lower IFN-γ responses were generated in the CBA/J mice regardless of the immunogen, CBA/J and CBA/CaJ mice were immunized subcutaneously with maleylated ovalbumin (mOVA) in complete Freund's adjuvant. One week later, draining lymph node CD69 depleted CD4 T cells were purified and re-stimulated with CBA/CaJ DCs in presence or absence of 100 μg/ml mOVA. After 16h, CD69 upregulation was assessed on responding effector $CD44^{high}$ CD4 cells **(Fig. 34A)**. No differences in the frequencies of mOVA-specific CD4 cells were found between the two strains. Further, when supernatant IFN-γ levels were assayed from these cultures at 24 and 48 h post-re-stimulation, no differences were evident in the levels of the cytokine induced in the two strains **(Fig. 34B)**. Frequencies of IFN-γ making CD4 cells were analyzed by intracellular IFN-γ staining and were found to be similar in CBA/J and CBA/CaJ cultures **(Fig. 34B)**.

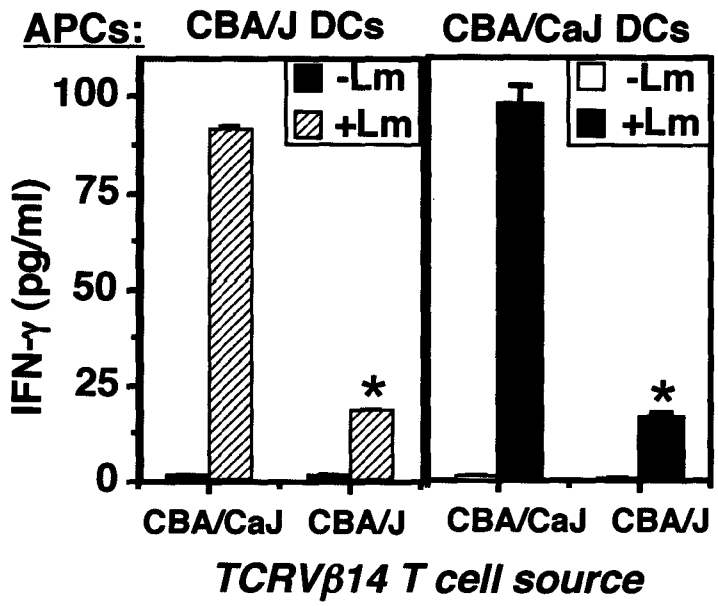
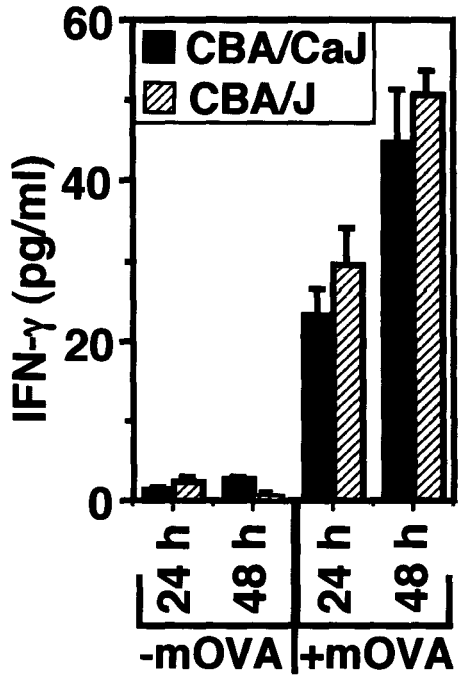



***Figure 33: Low IFN-γ responses generated by CBA/J T cells was independent of recall APC***

TCRVβ14 T cells from infected CBA/J or CBA/CaJ mice were re-stimulated with both CBA/J and CBA/CaJ DCs, uninfected or infected with Lm, and supernatant IFN-γ levels estimated at 48 h of co-culture.

**A**




**B**

| *1 week post immunization* | *CBA/CaJ* | *CBA/J* |
|---|---|---|
| % CD69 upregulation on $CD44^{high}$ CD4 T cells | 4.2 ± 1.6 % | 3.9 ± 1.7 % |
| % IFN-γ positive CD4 T cells | 2.0 ± 0.5 % | 2.2 ± 0.5 % |

***Figure 34: Altered T cell priming by CBA/J BMDCs was not immunogen independent***

CBA/J and CBA/CaJ mice were immunized with mOVA (100μg per mouse) in CFA. 1 week later, CD69 depleted CD4 T cells were purified and co-cultured with CBA/CaJ BMDCs in the presence or absence of 100μg mOVA. At 24 and 48 h of culture, supernatant IFN-γ levels were estimated by ELISA **(A)**. Data represent pooled mean ± SE values of three independent experiments, run in triplicates. Frequency of CD69 upregulating and IFN-γ making CD4 T cells was also estimated at 16 h and 48 h of co-culture, respectively **(B)**.

# Discussion

# *Discussion*

Disease phenotypes are a manifestation of the eventual outcome of multiple interactions betweens various cell lineages involved in an inflammatory process, driven by cellular signaling intermediates. Pathogen recognition by host cells initiates the activity of a series of cellular and molecular signaling components of the immune system, designed to achieve elimination of the invading pathogen and repair the tissue damage caused. Many signaling molecules have phylogenetically ordered functions, across cell lineages and in some cases, even across species. Toll like receptor (TLR) signaling and mitogen activated protein kinase (MAPK) signaling pathways are prime examples, showing conservation across species from invertebrates to vertebrates, and even in plant species (Hamel et al., 2006; Janeway and Medzhitov, 2002; Kultz, 2001). In addition to their role in cells of the immune system, such signaling molecules can also have regulatory roles in controlling development and functions of other lineages. Since the various cell lineages of the immune system are developmentally related, they share many common cellular signaling intermediates, increasing the probability that any given signaling molecule will be used in multiple signaling contexts. Hence, any defect in such intermediates, will be manifested by several cell lineages during an immune response.

The present work is an attempt to address, in a number of instances, the putative roles for such signaling intermediates in various cellular components involved in immune inflammatory cross-talk.

## *Bruton's tyrosine kinase (BTK) in myeloid cells: Role in eosinophil lineage*

One such intermediate, Bruton's tyrosine kinase (BTK), functions as a major intermediate in signal transduction and is expressed in both lymphoid and myeloid lineage cells (Smith et al., 1994). BTK has previously been shown, to participate in macrophage and neutrophil effector functions, and modulate neutrophil development (Horwood et al., 2003; Kawakami et al., 1998; Kawakami et al., 2006; Mangla et al., 2004; Schmidt et al., 2004).

Neutrophils comprise the vast majority of circulating granulocytes and form the first line of defense of the innate immune system. They are recruited by many inflammatory mediators such as IL-1β, TNF-α, or clotting factors, and act by phagocytosing, killing, and degrading microbial pathogens such as bacteria and fungi. Killing is accomplished in the phagocytic vacuole, where free oxygen radicals and other reactive oxygen species generated by the translocated NADPH oxidase activity, coupled with the activity of proteolytic enzymes, results in microbial killing. Eosinophils normally constitute a minority of the circulating granulocyte pool, with the majority sequestered in the intestinal lamina propria. They are recruited in specific response to the generation of Th2 (and endothelial) cytokines such as IL-4, IL-5, or IL-13, and appear to mediate host protection against parasites, via the release of an array of cytotoxic granule cationic proteins, with some role for ROI generation as well. Though eosinophils and neutrophils form component cells of the granulocyte lineage and are characterized by their ability to phagocytose and eliminate microbial pathogens, they differ in the choice of stimulus as well as in the mode of pathogen destruction utilized. Hence, given the reported role of

BTK in modulating neutrophil effector function, it was of interest to elucidate the possible role of BTK in the eosinophil lineage.

The relatively low proportion of eosinophils in the total granulocyte pool has been one of the major limitations for initiating studies on eosinophils. The IL-5 Tg mice, in which generation of IL-5, a major inducer of eosinophil recruitment is controlled by an inducible metallothionein promoter, have proved to be a useful tool to address this problem (Tominaga et al., 1991). In these mice constitutive expression of IL-5 transgene observed in the liver and spleen, possibly as a result of trace zinc in drinking water, since zinc is also known to trigger the metallothionein promoter (Alhonen et al., 1996), resulted in 16,000 fold higher levels of IL-5 as compared to heterozygous littermates, which was further increased by another five-fold on induction with cadmium injection (Tominaga et al., 1991). Consistent with the previous report, a high proportion of the circulating granulocytes in the IL-5 Tg mice were eosinophils **(Fig. 1A&B).**

Further, in keeping with their frequency in circulation, a high proportion of the granulocytes recruited to sites of acute inflammation, were eosinophils **(Fig. 1C).**

To examine the role of BTK in modulating eosinophil functions, male X-linked immunodeficient (XID) mice lacking functional BTK were crossed with female IL-5 Tg mice. The male progeny which carried the XID mutation were used as XID/IL-5 Tg mice and the littermate female mice were used as WT/IL-5 Tg. Though this approach has previously been reported in a study on XID eosinophils (Koike et al., 1995), there remains a possibility of gender based differences which may contribute to the differences

observed. Macrophages from male and female mice have been reported to show differences in eicosanoid production in collagen induced arthritis (Leslie et al., 1987), androgens have been reported to influence lymphoid development (Olsen and Kovacs, 2001), susceptibility to various autoimmune diseases including multiple sclerosis and systemic lupus erythematosis (Yu and Whitacre, 2004) have also been reported to be gender based. In view of this, the findings in the XID eosinophils reported here will need further validation in gender matched mice.

Peripheral blood leukocytes from the XID/IL-5 Tg mice showed a higher frequency of eosinophils when compared to the WT **(Fig. 2B)**. When considered in context of the reported mild defect in PMN development in XID mice, it is possible to speculate that eosinophil generation could be less affected by the XID mutation than PMN generation. However, further studies on eosinophil development will be needed to confirm this.

Compared to WT eosinophils with functional BTK, XID eosinophils displayed compromised recruitment to sites of inflammation **(Fig. 2A&C)**, similar to the defect shown by XID macrophages and neutrophils.

Thioglycollate induces a non-infectious inflammation, resulting in recruitment of large numbers of neutrophils, followed by macrophages. Initial granulocyte recruitment is followed by release of chemokines, resulting in upregulation of surface adhesion molecules and further chemokine release, which is dependent on their transcriptional induction. In XID macrophages, induction of some of these transcription factors is compromised, possibly resulting in poorer recruitment. Further, in XID neutrophils, poor recruitment is compounded by reduction on the number of granulocytic lineage cells in

the XID bone marrow. In view of the possible lack of an effect of the XID mutation on eosinophil generation, activation of chemokine and surface adhesion molecules appear to have a greater influence on eosinophil recruitment in the XID mice. However, the levels of Siglec-F, which is induced on eosinophil activation (Yamada et al., 2007), appear unchanged on XID eosinophils.

Generation of reactive oxygen species (ROI) was also compromised in the XID eosinophils **(Fig. 2D&E)**, again reminiscent of a similar deficiency exhibited by XID macrophages and neutrophils.

ROI generation is elicited in phagocytes, by microbial products (LPS, lipoproteins) and cytokines (IFN-γ, IL-18). NADPH (nicotinamide adenine dinucleotide phosphate) oxidase is one of the major sources of ROIs; membrane recruitment of its subunits, followed by assembly into the catalytic NADPH oxidase results in activation of a redox signaling pathway which generates reactive oxygen free radicals as reductive products from cellular oxygen. LPS signaling induced TLR4 mediated ROI induction has been reported to involve activation of protein kinase C (PKC), such that inhibition of PKC activation results in abrogation of ROI generation (Zheng et al., 1995). A constitutive association has been shown between BTK and PKC in B cells (Johannes et al., 1999) and murine mast cells (Yao et al., 1994). BTK has been shown in these studies to be a substrate of PKC isoforms and its enzymatic activity to be downregulated by PKC mediated phosphorylation (Yao et al., 1994). In turn BTK has been shown to regulate the membrane translocation and activation of PKC by direct interaction with this kinase (Kawakami et al., 2000). In XID cells, macrophages, neutrophils or granulocytes, the

disruption of this pathway could be responsible for the poorer ROI generation observed. This is further borne by the fact that upon direct PKC activation by stimulation with phorbol 12-myristate 13-acetate (PMA), thereby bypassing the requirement for BTK signaling, both XID and WT neutrophils generated equivalent levels of ROI (Mangla et al., 2004). These data thus indicate a role for BTK in controlling eosinophil effector functions, analogous to its function in neutrophils and macrophages.

## *Role of the BTK-targeted transcription factor c-Rel in macrophage effector functions*

Pathogen triggered signaling events culminate in generation of effector molecules geared at achieving pathogen elimination. Soluble signaling molecules including various cytokines which are generated, further act to amplify the myeloid immune response and also mediate proliferation and differentiation of lymphoid cells. Transcription factors of the NF-κB family act as integral second messengers in the enhancement of these multiple signaling events (Ghosh et al., 1998).

Previous work has shown poorer induction of Rel family members of NF-κB transcription factors in XID mouse macrophages. Of the three members studied, poorer induction of p-50 and complete abrogation of p-65 and c-Rel was seen in XID macrophages (Mukhopadhyay et al., 2002). c-Rel deficient mouse macrophages have also been reported to be deficient in induction of effector cytokine IL-12 (Sanjabi et al., 2000), an important factor controlling Th1 responses. c-Rel$^{-/-}$ DCs have also been reported to exhibit compromised ability to stimulate autologous T cell responses (Boffa et al., 2003). In view of these findings and the connection between BTK and c-Rel, a

possible role for c-Rel in controlling myeloid cell effector functions was investigated using the c-Rel deficient (c-Rel$^{-/-}$) mouse strain.

c-Rel$^{-/-}$ mice had normal differential and total leukocytes counts in peripheral blood **(Fig. 3A&B)**. c-Rel$^{-/-}$ mice also showed normal macrophage recruitment in response to peritoneal TG instillation and were comparable to WT in the induction of acute inflammatory response induced by s.c injection of carrageenan **(Fig. 3C&D)**. Generation of effector molecules by the c-Rel$^{-/-}$ macrophages was also seen to be normal as evidenced by similar levels of RNIs generated by c-Rel$^{-/-}$ and WT macrophages **(Fig. 4A)**. Phagocytic ability of myeloid cells, monocytes and granulocytes from c-Rel$^{-/-}$ mice was found to be normal **(Fig. 4B)**. Generation of ROI was also seen to be comparable to WT **(Fig. 5A)**, though at higher concentration of the stimulus, LPS, c-Rel$^{-/-}$ macrophages seemed to show a reduction **(Fig. 5B)**. Thus it was possible that for generation of the increased levels of ROIs induced at higher dose of the stimulus, initiation of fresh transcription and/or translation might be required. In such circumstances, absence of c-Rel might lead to compromised transcriptional induction of ROI generation, thus explaining the apparent deficit seen in the c-Rel$^{-/-}$ mice. To investigate this, WT macrophages treated with inhibitors of transcription (Actinomycin D) **(Fig. 6B)** and translation (Cycloheximide) **(Fig. 6A)**, were stimulated with high concentration of LPS used as a stimulus and ROI generation was estimated. ROI induction was unaffected on treatment with either inhibitor, indicating the absence of any role for fresh transcriptional or translational events in ROI generation. Induction of ROIs requires the membrane assembly of pre-formed subunits of the NADPH oxidase complex, which possibly have a long enough half life so that their levels remain unaffected by the inhibitor treatment. It

is thus possible that a deficiency of pre-formed NADPH subunits, due to lack of constitutive c-Rel signaling, might be responsible for a lack of ROI induction at high LPS doses in c-Rel$^{-/-}$ macrophages.

Since c-Rel$^{-/-}$ macrophages did not reveal any compromise in myeloid cell effector functions, a simple explanation for this could be an absence of c-Rel induction subsequent to pathogen recognition. As mentioned previously, c-Rel, p-65 and p-50 have been shown to be induced on LPS stimulation, contradicting the above assumption.

Although the Rel/NF-κB family of transcription factors is crucial for the development and function of the immune system, the precise roles of each Rel/NF-κB member in various immune cells are not well understood. Moreover, many of the NF-kB family of transcription factors are known to have functionally redundant roles. However, distinct functions of individual members of the Rel family have been identified. c-Rel has been identified to be crucial in maintaining cell viability and cell cycle progression in B and T lymphocytes, controlling DC maturation, survival and ability to stimulate autologous T cells, but not in upregulation of surface co-stimulatory molecules on DCs. c-Rel expression is seen only in mature macrophages, where it has a non-redundant role in IL-12 induction. In dendritic cells, c-Rel has been shown to be crucial in regulating maturation and stimulation of autologous T cells, without any role in co-stimulatory molecule upreglation (Boffa et al., 2003). Given these roles, c-Rel requirement has been suggested to be specific to the function of IL-12, which is essential for an effective response against intracellular infections, requiring the induction of a strong Th1 response (Sanjabi et al., 2000). Since most of the responses studied here were mimics of

extracellular infections involving TLR mediated receptor signaling, c-Rel might be argued to be redundantly involved in mediating such responses.

### *A role for BTK in T-B cell developmental cross-talk*

Mutations in the gene encoding BTK causes an X-linked immunodeficiency, X-linked agammaglobulinemia (XLA) in humans, and X-linked immunodeficiency (XID) in mice. XLA patients exhibit an early B cell developmental arrest associated with severe agammaglobulinemia involving immunoglobulin (Ig) of all istoypes (Campana et al., 1990; Milili et al., 1993). The XID defect, however, is less severe, manifested by abrogation of the peripheral B cell maturation in the spleen resulting in accumulation of immature $IgM^+$ B cells in peripheral lymphoid organs, associated with a decresase in $IgD^+$ mature B cell numbers to 50% of normal. (Hardy et al., 1983; Kerner et al., 1995; Khan et al., 1995). The resultant hypogammaglobulinemia affects levels of serum IgM and IgG3, with the levels of other isotypes being unaffected. The reason(s) for the different severity of the clinical phenotypes of similar *Btk* mutations in humans and mice are not yet elucidated.

Human XLA results from different mutations spanning the human *Btk* gene, including an R28C mutation, which is the mutation carried by the XID mice. Thus greater diversity in *Btk* mutations was not explanatory of the relative severity of human XLA. Crossing the XID mutation into the FOXN1-null CD40-null or *Tec*-null background leads to a worsening of the B cell immunodeficiency resulting in a disease severity comparable to human XLA (Ellmeier et al., 2000; Oka et al., 1996; Wortis et al., 1982). These reports indicated that developing B cells in the XID marrow might normally be rescued from an

otherwise more severe developmental block akin to that seen in XLA. Surface $IgM^+$ immature B cells, which undergo further development in the spleen, are completely dependent on signaling through the IgM-BCR (Benschop and Cambier, 1999). It is at this stage that a block in the B cell development is evident in the XID mice, indicating that BTK is indispensable for signaling through the BCR. Given these findings, it is possible to speculate that the pathway for the B cell development rescue at the pre-B cell stage in XID marrow might involve a substitution of BTK function by TEC when triggered by CD40 through a pathway involving stromal epithelial cells. Further, mouse pre B cells have been reported to express surface CD40 (Castigli et al., 1996), supporting such a possibility.

The stromal epithelial component crucial for amelioration of the XLA phenotype in mice involves the FOXN1 transcription factor, deficiency of which gives rise to the *nude* defect in mice (Su et al., 2003). Since FOXN1 deficiency leads to thymic aplasia and resultant T cell deficiency, it is plausible to speculate that T cells are a major component involves in amelioration of the XLA phenotype during B cell development in mice.

In order to investigate this hypothesis, the previous observations of severe agammaglobulinemia involving all Ig isotypes observed in T cell deficient athymic-XID/*nude* mice (Oka et al., 1996; Wortis et al., 1982) were first replicated. Serum IgM levels in the XID/*nu* **(Fig. 7A)** were lower than that observed in XID mice consistent with earlier reports, however, variable levels of serum IgG **(Fig. 7B)** were observed in these mice, with a few mice exhibiting very low serum IgG ($IgM^{low}IgG^{low}$) and the remaining ($IgM^{low}IgG^{high}$) showing IgG levels similar to that observed in XID and wild-

type mice. This contrasted with previous reports in which uniformly low levels of both serum IgM and IgG were observed in all XID/*nu* mice examined (Oka et al., 1996). The phenotype of T cell absence in nude mice has been reported to be "leaky", resulting in small numbers of T cells gaining access into the circulation (Cui et al., 2003). It was then possible that these leaky T cells, in case of the $IgM^{low}IgG^{high}$ XID/*nu* mice, affect a rescue to developing B cells resulting in normal levels of serum IgG. This was consistent with the findings when splenic total T cell yields were estimated. The $IgM^{low}IgG^{high}$ XID/*nu* mice had two-fold higher total splenic T cell numbers **(Fig. 8B)** as compared to the $IgM^{low}IgG^{low}$ XID/*nu* mice or the WT/*nu,* though in comparison to CBA/CaJ or the CBA/N, these T cell numbers were still about 6-fold lower, they appeared to be proficient at rescuing developing B cells, resulting in a splenic B cell frequency close to that seen in the CBA/N mice **(Fig. 8A-upper panel)**. $IgM^{low}IgG^{low}$ XID/*nu* mice displayed very low splenic T and B cell frequency and cell yields, with a complete absence of mature $IgD^{+}$ B cells **(Fig. 8A-lower panel)**. The B cell phenotype in the $IgM^{low}IgG^{high}$ XID/*nu* mice was similar to that seen in CBA/N mice with an apparent immature-mature developmental block resulting in accumulation of $IgM^{+}$ B cells and reduced frequencies of $IgD^{+}$ B cells **(Fig. 8A-lower panel)**.

In light of these findings, the XID mutation was bred onto a T cell receptor (TCR) β chain null background, where due to the lack of the TCR β chain, complete ablation of αβ T cells could be achieved. Further, since the *nude* defect in FOXN1 is an epithelial cell defect manifest in all stromal epithelial cells, this approach would also enable identification of the B cell developmental rescue as being completely T cell intrinsic.

On estimation of total circulating Ig, compared to CBA/N or XID/TCR $\beta^{-/+}$ littermates, a further 10-fold lower serum IgM levels **(Fig. 9A)** and a 400-fold reduction in serum IgG **(Fig. 9B)** levels were consistently observed in all the XID/TCR $\beta^{-/-}$ mice analyzed. Remarkably low total splenic and lymph node yields at were observed in the XID/TCR $\beta^{-/-}$ mice. Bone marrow B cell numbers were also exceptionally low **(Fig. 10B)** with very low frequency of $IgM^+$ immature B cells **(Fig. 10A-lower panel)**. On staining for the developing B cell subsets in the bone marrow, all the B cells in the XID/ TCR $\beta^{-/-}$ mice were found to be in the pro/pre B cell ($CD43^+B220^+$) subset, compared to CBA/N in which the frequencies of B cells in the pro-/pre- and the immature B cell ($CD43^-B220^+$) subsets were similar to that seen in WT-CBA/CaJ. This phenotype perfectly mimics the pro/pre B cell developmental block observed in human XLA.

Previous reports have shown contradicting results on the bone marrow B lineage development in the XID/*nu* mice. While one report demonstrates an early B lineage developmental arrest (Wortis et al., 1982), the other report suggests the XID/*nu* marrow shows no impairment in B cell development in bone marrow beyond the defects seen with the single XID mutation (Oka et al., 1996). The data reported here demonstrate that in specific conditions of T cell sufficiency, both these contradictory findings were reproducible. While in the $IgM^{low}IgG^{low}$ XID/*nu* mice, there was a severe B cell deficiency, the T cell numbers were low, in the $IgM^{low}IgG^{high}$ XID/*nu*, which showed a less severe B cell deficiency similar to that seen in XID mice, higher number of mature T cells was observed. Therefore, it is possible to suggest that the discordance in previous literature is due to the leakiness of the *nude* phenotype.

A similar developmental block at the pro/pre B cell stage has been reported in Tec/Btk double-deficient mice (Ellmeier et al., 2000). Together, these data can be interpreted to suggest that, in the XID mice, T cells effect rescue of developing XID B lineage cells from an otherwise inevitable developmental block at the pro/pre B cell stage, as seen in human XLA, via induction of TEC-mediated substitution of signaling. Early B cell development is dependent on various cytokines including IL-3 and IL-7 (Marshall et al., 1998). T cells and TEC could thus have a novel role in the secretion and signaling, respectively, of these cytokines. While bone marrow stromal cells from XID and WT mice have been reported to posses similar capabilities for driving B cell differentiation, the use of SV-40-mediated transformation in those experiments could have substituted for the lack of T cell (Dong and Wortis, 1994). Non-functioning of these signaling pathways in vivo could thus explain the more severe and early B cell developmental arrest seen in the absence of T cells in XID/*nu*, *Btk/Tec* deficient and XID/TCR $\beta^{-/-}$ mice.

The data from the XID/TCR-$\beta^{-/-}$ mice are in contrast to previous reports where in *Btk*/CD40 double-deficient mice, normal bone marrow B cell development was observed (Oka et al., 1996).

The developmental arrest in the XID/CD40 deficient mice was at the same stage as in the XID mice. Therefore, CD40 signaling mediated either by T cells or by other stromal cells known to express CD40 ligand (Abe et al., 2002), might be argued to assist progression to the $IgM^+$ B cell stage, at which stage the B cell development is completely dependent on BCR mediated signaling via the surface IgM receptor (Benschop and Cambier, 1999). Again, CD40-driven signals may be critical for driving TEC-mediated

substitution of BTK function at this stage. In CD40 null mice, CD40/TEC would then no longer be able to compensate for the absence of BTK, leading to a developmental arrest at the immature B cell stage. Thus, in the XID mice, CD40 mediated signals can further improve B cell responsiveness, in the absence of *Btk*, leading to almost complete rescue of serum IgG levels and only partial restoration of IgM levels. Therefore, an absence of CD40 would manifest as a splenic maturation defect, but still result in a severe hypogammaglobulinemia, due to the absence of its effect on splenic immature B cells.

### *The role of lysosomal trafficking regulator LYST in myeloid cell functions*

The endo-lysosomal compartments are key players in mediating both the effector and antigen presentation functions of myeloid cells. Engulfed pathogens are trafficked via vesicular maturation, through a series of increasingly acidic compartments of the endo-lysosomal system, where they simultaneously undergo degradation and processing into peptide fragments for presentation on MHCII molecules. Coordinated movement of cargo through this vesicular system is achieved by the association of small protein GTPases of the Rab family. LYST, a lysosomal transport protein whose precise molecular role is still uncertain, is critical for regulating lysosomal maturation (Stinchcombe et al., 2000). A mutation in the gene encoding the LYST protein has been implicated in a human immunodeficiency disorder, the Chediak-Higashi Syndrome (CHS) (Nagle et al., 1996). Both CHS and the mouse model for CHS, the beige (bg/bg) mouse strain, are characterized by accumulation of giant dysfunctional lysosomes, delayed antigen presentation and decreased cytolytic granule release, resulting in recurrent bacterial infections (Ward et al., 2000). *Lyst* mutant neutrophil functions have been shown to be compromised due to defective lysosomal protease activity (Takeuchi et

al., 1986) contributing to the failure to clear bacterial infections (Gallin et al., 1974). In view of these defects, it was of interest to examine the effects of the LYST mutation on macrophage functions.

Peritoneal macrophage recruitment in response to TG instillation **(Fig. 11A)** as well as carrageenan induced footpad edema **(Fig. 11B)** was similar between wild-type C57BL/6 and bg/bg mice. This was contrary to a previous report (Gallin et al., 1974) which showed compromised granulocyte recruitment in response to peritoneal instillation of chemotactic factor. This difference could be due to the trafficking pathways utilized by PAMPs and chemokine receptors, and therefore their differential dependence on LYST. Chemokine receptors undergo basal levels of internalization and recycling, which is greatly enhanced on receptor ligation (Neel et al., 2005). The balance between receptor degradation in the endo-lysosomal vesicles versus its recycling to the surface determines the eventual fate of receptor ligand stimulation. bg/bg mice show a prolonged transit time in transport to the late endosomes, mis-sorting of the late endosomes, with increased transit to lysosomal degradation rather than membrane recycling (Faigle et al., 1998). Together, they could lead to rapid downregulation of surface receptor levels, resulting in lower recruitment in response to chemotactic factor exhibited by bg/bg neutrophils. PAMPs have a long surface residence and on signal initiation get translocated to lipid rafts, from where they dissociate on raft disruption. Hence, endo-lysosomal trafficking defects of bg/bg mice do not affect their signaling outcomes.

Macrophage and neutrophil phagocytosis **(Fig. 12)** was unaffected in the bg/bg consistent with previous reports (Gallin et al., 1974). Encounter with pathogen results in

a series of events starting with pathogen recognition by cell surface receptors such as pattern recognition receptors (PRRs), Fc receptors, complement receptors, followed by the formation of actin-containing membrane extensions which engulfe the particle to form the phagosome (Henneke and Golenbock, 2004). Following microbial internalization, phagosomes have to undergo sequential steps of vesicle maturation, to culminate in phago-lysosome formation, so as to facilitate microbial degradation (Ismail et al., 2002). Thus, pathogen elimination depends on these two distinct, yet closely inter-related steps of host defense. In view of these data and previous reports, *Lyst* mutation appears to result in a defect in the later processes, with no evident compromise in pathogen uptake.

The process of phagosome maturation is mediated by the association of series proteins of the Rab family, the SNAREs, LYST, which regulate the rate of progression of maturation to the lysosomal compartments. Differential association of Rab5a to phagosomes has been reported to regulate trafficking to lysosomal compartments for degradation in case of extracellular bacteria, or prevent phago-lysosomal fusion in case of facultative intracellular pathogen, with the phagocytic ability of the cell remaining unaltered (Perskvist et al., 2002). Further, facultative intracellular pathogens employ various methods to escape trafficking to, or degradation in the lysosomes. *Listeria, Rickettsia, Shigella, Orienta* escape into the cytosol from the late endocytic compartments (Ismail et al., 2002), *Leishmania* parasite on the other hand delays the phagosome maturation in order to transform from the lysosomal degradation sensitive promastigotes to the resistant amastigote form (Scianimanico et al., 1999). In view of the known endo-lysosomal trafficking defect in the *Lyst* mutant bg/bg mice, it was of interest

to analyze the effect of such a defect on the clinical course of a leishmanial infection these mice. The model system chosen was that of *Leishmania major* (Lm) induced cutaneous leishmanial infection.

Lm parasites have a biphasic life-cycle, residing in the insect host as flagellated promastigotes and in the vertebrate host macrophages as aflagellar amastigotes. Subsequent to entering as the promastigote form into the vertebrate host macrophages, the parasites travel through the maturing endo-lysosomal vesicular system where they reside and multiply, before emerging from the host cell. The promastigote stage of Lm is susceptible to lysosomal hydrolases, but the amastigote stage is resistant to these enzymes (Scianimanico et al., 1999). The efficacy of parasite clearance upon initial exposure to promastigotes via the insect vector would therefore depend on the efficiency of transport to the lysosomal compartments before the promastigotes convert into the resistant amastigote stage.

Also, the generation of important toxic effector molecules, nitrite (NO) and ROIs, are critical for parasite clearance from infected cells. Induction of a strong Th (T-helper)-1 response characterized by the generation of IFN-γ, which in turn is a major inducer of macrophage nitrite production, is also a component of effective Lm clearance *in vivo*. Not surprisingly, a complex mosaic of genetic background effects can determine the degree of susceptibility to Lm infection, with the genetically susceptible BALB/c and resistant C57BL/6 mice comprising two ends of the spectrum (Sacks and Noben-Trauth, 2002).

On Lm infection *in vivo*, bg/bg mice, despite being on a genetically resistant C57BL/6 background, developed progressive lesions with extensive inflammation, accumulation of large numbers of Lm laden infected macrophages and associated loss of tissue architecture, unlike C57BL/6 mice **(Fig. 14)**. However, the extent of lesion progression and tissue damage was considerably less severe than observed in the BALB/c mice **(Fig. 13&14)**. In an *in vitro* clearance assay, bg/bg macrophages **(Fig. 15&16)** showed significantly compromised Lm clearance, comparable to that observed in the inducible nitric oxide synthase- deficient ($iNOS^{-/-}$) mouse macrophages. IFN-γ mediated induction of iNOS in macrophages is responsible for the bulk of the nitrite synthesis in Lm infected macrophages, and is essential for successful Lm clearance. However, unlike the $iNOS^{-/-}$, bg/bg macrophages were not deficient in IFN-γ induced nitrite generation, producing NO levels similar to that induced in wild-type BL/6 macrophages **(Fig. 17A)**. IFN-γ pre-treatment resulted in a significant enhancement in the Lm clearance exhibited by the bg/bg and BL/6 macrophages, but not in the $iNOS^{-/-}$ macrophages. Notably, bg/bg macrophages showed enhanced IFN-γ mediated Lm clearance only at later time-points, unlike WT mice **(Fig. 17B)**

In addition to the ability to induce macrophage effector molecule synthesis; IFN-γ has also been reported to effect phagosome maturation. Virulent strains of intracellular pathogens like Lm and *Coxiella burnetti* have been reported to escape clearance by preventing maturation of late-endosomes into lysosomes (Ghigo et al., 2002), where they can be more effectively eliminated. IFN-γ treatment of cells before *Coxiella burnetti* infection has been reported to counter this survival strategy, by counteracting the pathogen induced phagosome maturation block (Ghigo et al., 2002). The LYST mutation

is known to prevent the conversion of early endosomes into late multi-vesicular endosomes. This maturation block in Lm infected macrophages can therefore plausibly result, in enormously compromised Lm clearance by bg/bg macrophages, despite normal levels of NO induction. Further, it is possible to speculate that due to the effect of the *Lyst* mutation, IFN-γ mediated abrogation of the Lm infection induced phagosome maturation block is delayed so that, improved Lm clearance in IFN-γ primed bg/bg macrophages is evident only at the later time point of 48 h, compared to C57BL/6 macrophages which begin showing improved clearance even by 24 h of infection **(Fig. 17B)**.

Previous work has demonstrated that the Th1/Th2 balance of an immune response can be modulated by alterations in the antigen processing and presentation on MHCII. Increasing the persistence of a cognate antigen resulted in a Th1 biased response, in case of a protein antigen (Singh et al., 1998). On the other hand, despite shorter residence in the host cell, a pathogenic strain of salmonella induced a Th1 response, rather than a more persistent infection with a non-pathogenic strain (Pashine et al., 1999). These data emphasize the circumstance specific modulatory roles exercised by similar variables of the antigen processing and presentation pathways. Since the *Lyst* mutation has been reported to result in deficient peptide loading and MHCII endosomal sorting (Faigle et al., 1998), investigating the resultant Lm-specific T cell response in the bg/bg mice was of interest.

Frequencies of Lm-specific CD4 T cells from lesion draining lymph nodes were similar in bg/bg, BL/6 and BALB/c mice **(Fig. 18A&B)**. On examining for Lm induced recall

cytokine responses, CD4 T cells from Lm-infected bg/bg mice showed a dominant Th1 response and made similar levels of IFN-γ as C57BL/6 CD4 T cells, whereas the BALB/c cultures made low levels of IFN-γ **(Fig. 18C)**. Expectedly, BALB/c CD4 T cells secreted large amounts of IL-4, unlike C57BL/6 CD4 T cells. However, despite the exquisite sensitivity to infection, the bg/bg CD4 T cells did not display any apparent shift to a Th2-like cytokine response, as evidenced by generation of similarly low levels of IL-4 as seen in the C57BL/6 cultures.

Therefore, the lack of an IFN-γ dominant Th1 response was not responsible for the increased Lm infection susceptibility exhibited by the bg/bg mice. Rather, compromised phagosome maturation resulting from the *Lyst* mutation, further accentuated by infecting Lm parasites, which could only be partially rescued by IFN-γ, eventually leading to compromised parasite clearance and increased disease severity. Interestingly, simply a delay in the macrophage response to IFN-γ thus appears to be sufficient to cause a transition from health to chronic disease, underlining the subtle and quantitative nature of the determinants of clinical disease.

## *The role of endogenous retroviral loci in controlling susceptibility to Lm infection*

The classical C57BL/6-BALB/c mouse model system used to study cutaneous leishmanial disease, while having been extremely useful in understanding the principles of control of infectious agents; it has not proven as useful for understanding the genetic basis of human leishmaniasis. One of the reasons is that BALB/c mice appear to have a

global, rather than a disease-specific, tendency to generate interleukin-4 (IL-4)-dominated Th2-like T cell responses (Hsieh et al., 1995). Also, while C57BL/6 mice show rapid and almost complete clearance of Lm infection, BALB/c mice succumb to a disseminating form of infection that is rapidly fatal (Howard et al., 1980), unlike human leishmaniasis which is generally exhibited as a chronic relapsing infection. Thus, while this model contributes to elucidating the genetic control of the global regulation of the Th1/Th2 balance, traits that specifically and quantitatively regulate this balance in response to leishmanial parasites are the genetic targets most likely to be relevant in human disease, and they still remain poorly understood (Lipoldova and Demant, 2006).

Endogenous replication deficient retroviral integrants have been identified in many mammalian genomes. Some of these proviral insertions are transcriptionally active and code for superantigen proteins (SAgs) that, in association with MHCII molecules (Korman et al., 1992) stimulate T cells bearing particular T cell receptor V-beta (TCRVβ) segments leading to the activation and/or deletion of these T cell subsets (Janeway, 1991). Similar insertions in humans, human endogenous retroviruses (HERVs), occupy up to 8% of the genome and have been reported to show associations with a number of autoimmune conditions, similarly endogenous murine tumor viruses (*mtv*s) are reported to mediate susceptibility to both infections (Bhadra et al., 2006; Gorgette et al., 2002) and tumors (Schirrmacher et al., 1998). Although the mechanisms involved in such a modulation of responses are still unclear, they seem to be a result of more than the T cell deleting function of the SAgs.

On this background, the course of Lm induced cutaneous leishmanial infection was studied in two closely related mouse strains in the genetically Lm resistant CBA background, namely, CBA/J and CBA/CaJ, which differ by the presence of integrations of the retroviruses *mtv-6* and *mtv-7* carried by the CBA/J in addition to *mtv-8* and *mtv-9* which are carried by both CBA/J and CBA/CaJ (Tomonari et al., 1993).

On footpad infection with Lm, while both strains were resistant in comparison to the BALB/c strain **(Fig.19B)**, only transient lesion formation was observed in CBA/CaJ mice, whereas low-grade chronic lesions were in evidence in the CBA/J mice **(Fig. 19A)**. Histopathological examination revealed persistent subcutaneous inflammatory infiltrates in the CBA/J, while CBA/CaJ mice showed relatively normal tissue morphology **(Fig. 20)**.

The magnitude of effector CD4 and CD8 T cell expansion induced in the draining lymph nodes in response to Lm infection was similar between CBA/J and CBA/CaJ mice **(Fig. 23)**, with the CD4 T cell subset being the dominant contributor to the expanded effector population in both the strains. Further, Lm-specific CD4 T cell frequencies, as analyzed by CD69 induction upon re-stimulation *in vitro*, were comparable in both the strains **(Fig. 24&25)**. Thus, the susceptibility of CBA/J mice to chronic Lm infection is not likely to be due to a smaller magnitude of the CD4 T cell response.

A major determinant of the eventual outcome of Lm infection clearance is the balance of Th1 and Th2 cytokine responses generated. IL-4-deficient BALB/c mice control leishmanial infection effectively (Kopf et al., 1996), while IFN-γ-deficient C57BL/6

mice do not (Wang et al., 1994). In both CBA/J and CBA/CaJ mice infected with Lm, no IL-4, IL-5 or IL-13 was detected in culture supernatants, similar to the profile reported for C57BL/6 (Wang et al., 1994). IL-10 has been recently implicated in homeostatic downregulation of the inflammatory response (Anderson et al., 2007). Generation of greater amounts of IL-10 by CBA/J as compared to CBA/CaJ mice might explain the persistent lesions observed in the former. However, no differences were detectable in the IL-10 levels generated by Lm-specific effector CD4 T cells from CBA/J and CBA/CaJ mice **(Fig. 26C)**. Recent studies have identified a third subset of IL-17 producing T-helper cells, Th17 cells, and characterized them to be pro-inflammatory in nature (Bettelli et al., 2007). Though the Lm specific IL-17 response in the CBA/J and CBA/CaJ has not been analyzed in this study, Th17 responses have been reported to result in massive inflammatory infiltrates leading to tissue destruction and a progressive worsening of the disease phenotype (Kolls and Linden, 2004), contrary to the phenotype of localized chronic indolent lesions exhibited by the CBA/J mice. Hence, Th17 responses are unlikely to be responsible for the chronic Lm infection in CBA/J.

On analyzing for IFN-γ responses; despite the presence of similar frequencies of Lm-specific CD4 T cells, only about half as many responding cells made IFN-γ in the CBA/J strain as in the CBA/CaJ strain **(Fig. 27&28)**. Further, the CBA/J T cell culture supernatants also showed significantly lower levels of IFN-γ as compared to the CBA/CaJ T cell cultures **(Fig. 26&28)**. Since, the difference in frequency of IFN-γ secreting CD4 T cells between the two strains was far less than the quantitative difference in the supernatant IFN-γ detected, a possibility of a higher per cell IFN-γ output from the CBA/CaJ CD4 T cells cannot be excluded. Therefore, a quantitative

reduction in the IFN-γ component of the T cell response, possibly associated with a greater per cell IFN-γ output, with no observable increase in Th2-like cytokines produced, appeared to have resulted in chronic indolent lesions in response to Lm infection in the CBA/J mice, similar in appearance to human clinical situation.

The major reported genetic difference between these two closely related strains of mice is the presence of proviral integrants for *mtv-6* and *mtv-7* in the CBA/J. On genetic intercross analysis, the (CBA/J x CBA/CaJ) F1 mice, which inherit both *mtv-6* & *mtv-7*, showed reduced resistance to Lm infection similar to parental CBA/J mice **(Fig. 21A)**. In the F2 generation, reduced Lm resistance was seen in mice inheriting the *mtv-7* integration, either alone or along with *mtv-6* **(Fig. 22)**. Also, while all F2-generation mice showed comparable frequencies of Lm-specific CD4 T cells **(Fig. 29A)**, these cells from F2 mice inheriting *mtv-7* made lower levels of IFN-γ, as seen in parental CBA/J mice **(Fig. 29B)**. These data implicate the *mtv-7* locus in the reduction of IFN-γ responses associated with increased susceptibility to chronic Lm infection.

Retroviral insertions in the mouse genome have been correlated with specific alterations of disease susceptibility; thus, an *mtv*-null mouse strain has been shown to be specifically more sensitive to infection by *Vibrio cholerae* but not other pathogens (Bhadra et al., 2006). More particularly, the *mtv-7* locus has also been shown to be associated with disease susceptibility in a number of situations. Tumor susceptibility is induced in a genetically tumor resistant mouse strain by crossing in the *mtv-7* locus (Schirrmacher et al., 1998). Inheritance of *mtv-7* also confers susceptibility to murine cerebral malaria by deleting the v-SAg-7-reactive TVRVβ8.1-using T cell population (Gorgette et al., 2002)

and to chronic graft-versus-host disease (Gorham et al., 1996). In Lm infection, a subset of T cells expressing TCRVβ4 TCRVα8 receptors which recognize the LACK protein of Lm and prominently make IL-4 in Lm infected BALB/c resulting in chronic progressive lesions, also recognize the vSAg of MMTV (SIM). BALB/c mice rendered TCRVβ4-deficient by MMTV (SIM) but not littermates rendered TCRVβ6-deficient by MMTV (SW) develop a Th1 response to Lm mimicking the response of C57BL/6 strain (Launois et al., 1997).

Thus, the simplest model which could explain the association of *mtv-7* and Lm susceptibility in CBA/J mice was that v-SAg-7-reactive TCRVβ-using T cells formed the dominant immunoreactive subset responsible for an effective anti-Lm immune response. Thereby, developmental deletion of this subset in the CBA/J mice would result in a poor Lm-specific T cell response generation leading to a chronic Lm infection. However, this was not the case, since the magnitude of the anti-Lm CD4 T cell response was similar between CBA/J and CBA/CaJ mice **(Fig. 24&25)**, as it was between the various F2 groups irrespective of the *mtv-7* inheritance patterns **(Fig. 29A)**. Further, the anti-Lm CD4 T cell response was also found to be highly heterogeneous in the TCRVβ subset representation, since all of the TCRVβ subsets tested, irrespective of v-SAg reactivity, showed comparable frequencies of Lm-specific CD4 T cells **(Fig. 30A)**.

It was also possible that the v-SAg-7-reactive TCRVβ subsets might display comparatively higher IFN-γ commitment, resulting in a proportionately higher IFN-γ commitment in CD4 T cells from CBA/CaJ or the *mtv-7* non-integrant F2 mice. However, all TCRVβ-using CD4 T cell subpopulations in a given strain, irrespective of

*mtv-7* reactivity, showed equivalent IFN-γ responses. On the other hand, the same TCRVβ-using CD4 T cells from Lm-infected CBA/J mice made less IFN-γ than those from CBA/CaJ mice or *mtv-7* non-integrant F2 mice **(Fig. 29B&30B)**. These data suggested that, independent of any TCRVβ subset specificity, all T cells displayed poor IFN-γ commitment if primed in the presence of *mtv-7* integrant APCs. Further, a similar modulation was apparent during the re-stimulation of already primed Lm-specific T cells, since the IFN-γ response of primed T cells was similarly recalled whether CBA/J or CBA/CaJ APCs are used for re-stimulation **(Fig. 33)**.

Additionally, there was no associated reduction in the ability of *mtv-7*-bearing macrophages to kill Lm in vitro **(Fig. 31)**. Macrophages treated with *Leishmania* surface glycoproteins have been reported to lose their ability to induce iNOS or generate an NO response on stimulation with IFN-γ and/or LPS (Proudfoot et al., 1996; Proudfoot et al., 1995). A similar level of inhibition on NO generation was observed in CBA/J or CBA/CaJ BMDCs, subsequent to Lm infection, *in vitro* **(Fig. 32 A)**. Notably, a dramatic reduction in nitrite generation was observed when IFN-γ was added at 24 h of infection **(Fig. 32A)**, consistent with previous reports of such an inhibition in LPG (lipophosphoglycan) pre-treated macrophages (Proudfoot et al., 1995). Similarly, repression of IL-12 induction has also been reported in Lm infected (Carrera et al., 1996) macrophages, consistent with the observations from CBA/J and CBA/CaJ cultures. However, no observable differences in the levels of IL-12 **(Fig. 32B)** or TNF-α **(Fig. 32C)** were seen between CBA/J and CBA/CaJ DCs, confirming the absence of a difference in the macrophage effector functions between the two strains.

These data suggested that the presence of *mtv-7* leads to a modulation of APC function during T cell activation, resulting in poor signals for IFN-γ commitment during priming, and/or that the presence of *mtv-7* during T cell development conditions them for poor IFN-γ responses later. An analogy would be the BALB/c mouse strain, where the IL-4-dominance in the T cell response is not restricted to Lm infection alone, but is seen in response to other immunizations as well (Gorham et al., 1996; Guery et al., 1996; Hsieh et al., 1995). However, CBA/J and CBA/CaJ mice responded similarly, both in terms of the magnitude and the IFN-γ component of the T cell response, when immunized with a protein antigen in adjuvant **(Fig. 34A&B)**. Thus, *mtv-7* appears to provide a specific alteration in the commitment of Lm-specific T cells to IFN-γ in the CBA mouse strain during Lm infection, probably in *mtv-7*-expressing myeloid APCs, and this alteration is subtle enough to make only a quantitative difference, contributing to indolent chronicity of Lm infection.

The eventual outcome of leishmanial infection depends on the cytokine pattern of the CD4 T response mounted, so that an IFN-γ-dominated response results in effective control of infection while its absence is likely to permit progressive disease. On extrapolating these data to the chronic disease situation, a likely hypothesis would be that a quantitative reduction in the IFN-γ component of the T cells response may contribute to a state of chronic low-grade infection with chronically persisting lesions.

Such a subtle effect of retroviral insertions generate the possibility that the known retroviral insertions in the human genome (Bannert and Kurth, 2004) may also modify immune responses to particular pathogens in this subtle fashion, and may contribute to

small but significant increments in susceptibility that would contribute to the difference between clearance versus clinical disease.

Further, most of the genome integrant retroviruses remain replication deficient and a further more transcriptionally inactive, due to obvious inactivating mutations. The possibility of these hitherto unknown ghost viruses gaining replication competency and becoming infectious in turn by utilizing surrogate sequences from existing active retroviruses like HIV acting as donors, is very real (Lee and Bieniasz, 2007).

Thus, the studies reported here highlight the complex and quantitative role played by the various components of the cellular signaling environment in myeloid cells, controlling both effector and antigen presenting functions, resulting in subtle but decisive modulations in the eventual innate and acquired responses so as to be deterministic in fine-tuning the balance between health and disease.

# *Summary*

*The studies presented here are an effort to use genetic models to study the interactions between the various cellular and molecular signaling interactions components involved in regulating an immune inflammatory response, and their eventual role in determining the balance between disease and health.*

## *A. Role of signaling intermediate Btk in regulating cellular interactions in inflammation*

### *1. Bruton's tyrosine kinase (BTK) in myeloid cells:*

#### *1.1 Role in eosinophil lineage*

- *Eosinophils constitute a high proportion of granulocytes in the XID/IL-5 Tg mice*
- *Compromised eosinophil recruitment and effector functions in XID/IL-5 Tg mice*

*Similar to its reported role in other myeloid cells, BTK seems to have a role in regulating eosinophil effector functions.*

#### *1.2 Role of the BTK-targeted transcription factor c-Rel in macrophage effector functions*

- *Peripheral blood circulating leukocytes frequencies in the c-Rel$^{-/-}$ mice, remain unaltered*
- *Myeloid cell recruitment to sites of inflammation is unaltered in the c-Rel$^{-/-}$ mice*
- *No compromise in myeloid cell effector functions in the c-Rel$^{-/-}$ mice*

*Thus, indicating that c-Rel does not have a non-redundant role in mediating macrophage effector functions.*

### *2. A role for BTK in T-B cell developmental cross-talk*

- *Severe reduction serum immunoglobulin levels in XID/nu and XID/TCRβ-/- mice, with profound reduction in mature circulating B cells*

- *Early B cell developmental arrest at the pro/pre B cell stage in XID/TCRβ-/-*
- *B cell development is rescued with even a marginal increase in circulating T cells in the XID/nu, but peripheral maturation arrest seen in XID mice remains unaltered*

*XID mice, in the absence of circulating T cells, show severe agammaglobulinemia, profoundly reduced mature B cell frequency and an almost complete B cell developmental arrest, closely resembling XLA.*

## *B. Role of lysosomal transporter LYST, in regulating myeloid cell functions*

- *Myeloid cells of bg/bg mice show no defect in recruitment to sites of inflammation*
- *bg/bg mice show increased susceptibility to cutaneous leishmaniasis*
- *bg/bg macrophages show compromised leishmanial clearance*
- *bg/bg mice show a Th1 biased T cells response on leishmanial infection*
- *IFN-γ primed bg/bg macrophages show enhanced leishmanial clearance*

*Thus, the enhanced susceptibility of bg/bg mice to cutaneous leishmaniasis does not result from an inability to mount an IFN-γ dominant-Th1 response, but is due to compromised parasite elimination by bg/bg macrophages.*

## *C. The role of endogenous retroviral loci in controlling susceptibility to Lm infection*

- *CBA/J mice show chronic low-grade cutaneous leishmanial infection*
- *Chronicity of Lm infection is associated with inheritance of mtv-7*

- *Leishmania-specific T cell frequencies are equivalent in CBA/J and CBA/CaJ mice*
- *Leishmania-specific CBA/J T cells make lower levels of IFN-γ than CBA/CaJ T cells*
- *Low IFN-γ levels made by CBA/J T cells are not due to absence of v-SAg-7-reactive TCRVβ subsets*
- *Leishmania-directed CBA/J and CBA/CaJ macrophage functions are similar*
- *APC identity in recall does not alter the level of IFN-γ production*
- *Altered T cell priming by CBA/J BMDCs is not immunogen independent*

*Thus, inheritance of the mtv-7 locus confers a disease specific increase in susceptibility to leishmaniasis, independent of its T cell deletion function and mediated by a quantitative rather than a qualitative modulation of the immune response.*

# *Bibliography*

Abe, M., Shintani, Y., Eto, Y., Harada, K., Kosaka, M., and Matsumoto, T. (2002). Potent induction of activin A secretion from monocytes and bone marrow stromal fibroblasts by cognate interaction with activated T cells. J Leukoc Biol *72*, 347-352.

Abraham, R., Singh, N., Mukhopadhyay, A., Basu, S. K., Bal, V., and Rath, S. (1995). Modulation of immunogenicity and antigenicity of proteins by maleylation to target scavenger receptors on macrophages. J Immunol *154*, 1-8.

Acha-Orbea, H., and Palmer, E. (1991). Mls--a retrovirus exploits the immune system. Immunol Today *12*, 356-361.

Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L., and Murphy, K. M. (2002). T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. Nat Immunol *3*, 549-557.

Akimzhanov, A. M., Yang, X. O., and Dong, C. (2007). Chromatin remodeling of interleukin-17 (IL-17)-IL-17F cytokine gene locus during inflammatory helper T cell differentiation. J Biol Chem *282*, 5969-5972.

Akira, S. (2006). TLR signaling. Curr Top Microbiol Immunol *311*, 1-16.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nat Rev Immunol *4*, 499-511.

Alexander, J., and Russell, D. G. (1992). The interaction of Leishmania species with macrophages. Adv Parasitol *31*, 175-254.

Alhonen, L., Heikkinen, S., Sinervirta, R., Halmekyto, M., Alakuijala, P., and Janne, J. (1996). Transgenic mice expressing the human ornithine decarboxylase gene under the control of mouse metallothionein I promoter. Biochem J *314 ( Pt 2)*, 405-408.

Allen, R. D., Slayback, D. L., Harper, J. M., Aguirre, T. L., and Dobkins, J. A. (2000). A locus closely linked to Mtv7 on mouse chromosome 1 influences development of acute versus chronic graft-versus-host disease in a murine model. Clin Immunol *95*, 9-19.

Alugupalli, K. R., Akira, S., Lien, E., and Leong, J. M. (2007). MyD88- and Bruton's tyrosine kinase-mediated signals are essential for T cell-independent pathogen-specific IgM responses. J Immunol *178*, 3740-3749.

Amoras, A. L., Kanegane, H., Miyawaki, T., and Vilela, M. M. (2003). Defective Fc-, CR1- and CR3-mediated monocyte phagocytosis and chemotaxis in common variable immunodeficiency and X-linked agammaglobulinemia patients. J Investig Allergol Clin Immunol *13*, 181-188.

Anderson, C. F., Oukka, M., Kuchroo, V. J., and Sacks, D. (2007). CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis. J Exp Med *204*, 285-297.

Antoine, J. C., Lang, T., Prina, E., Courret, N., and Hellio, R. (1999). H-2M molecules, like MHC class II molecules, are targeted to parasitophorous vacuoles of Leishmania-

infected macrophages and internalized by amastigotes of L. amazonensis and L. mexicana. J Cell Sci *112 ( Pt 15)*, 2559-2570.

Awasthi, A., Mathur, R., Khan, A., Joshi, B. N., Jain, N., Sawant, S., Boppana, R., Mitra, D., and Saha, B. (2003). CD40 signaling is impaired in L. major-infected macrophages and is rescued by a p38MAPK activator establishing a host-protective memory T cell response. J Exp Med *197*, 1037-1043.

Baetz, K., Isaaz, S., and Griffiths, G. M. (1995). Loss of cytotoxic T lymphocyte function in Chediak-Higashi syndrome arises from a secretory defect that prevents lytic granule exocytosis. J Immunol *154*, 6122-6131.

Bania, J., Gatti, E., Lelouard, H., David, A., Cappello, F., Weber, E., Camosseto, V., and Pierre, P. (2003). Human cathepsin S, but not cathepsin L, degrades efficiently MHC class II-associated invariant chain in nonprofessional APCs. Proc Natl Acad Sci U S A *100*, 6664-6669.

Bannert, N., and Kurth, R. (2004). Retroelements and the human genome: new perspectives on an old relation. Proc Natl Acad Sci U S A *101 Suppl 2*, 14572-14579.

Barbosa, M. D., Nguyen, Q. A., Tchernev, V. T., Ashley, J. A., Detter, J. C., Blaydes, S. M., Brandt, S. J., Chotai, D., Hodgman, C., Solari, R. C., *et al.* (1996). Identification of the homologous beige and Chediak-Higashi syndrome genes. Nature *382*, 262-265.

Baribaud, F., Maillard, I., Vacheron, S., Brocker, T., Diggelmann, H., and Acha-Orbea, H. (1999). Role of dendritic cells in the immune response induced by mouse mammary tumor virus superantigen. J Virol *73*, 8403-8410.

Barral, A., Barral-Netto, M., Yong, E. C., Brownell, C. E., Twardzik, D. R., and Reed, S. G. (1993). Transforming growth factor beta as a virulence mechanism for Leishmania braziliensis. Proc Natl Acad Sci U S A *90*, 3442-3446.

Batten, M., Li, J., Yi, S., Kljavin, N. M., Danilenko, D. M., Lucas, S., Lee, J., de Sauvage, F. J., and Ghilardi, N. (2006). Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. Nat Immunol *7*, 929-936.

Bedell, M. A., Jenkins, N. A., and Copeland, N. G. (1997a). Mouse models of human disease. Part I: techniques and resources for genetic analysis in mice. Genes Dev *11*, 1-10.

Bedell, M. A., Largaespada, D. A., Jenkins, N. A., and Copeland, N. G. (1997b). Mouse models of human disease. Part II: recent progress and future directions. Genes Dev *11*, 11-43.

Belkaid, Y., Mendez, S., Lira, R., Kadambi, N., Milon, G., and Sacks, D. (2000). A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. J Immunol *165*, 969-977.

Belkaid, Y., Von Stebut, E., Mendez, S., Lira, R., Caler, E., Bertholet, S., Udey, M. C., and Sacks, D. (2002). CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with Leishmania major. J Immunol *168*, 3992-4000.

Bengtsson, A., Blomberg, J., Nived, O., Pipkorn, R., Toth, L., and Sturfelt, G. (1996). Selective antibody reactivity with peptides from human endogenous retroviruses and nonviral poly(amino acids) in patients with systemic lupus erythematosus. Arthritis Rheum *39*, 1654-1663.

Benschop, R. J., and Cambier, J. C. (1999). B cell development: signal transduction by antigen receptors and their surrogates. Curr Opin Immunol *11*, 143-151.

Berberich, C., Ramirez-Pineda, J. R., Hambrecht, C., Alber, G., Skeiky, Y. A., and Moll, H. (2003). Dendritic cell (DC)-based protection against an intracellular pathogen is dependent upon DC-derived IL-12 and can be induced by molecularly defined antigens. J Immunol *170*, 3171-3179.

Bettelli, E., Oukka, M., and Kuchroo, V. K. (2007). T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol *8*, 345-350.

Bhadra, S., Lozano, M. M., Payne, S. M., and Dudley, J. P. (2006). Endogenous MMTV Proviruses Induce Susceptibility to Both Viral and Bacterial Pathogens. PLoS Pathog *2*, e128.

Bharadwaj, D., Mold, C., Markham, E., and Du Clos, T. W. (2001). Serum amyloid P component binds to Fc gamma receptors and opsonizes particles for phagocytosis. J Immunol *166*, 6735-6741.

Blander, J. M., and Medzhitov, R. (2004). Regulation of phagosome maturation by signals from toll-like receptors. Science *304*, 1014-1018.

Blander, J. M., and Medzhitov, R. (2006). On regulation of phagosome maturation and antigen presentation. Nat Immunol *7*, 1029-1035.

Bliss, S. K., Butcher, B. A., and Denkers, E. Y. (2000). Rapid recruitment of neutrophils containing prestored IL-12 during microbial infection. J Immunol *165*, 4515-4521.

Boffa, D. J., Feng, B., Sharma, V., Dematteo, R., Miller, G., Suthanthiran, M., Nunez, R., and Liou, H. C. (2003). Selective loss of c-Rel compromises dendritic cell activation of T lymphocytes. Cell Immunol *222*, 105-115.

Bogdan, C., Rollinghoff, M., and Diefenbach, A. (2000). Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol *12*, 64-76.

Boyce, J. A., Friend, D., Matsumoto, R., Austen, K. F., and Owen, W. F. (1995). Differentiation in vitro of hybrid eosinophil/basophil granulocytes: autocrine function of an eosinophil developmental intermediate. J Exp Med *182*, 49-57.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., and Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. Science *303*, 1532-1535.

Brittingham, A., Morrison, C. J., McMaster, W. R., McGwire, B. S., Chang, K. P., and Mosser, D. M. (1995). Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. J Immunol *155*, 3102-3111.

Bullock, E. D., and Johnson, E. M., Jr. (1996). Nerve growth factor induces the expression of certain cytokine genes and bcl-2 in mast cells. Potential role in survival promotion. J Biol Chem *271*, 27500-27508.

Bush, K. A., Farmer, K. M., Walker, J. S., and Kirkham, B. W. (2002). Reduction of joint inflammation and bone erosion in rat adjuvant arthritis by treatment with interleukin-17 receptor IgG1 Fc fusion protein. Arthritis Rheum *46*, 802-805.

Buxbaum, L. U., and Scott, P. (2005). Interleukin 10- and Fcgamma receptor-deficient mice resolve Leishmania mexicana lesions. Infect Immun *73*, 2101-2108.

Campana, D., Farrant, J., Inamdar, N., Webster, A. D., and Janossy, G. (1990). Phenotypic features and proliferative activity of B cell progenitors in X-linked agammaglobulinemia. J Immunol *145*, 1675-1680.

Campbell, K. A., Ovendale, P. J., Kennedy, M. K., Fanslow, W. C., Reed, S. G., and Maliszewski, C. R. (1996). CD40 ligand is required for protective cell-mediated immunity to Leishmania major. Immunity *4*, 283-289.

Cancro, M. P., Sah, A. P., Levy, S. L., Allman, D. M., Schmidt, M. R., and Woodland, R. T. (2001). xid mice reveal the interplay of homeostasis and Bruton's tyrosine kinase-mediated selection at multiple stages of B cell development. Int Immunol *13*, 1501-1514.

Carrera, L., Gazzinelli, R. T., Badolato, R., Hieny, S., Muller, W., Kuhn, R., and Sacks, D. L. (1996). Leishmania promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. J Exp Med *183*, 515-526.

Carrington, M., Dean, M., Martin, M. P., and O'Brien, S. J. (1999). Genetics of HIV-1 infection: chemokine receptor CCR5 polymorphism and its consequences. Hum Mol Genet *8*, 1939-1945.

Casanova, J. L., and Abel, L. (2004). The human model: a genetic dissection of immunity to infection in natural conditions. Nat Rev Immunol *4*, 55-66.

Castellano, F., Montcourrier, P., and Chavrier, P. (2000). Membrane recruitment of Rac1 triggers phagocytosis. J Cell Sci *113 ( Pt 17)*, 2955-2961.

Castigli, E., Young, F., Carossino, A. M., Alt, F. W., and Geha, R. S. (1996). CD40 expression and function in murine B cell ontogeny. Int Immunol *8*, 405-411.

Chapman, A. L., Hampton, M. B., Senthilmohan, R., Winterbourn, C. C., and Kettle, A. J. (2002). Chlorination of bacterial and neutrophil proteins during phagocytosis and killing of Staphylococcus aureus. J Biol Chem *277*, 9757-9762.

Chatelain, R., Varkila, K., and Coffman, R. L. (1992). IL-4 induces a Th2 response in Leishmania major-infected mice. J Immunol *148*, 1182-1187.

Chaudhry, A., Das, S. R., Hussain, A., Mayor, S., George, A., Bal, V., Jameel, S., and Rath, S. (2005). The Nef protein of HIV-1 induces loss of cell surface costimulatory molecules CD80 and CD86 in APCs. J Immunol *175*, 4566-4574.

Chen, L., Watanabe, T., Watanabe, H., and Sendo, F. (2001). Neutrophil depletion exacerbates experimental Chagas' disease in BALB/c, but protects C57BL/6 mice through modulating the Th1/Th2 dichotomy in different directions. Eur J Immunol *31*, 265-275.

Clausen, J. (2003). Endogenous retroviruses and MS: using ERVs as disease markers. Int MS J *10*, 22-28.

Cohen, G. B., Gandhi, R. T., Davis, D. M., Mandelboim, O., Chen, B. K., Strominger, J. L., and Baltimore, D. (1999). The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity *10*, 661-671.

Collins, P. D., Marleau, S., Griffiths-Johnson, D. A., Jose, P. J., and Williams, T. J. (1995). Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. J Exp Med *182*, 1169-1174.

Conley, M. E. (1985). B cells in patients with X-linked agammaglobulinemia. J Immunol *134*, 3070-3074.

Cornillon, S., Dubois, A., Bruckert, F., Lefkir, Y., Marchetti, A., Benghezal, M., De Lozanne, A., Letourneur, F., and Cosson, P. (2002). Two members of the beige/CHS (BEACH) family are involved at different stages in the organization of the endocytic pathway in Dictyostelium. J Cell Sci *115*, 737-744.

Courret, N., Frehel, C., Gouhier, N., Pouchelet, M., Prina, E., Roux, P., and Antoine, J. C. (2002). Biogenesis of Leishmania-harbouring parasitophorous vacuoles following phagocytosis of the metacyclic promastigote or amastigote stages of the parasites. J Cell Sci *115*, 2303-2316.

Cox, D., Chang, P., Zhang, Q., Reddy, P. G., Bokoch, G. M., and Greenberg, S. (1997). Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. J Exp Med *186*, 1487-1494.

Cui, Z., Willingham, M. C., Hicks, A. M., Alexander-Miller, M. A., Howard, T. D., Hawkins, G. A., Miller, M. S., Weir, H. M., Du, W., and DeLong, C. J. (2003). Spontaneous regression of advanced cancer: identification of a unique genetically determined, age-dependent trait in mice. Proc Natl Acad Sci U S A *100*, 6682-6687.

Daugherty, B. L., Siciliano, S. J., DeMartino, J. A., Malkowitz, L., Sirotina, A., and Springer, M. S. (1996). Cloning, expression, and characterization of the human eosinophil eotaxin receptor. J Exp Med *183*, 2349-2354.

de Gorter, D. J., Beuling, E. A., Kersseboom, R., Middendorp, S., van Gils, J. M., Hendriks, R. W., Pals, S. T., and Spaargaren, M. (2007). Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. Immunity *26*, 93-104.

Dermine, J. F., Duclos, S., Garin, J., St-Louis, F., Rea, S., Parton, R. G., and Desjardins, M. (2001). Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. J Biol Chem *276*, 18507-18512.

Dermine, J. F., Scianimanico, S., Prive, C., Descoteaux, A., and Desjardins, M. (2000). Leishmania promastigotes require lipophosphoglycan to actively modulate the fusion properties of phagosomes at an early step of phagocytosis. Cell Microbiol *2*, 115-126.

Desjardins, M. (1995). Biogenesis of phagolysosomes: the 'kiss and run' hypothesis. Trends Cell Biol *5*, 183-186.

Desjardins, M., and Descoteaux, A. (1997). Inhibition of phagolysosomal biogenesis by the Leishmania lipophosphoglycan. J Exp Med *185*, 2061-2068.

Desjardins, M., Huber, L. A., Parton, R. G., and Griffiths, G. (1994). Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. J Cell Biol *124*, 677-688.

Desjardins, M., Nzala, N. N., Corsini, R., and Rondeau, C. (1997). Maturation of phagosomes is accompanied by changes in their fusion properties and size-selective acquisition of solute materials from endosomes. J Cell Sci *110 ( Pt 18)*, 2303-2314.

Dillon, S., Agrawal, A., Van Dyke, T., Landreth, G., McCauley, L., Koh, A., Maliszewski, C., Akira, S., and Pulendran, B. (2004). A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. J Immunol *172*, 4733-4743.

Dong, Z., and Wortis, H. H. (1994). Function of bone marrow stromal cell lines derived from nude mice. J Immunol *153*, 1441-1448.

Doyle, S. E., O'Connell, R. M., Miranda, G. A., Vaidya, S. A., Chow, E. K., Liu, P. T., Suzuki, S., Suzuki, N., Modlin, R. L., Yeh, W. C., *et al.* (2004). Toll-like receptors induce a phagocytic gene program through p38. J Exp Med *199*, 81-90.

Du, J., Stankiewicz, M. J., Liu, Y., Xi, Q., Schmitz, J. E., Lekstrom-Himes, J. A., and Ackerman, S. J. (2002). Novel combinatorial interactions of GATA-1, PU.1, and C/EBPepsilon isoforms regulate transcription of the gene encoding eosinophil granule major basic protein. J Biol Chem *277*, 43481-43494.

Duclos, S., Diez, R., Garin, J., Papadopoulou, B., Descoteaux, A., Stenmark, H., and Desjardins, M. (2000). Rab5 regulates the kiss and run fusion between phagosomes and

endosomes and the acquisition of phagosome leishmanicidal properties in RAW 264.7 macrophages. J Cell Sci *113 Pt 19*, 3531-3541.

Dvorak, A. M., Furitsu, T., Letourneau, L., Ishizaka, T., and Ackerman, S. J. (1991). Mature eosinophils stimulated to develop in human cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. Part I. Piecemeal degranulation of specific granules and distribution of Charcot-Leyden crystal protein. Am J Pathol *138*, 69-82.

Ellmeier, W., Jung, S., Sunshine, M. J., Hatam, F., Xu, Y., Baltimore, D., Mano, H., and Littman, D. R. (2000). Severe B cell deficiency in mice lacking the tec kinase family members Tec and Btk. J Exp Med *192*, 1611-1624.

Faigle, W., Raposo, G., Tenza, D., Pinet, V., Vogt, A. B., Kropshofer, H., Fischer, A., de Saint-Basile, G., and Amigorena, S. (1998). Deficient peptide loading and MHC class II endosomal sorting in a human genetic immunodeficiency disease: the Chediak-Higashi syndrome. J Cell Biol *141*, 1121-1134.

Fischer, A. (2001). Primary immunodeficiency diseases: an experimental model for molecular medicine. Lancet *357*, 1863-1869.

Fluckiger, A. C., Li, Z., Kato, R. M., Wahl, M. I., Ochs, H. D., Longnecker, R., Kinet, J. P., Witte, O. N., Scharenberg, A. M., and Rawlings, D. J. (1998). Btk/Tec kinases regulate sustained increases in intracellular Ca2+ following B-cell receptor activation. Embo J *17*, 1973-1985.

Fortier, A., Min-Oo, G., Forbes, J., Lam-Yuk-Tseung, S., and Gros, P. (2005). Single gene effects in mouse models of host: pathogen interactions. J Leukoc Biol *77*, 868-877.

Foy, T. M., Aruffo, A., Bajorath, J., Buhlmann, J. E., and Noelle, R. J. (1996). Immune regulation by CD40 and its ligand GP39. Annu Rev Immunol *14*, 591-617.

Frankel, W. N., Rudy, C., Coffin, J. M., and Huber, B. T. (1991). Linkage of Mls genes to endogenous mammary tumour viruses of inbred mice. Nature *349*, 526-528.

Friedland, J. S., Shattock, R. J., and Griffin, G. E. (1993). Phagocytosis of Mycobacterium tuberculosis or particulate stimuli by human monocytic cells induces equivalent monocyte chemotactic protein-1 gene expression. Cytokine *5*, 150-156.

Gallin, J. I., Bujak, J. S., Patten, E., and Wolff, S. M. (1974). Granulocyte function in the Chediak-Higashi syndrome of mice. Blood *43*, 201-206.

Ganz, T., Metcalf, J. A., Gallin, J. I., Boxer, L. A., and Lehrer, R. I. (1988). Microbicidal/cytotoxic proteins of neutrophils are deficient in two disorders: Chediak-Higashi syndrome and "specific" granule deficiency. J Clin Invest *82*, 552-556.

Gauchat, J. F., Henchoz, S., Fattah, D., Mazzei, G., Aubry, J. P., Jomotte, T., Dash, L., Page, K., Solari, R., Aldebert, D., and et al. (1995). CD40 ligand is functionally expressed on human eosinophils. Eur J Immunol *25*, 863-865.

Ghigo, E., Capo, C., Tung, C. H., Raoult, D., Gorvel, J. P., and Mege, J. L. (2002). Coxiella burnetii survival in THP-1 monocytes involves the impairment of phagosome maturation: IFN-gamma mediates its restoration and bacterial killing. J Immunol *169*, 4488-4495.

Ghosh, S., May, M. J., and Kopp, E. B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol *16*, 225-260.

Gilbert, C., Levasseur, S., Desaulniers, P., Dusseault, A. A., Thibault, N., Bourgoin, S. G., and Naccache, P. H. (2003). Chemotactic factor-induced recruitment and activation of Tec family kinases in human neutrophils. II. Effects of LFM-A13, a specific Btk inhibitor. J Immunol *170*, 5235-5243.

Goff, S. P. (1996). Operating under a Gag order: a block against incoming virus by the Fv1 gene. Cell *86*, 691-693.

Golovkina, T. V., Shlomchik, M., Hannum, L., and Chervonsky, A. (1999). Organogenic role of B lymphocytes in mucosal immunity. Science *286*, 1965-1968.

Gorgette, O., Existe, A., Boubou, M. I., Bagot, S., Guenet, J. L., Mazier, D., Cazenave, P. A., and Pied, S. (2002). Deletion of T cells bearing the V beta8.1 T-cell receptor following mouse mammary tumor virus 7 integration confers resistance to murine cerebral malaria. Infect Immun *70*, 3701-3706.

Gorham, J. D., Guler, M. L., Steen, R. G., Mackey, A. J., Daly, M. J., Frederick, K., Dietrich, W. F., and Murphy, K. M. (1996). Genetic mapping of a murine locus controlling development of T helper 1/T helper 2 type responses. Proc Natl Acad Sci U S A *93*, 12467-12472.

Gray, P. M., Reiner, S. L., Smith, D. F., Kaye, P. M., and Scott, P. (2006). Antigen-experienced T cells limit the priming of naive T cells during infection with Leishmania major. J Immunol *177*, 925-933.

Guery, J. C., Galbiati, F., Smiroldo, S., and Adorini, L. (1996). Selective development of T helper (Th)2 cells induced by continuous administration of low dose soluble proteins to normal and beta(2)-microglobulin-deficient BALB/c mice. J Exp Med *183*, 485-497.

Haak-Frendscho, M., Marsters, S. A., Mordenti, J., Brady, S., Gillett, N. A., Chen, S. A., and Ashkenazi, A. (1994). Inhibition of TNF by a TNF receptor immunoadhesin. Comparison to an anti-TNF monoclonal antibody. J Immunol *152*, 1347-1353.

Hackam, D. J., Rotstein, O. D., Schreiber, A., Zhang, W., and Grinstein, S. (1997). Rho is required for the initiation of calcium signaling and phagocytosis by Fcgamma receptors in macrophages. J Exp Med *186*, 955-966.

Haliotis, T., Roder, J., Klein, M., Ortaldo, J., Fauci, A. S., and Herberman, R. B. (1980). Chediak-Higashi gene in humans I. Impairment of natural-killer function. J Exp Med *151*, 1039-1048.

Hamel, L. P., Nicole, M. C., Sritubtim, S., Morency, M. J., Ellis, M., Ehlting, J., Beaudoin, N., Barbazuk, B., Klessig, D., Lee, J., *et al.* (2006). Ancient signals: comparative genomics of plant MAPK and MAPKK gene families. Trends Plant Sci *11*, 192-198.

Hardy, R. R., Hayakawa, K., Parks, D. R., and Herzenberg, L. A. (1983). Demonstration of B-cell maturation in X-linked immunodeficient mice by simultaneous three-colour immunofluorescence. Nature *306*, 270-272.

Hardy, R. R., Hayakawa, K., Parks, D. R., and Herzenberg, L. A. (1984). Murine B cell differentiation lineages. J Exp Med *159*, 1169-1188.

Hata, D., Kawakami, Y., Inagaki, N., Lantz, C. S., Kitamura, T., Khan, W. N., Maeda-Yamamoto, M., Miura, T., Han, W., Hartman, S. E., *et al.* (1998). Involvement of Bruton's tyrosine kinase in FcepsilonRI-dependent mast cell degranulation and cytokine production. J Exp Med *187*, 1235-1247.

Hatzigeorgiou, D. E., Geng, J., Zhu, B., Zhang, Y., Liu, K., Rom, W. N., Fenton, M. J., Turco, S. J., and Ho, J. L. (1996). Lipophosphoglycan from Leishmania suppresses agonist-induced interleukin 1 beta gene expression in human monocytes via a unique promoter sequence. Proc Natl Acad Sci U S A *93*, 14708-14713.

Hayashi, F., Means, T. K., and Luster, A. D. (2003). Toll-like receptors stimulate human neutrophil function
10.1182/blood-2003-04-1078. Blood *102*, 2660-2669.

Henneke, P., and Golenbock, D. T. (2004). Phagocytosis, innate immunity, and host-pathogen specificity. J Exp Med *199*, 1-4.

Herath, S., Kropf, P., and Muller, I. (2003). Cross-talk between CD8(+) and CD4(+) T cells in experimental cutaneous leishmaniasis: CD8(+) T cells are required for optimal IFN-gamma production by CD4(+) T cells. Parasite Immunol *25*, 559-567.

Himmelrich, H., Launois, P., Maillard, I., Biedermann, T., Tacchini-Cottier, F., Locksley, R. M., Rocken, M., and Louis, J. A. (2000). In BALB/c mice, IL-4 production during the initial phase of infection with Leishmania major is necessary and sufficient to instruct Th2 cell development resulting in progressive disease. J Immunol *164*, 4819-4825.

Holaday, B. J., Sadick, M. D., Wang, Z. E., Reiner, S. L., Heinzel, F. P., Parslow, T. G., and Locksley, R. M. (1991). Reconstitution of Leishmania immunity in severe combined immunodeficient mice using Th1- and Th2-like cell lines. J Immunol *147*, 1653-1658.

Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanaho, Y. (1999). Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell *99*, 521-532.

Hondowicz, B. D., Scharton-Kersten, T. M., Jones, D. E., and Scott, P. (1997). Leishmania major-infected C3H mice treated with anti-IL-12 mAb develop but do not maintain a Th2 response. J Immunol *159*, 5024-5031.

Horwood, N. J., Mahon, T., McDaid, J. P., Campbell, J., Mano, H., Brennan, F. M., Webster, D., and Foxwell, B. M. J. (2003). Bruton's Tyrosine Kinase Is Required For Lipopolysaccharide-induced Tumor Necrosis Factor {alpha} Production 10.1084/jem.20021845. J Exp Med *197*, 1603-1611.

Horwood, N. J., Page, T. H., McDaid, J. P., Palmer, C. D., Campbell, J., Mahon, T., Brennan, F. M., Webster, D., and Foxwell, B. M. (2006). Bruton's tyrosine kinase is required for TLR2 and TLR4-induced TNF, but not IL-6, production. J Immunol *176*, 3635-3641.

Hostager, B. S., Haxhinasto, S. A., Rowland, S. L., and Bishop, G. A. (2003). Tumor necrosis factor receptor-associated factor 2 (TRAF2)-deficient B lymphocytes reveal novel roles for TRAF2 in CD40 signaling. J Biol Chem *278*, 45382-45390.

Howard, J. G., Hale, C., and Chan-Liew, W. L. (1980). Immunological regulation of experimental cutaneous leishmaniasis. 1. Immunogenetic aspects of susceptibility to Leishmania tropica in mice. Parasite Immunol *2*, 303-314.

Howell, C. D., Li, J., Roper, E., and Kotzin, B. L. (1995). Biased liver T cell receptor V beta repertoire in a murine graft-versus-host disease model. J Immunol *155*, 2350-2358.

Hsieh, C. S., Macatonia, S. E., O'Garra, A., and Murphy, K. M. (1995). T cell genetic background determines default T helper phenotype development in vitro. J Exp Med *181*, 713-721.

Hunter, C. A. (2005). New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. Nat Rev Immunol *5*, 521-531.

Huynh, C., Roth, D., Ward, D. M., Kaplan, J., and Andrews, N. W. (2004). Defective lysosomal exocytosis and plasma membrane repair in Chediak-Higashi/beige cells. Proc Natl Acad Sci U S A *101*, 16795-16800.

Ismail, N., Olano, J. P., Feng, H. M., and Walker, D. H. (2002). Current status of immune mechanisms of killing of intracellular microorganisms. FEMS Microbiol Lett *207*, 111-120.

James, S. L. (1995). Role of nitric oxide in parasitic infections. Microbiol Rev *59*, 533-547.

Janeway, C. (1991). Immune recognition. Mls: makes a little sense. Nature *349*, 459-461.

Janeway, C. A., Jr., and Medzhitov, R. (2002). Innate immune recognition. Annu Rev Immunol *20*, 197-216.

Jankovic, D., Kullberg, M. C., Feng, C. G., Goldszmid, R. S., Collazo, C. M., Wilson, M., Wynn, T. A., Kamanaka, M., Flavell, R. A., and Sher, A. (2007). Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. J Exp Med *204*, 273-283.

Jefferies, C. A., Doyle, S., Brunner, C., Dunne, A., Brint, E., Wietek, C., Walch, E., Wirth, T., and O'Neill, L. A. (2003). Bruton's tyrosine kinase is a Toll/interleukin-1 receptor domain-binding protein that participates in nuclear factor kappaB activation by Toll-like receptor 4. J Biol Chem *278*, 26258-26264.

Ji, J., Sun, J., Qi, H., and Soong, L. (2002). Analysis of T helper cell responses during infection with Leishmania amazonensis. Am J Trop Med Hyg *66*, 338-345.

Johannes, F. J., Hausser, A., Storz, P., Truckenmuller, L., Link, G., Kawakami, T., and Pfizenmaier, K. (1999). Bruton's tyrosine kinase (Btk) associates with protein kinase C mu. FEBS Lett *461*, 68-72.

Kaiser, S. M., Malik, H. S., and Emerman, M. (2007). Restriction of an Extinct Retrovirus by the Human TRIM5{alpha} Antiviral Protein 10.1126/science.1140579. Science *316*, 1756-1758.

Kamanaka, M., Yu, P., Yasui, T., Yoshida, K., Kawabe, T., Horii, T., Kishimoto, T., and Kikutani, H. (1996). Protective role of CD40 in Leishmania major infection at two distinct phases of cell-mediated immunity. Immunity *4*, 275-281.

Kane, M. M., and Mosser, D. M. (2001). The role of IL-10 in promoting disease progression in leishmaniasis. J Immunol *166*, 1141-1147.

Karpus, W. J., Lukacs, N. W., Kennedy, K. J., Smith, W. S., Hurst, S. D., and Barrett, T. A. (1997). Differential CC chemokine-induced enhancement of T helper cell cytokine production. J Immunol *158*, 4129-4136.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999). Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity *11*, 115-122.

Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P. F., Sato, S., Hoshino, K., and Akira, S. (2001). Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J Immunol *167*, 5887-5894.

Kawakami, Y., Hartman, S. E., Holland, P. M., Cooper, J. A., and Kawakami, T. (1998). Multiple signaling pathways for the activation of JNK in mast cells: involvement of Bruton's tyrosine kinase, protein kinase C, and JNK kinases, SEK1 and MKK7. J Immunol *161*, 1795-1802.

Kawakami, Y., Inagaki, N., Salek-Ardakani, S., Kitaura, J., Tanaka, H., Nagao, K., Xiao, W., Nagai, H., Croft, M., and Kawakami, T. (2006). Regulation of dendritic cell maturation and function by Bruton's tyrosine kinase via IL-10 and Stat3. Proc Natl Acad Sci U S A *103*, 153-158.

Kawakami, Y., Kitaura, J., Hartman, S. E., Lowell, C. A., Siraganian, R. P., and Kawakami, T. (2000a). Regulation of protein kinase Cbeta I by two protein-tyrosine kinases, Btk and Syk 10.1073/pnas.120175097. PNAS *97*, 7423-7428.

Kawakami, Y., Kitaura, J., Satterthwaite, A. B., Kato, R. M., Asai, K., Hartman, S. E., Maeda-Yamamoto, M., Lowell, C. A., Rawlings, D. J., Witte, O. N., and Kawakami, T. (2000b). Redundant and opposing functions of two tyrosine kinases, Btk and Lyn, in mast cell activation. J Immunol *165*, 1210-1219.

Kerner, J. D., Appleby, M. W., Mohr, R. N., Chien, S., Rawlings, D. J., Maliszewski, C. R., Witte, O. N., and Perlmutter, R. M. (1995). Impaired expansion of mouse B cell progenitors lacking Btk. Immunity *3*, 301-312.

Khan, W. N., Alt, F. W., Gerstein, R. M., Malynn, B. A., Larsson, I., Rathbun, G., Davidson, L., Muller, S., Kantor, A. B., Herzenberg, L. A., and et al. (1995). Defective B cell development and function in Btk-deficient mice. Immunity *3*, 283-299.

Khan, W. N., Nilsson, A., Mizoguchi, E., Castigli, E., Forsell, J., Bhan, A. K., Geha, R., Sideras, P., and Alt, F. W. (1997). Impaired B cell maturation in mice lacking Bruton's tyrosine kinase (Btk) and CD40. Int Immunol *9*, 395-405.

Kirkpatrick, C. E., and Farrell, J. P. (1982). Leishmaniasis in beige mice. Infect Immun *38*, 1208-1216.

Kitanaka, A., Mano, H., Conley, M. E., and Campana, D. (1998). Expression and activation of the nonreceptor tyrosine kinase Tec in human B cells. Blood *91*, 940-948.

Kjeken, R., Egeberg, M., Habermann, A., Kuehnel, M., Peyron, P., Floetenmeyer, M., Walther, P., Jahraus, A., Defacque, H., Kuznetsov, S. A., and Griffiths, G. (2004). Fusion between phagosomes, early and late endosomes: a role for actin in fusion between late, but not early endocytic organelles. Mol Biol Cell *15*, 345-358.

Koike, M., Kikuchi, Y., Tominaga, A., Takaki, S., Akagi, K., Miyazaki, J., Yamamura, K., and Takatsu, K. (1995). Defective IL-5-receptor-mediated signaling in B cells of X-linked immunodeficient mice. Int Immunol *7*, 21-30.

Kolls, J. K., and Linden, A. (2004). Interleukin-17 family members and inflammation. Immunity *21*, 467-476.

Komiyama, Y., Nakae, S., Matsuki, T., Nambu, A., Ishigame, H., Kakuta, S., Sudo, K., and Iwakura, Y. (2006). IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol *177*, 566-573.

Konecny, P., Stagg, A. J., Jebbari, H., English, N., Davidson, R. N., and Knight, S. C. (1999). Murine dendritic cells internalize Leishmania major promastigotes, produce IL-12 p40 and stimulate primary T cell proliferation in vitro. Eur J Immunol *29*, 1803-1811.

Kopf, M., Brombacher, F., Kohler, G., Kienzle, G., Widmann, K. H., Lefrang, K., Humborg, C., Ledermann, B., and Solbach, W. (1996). IL-4-deficient Balb/c mice resist infection with Leishmania major. J Exp Med *184*, 1127-1136.

Korman, A. J., Bourgarel, P., Meo, T., and Rieckhof, G. E. (1992). The mouse mammary tumour virus long terminal repeat encodes a type II transmembrane glycoprotein. Embo J *11*, 1901-1905.

Kozak, C., Peters, G., Pauley, R., Morris, V., Michalides, R., Dudley, J., Green, M., Davisson, M., Prakash, O., Vaidya, A., and et al. (1987). A standardized nomenclature for endogenous mouse mammary tumor viruses. J Virol *61*, 1651-1654.

Krakowski, M., and Owens, T. (1996). Interferon-gamma confers resistance to experimental allergic encephalomyelitis. Eur J Immunol *26*, 1641-1646.

Kropf, P., Etges, R., Schopf, L., Chung, C., Sypek, J., and Muller, I. (1997). Characterization of T cell-mediated responses in nonhealing and healing Leishmania major infections in the absence of endogenous IL-4. J Immunol *159*, 3434-3443.

Kultz, D. (2001). Evolution of Osmosensory MAP Kinase Signaling Pathways 10.1093/icb/41.4.743. Amer Zool *41*, 743-757.

Kurata, H., Lee, H. J., O'Garra, A., and Arai, N. (1999). Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. Immunity *11*, 677-688.

Lacy, P., Mahmudi-Azer, S., Bablitz, B., Hagen, S. C., Velazquez, J. R., Man, S. F., and Moqbel, R. (1999). Rapid mobilization of intracellularly stored RANTES in response to interferon-gamma in human eosinophils. Blood *94*, 23-32.

Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A., and Cua, D. J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med *201*, 233-240.

Launois, P., Maillard, I., Pingel, S., Swihart, K. G., Xenarios, I., Acha-Orbea, H., Diggelmann, H., Locksley, R. M., MacDonald, H. R., and Louis, J. A. (1997). IL-4 rapidly produced by V beta 4 V alpha 8 CD4+ T cells instructs Th2 development and susceptibility to Leishmania major in BALB/c mice. Immunity *6*, 541-549.

Lee, H. J., Takemoto, N., Kurata, H., Kamogawa, Y., Miyatake, S., O'Garra, A., and Arai, N. (2000). GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. J Exp Med *192*, 105-115.

Lee, Y. N., and Bieniasz, P. D. (2007). Reconstitution of an infectious human endogenous retrovirus. PLoS Pathog *3*, e10.

Leslie, C. A., Gonnerman, W. A., and Cathcart, E. S. (1987). Gender differences in eicosanoid production from macrophages of arthritis-susceptible mice. J Immunol *138*, 413-416.

Li, J., Hunter, C. A., and Farrell, J. P. (1999). Anti-TGF-beta treatment promotes rapid healing of Leishmania major infection in mice by enhancing in vivo nitric oxide production. J Immunol *162*, 974-979.

Lighvani, A. A., Frucht, D. M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B. D., Nguyen, B. V., Gadina, M., Sher, A., Paul, W. E., and O'Shea, J. J. (2001). T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. Proc Natl Acad Sci U S A *98*, 15137-15142.

Lindvall, J. M., Blomberg, K. E., Valiaho, J., Vargas, L., Heinonen, J. E., Berglof, A., Mohamed, A. J., Nore, B. F., Vihinen, M., and Smith, C. I. (2005). Bruton's tyrosine kinase: cell biology, sequence conservation, mutation spectrum, siRNA modifications, and expression profiling. Immunol Rev *203*, 200-215.

Lipoldova, M., and Demant, P. (2006). Genetic susceptibility to infectious disease: lessons from mouse models of leishmaniasis. Nat Rev Genet *7*, 294-305.

Locksley, R. M., Reiner, S. L., Hatam, F., Littman, D. R., and Killeen, N. (1993). Helper T cells without CD4: control of leishmaniasis in CD4-deficient mice. Science *261*, 1448-1451.

Logan, M. R., Lacy, P., Bablitz, B., and Moqbel, R. (2002). Expression of eosinophil target SNAREs as potential cognate receptors for vesicle-associated membrane protein-2 in exocytosis. J Allergy Clin Immunol *109*, 299-306.

MacKenzie, J. R., Mattes, J., Dent, L. A., and Foster, P. S. (2001). Eosinophils promote allergic disease of the lung by regulating CD4(+) Th2 lymphocyte function. J Immunol *167*, 3146-3155.

MacPherson, J. C., Comhair, S. A., Erzurum, S. C., Klein, D. F., Lipscomb, M. F., Kavuru, M. S., Samoszuk, M. K., and Hazen, S. L. (2001). Eosinophils are a major source of nitric oxide-derived oxidants in severe asthma: characterization of pathways available to eosinophils for generating reactive nitrogen species. J Immunol *166*, 5763-5772.

Maillard, I., Launois, P., Xenarios, I., Louis, J. A., Acha-Orbea, H., and Diggelmann, H. (1998). Immune response to mouse mammary tumor virus in mice lacking the alpha/beta interferon or the gamma interferon receptor. J Virol *72*, 2638-2646.

Manetti, R., Parronchi, P., Giudizi, M. G., Piccinni, M. P., Maggi, E., Trinchieri, G., and Romagnani, S. (1993). Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. J Exp Med *177*, 1199-1204.

Mangla, A., Khare, A., Vineeth, V., Panday, N. N., Mukhopadhyay, A., Ravindran, B., Bal, V., George, A., and Rath, S. (2004). Pleiotropic consequences of Bruton tyrosine kinase deficiency in myeloid lineages lead to poor inflammatory responses. Blood *104*, 1191-1197.

Marshall, A. J., Fleming, H. E., Wu, G. E., and Paige, C. J. (1998). Modulation of the IL-7 dose-response threshold during pro-B cell differentiation is dependent on pre-B cell receptor expression. J Immunol *161*, 6038-6045.

Matthews, D. J., Emson, C. L., McKenzie, G. J., Jolin, H. E., Blackwell, J. M., and McKenzie, A. N. (2000). IL-13 is a susceptibility factor for Leishmania major infection. J Immunol *164*, 1458-1462.

Mattner, F., Magram, J., Ferrante, J., Launois, P., Di Padova, K., Behin, R., Gately, M. K., Louis, J. A., and Alber, G. (1996). Genetically resistant mice lacking interleukin-12

are susceptible to infection with Leishmania major and mount a polarized Th2 cell response. Eur J Immunol *26*, 1553-1559.

McConville, M. J., Turco, S. J., Ferguson, M. A., and Sacks, D. L. (1992). Developmental modification of lipophosphoglycan during the differentiation of Leishmania major promastigotes to an infectious stage. Embo J *11*, 3593-3600.

McNagny, K., and Graf, T. (2002). Making eosinophils through subtle shifts in transcription factor expression. J Exp Med *195*, F43-47.

Milili, M., Le Deist, F., de Saint-Basile, G., Fischer, A., Fougereau, M., and Schiff, C. (1993). Bone marrow cells in X-linked agammaglobulinemia express pre-B-specific genes (lambda-like and V pre-B) and present immunoglobulin V-D-J gene usage strongly biased to a fetal-like repertoire. J Clin Invest *91*, 1616-1629.

Miller, A. T., and Berg, L. J. (2002). New insights into the regulation and functions of Tec family tyrosine kinases in the immune system. Curr Opin Immunol *14*, 331-340.

Mira, M. T., Alcais, A., Nguyen, V. T., Moraes, M. O., Di Flumeri, C., Vu, H. T., Mai, C. P., Nguyen, T. H., Nguyen, N. B., Pham, X. K., *et al.* (2004). Susceptibility to leprosy is associated with PARK2 and PACRG. Nature *427*, 636-640.

Mishra, A., Hogan, S. P., Lee, J. J., Foster, P. S., and Rothenberg, M. E. (1999). Fundamental signals that regulate eosinophil homing to the gastrointestinal tract. J Clin Invest *103*, 1719-1727.

Mizgerd, J. P., Meek, B. B., Kutkoski, G. J., Bullard, D. C., Beaudet, A. L., and Doerschuk, C. M. (1996). Selectins and neutrophil traffic: margination and Streptococcus pneumoniae-induced emigration in murine lungs. J Exp Med *184*, 639-645.

Modiano, D., Petrarca, V., Sirima, B. S., Nebie, I., Diallo, D., Esposito, F., and Coluzzi, M. (1996). Different response to Plasmodium falciparum malaria in west African sympatric ethnic groups. Proc Natl Acad Sci U S A *93*, 13206-13211.

Mohamed, A. J., Nore, B. F., Christensson, B., and Smith, C. I. (1999). Signalling of Bruton's tyrosine kinase, Btk. Scand J Immunol *49*, 113-118.

Moll, H., Fuchs, H., Blank, C., and Rollinghoff, M. (1993). Langerhans cells transport Leishmania major from the infected skin to the draining lymph node for presentation to antigen-specific T cells. Eur J Immunol *23*, 1595-1601.

Moll, H., Scollay, R., and Mitchell, G. F. (1988). Resistance to cutaneous leishmaniasis in nude mice injected with L3T4+ T cells but not with Ly-2+ T cells. Immunol Cell Biol *66 ( Pt 1)*, 57-63.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L., and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol *19*, 683-765.

Mosser, D. M., and Edelson, P. J. (1987). The third component of complement (C3) is responsible for the intracellular survival of Leishmania major. Nature *327*, 329-331.

Mukhopadhyay, S., George, A., Bal, V., Ravindran, B., and Rath, S. (1999a). Bruton's tyrosine kinase deficiency in macrophages inhibits nitric oxide generation leading to enhancement of IL-12 induction. J Immunol *163*, 1786-1792.

Mukhopadhyay, S., Mohanty, M., Mangla, A., George, A., Bal, V., Rath, S., and Ravindran, B. (2002). Macrophage effector functions controlled by Bruton's tyrosine kinase are more crucial than the cytokine balance of T cell responses for microfilarial clearance. J Immunol *168*, 2914-2921.

Mukhopadhyay, S., Sahoo, P. K., George, A., Bal, V., Rath, S., and Ravindran, B. (1999b). Delayed clearance of filarial infection and enhanced Th1 immunity due to modulation of macrophage APC functions in xid mice. J Immunol *163*, 875-883.

Murray, H. W., and Nathan, C. F. (1999). Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral Leishmania donovani. J Exp Med *189*, 741-746.

Nagle, D. L., Karim, M. A., Woolf, E. A., Holmgren, L., Bork, P., Misumi, D. J., McGrail, S. H., Dussault, B. J., Jr., Perou, C. M., Boissy, R. E., *et al.* (1996). Identification and mutation analysis of the complete gene for Chediak-Higashi syndrome. Nat Genet *14*, 307-311.

Nakae, S., Saijo, S., Horai, R., Sudo, K., Mori, S., and Iwakura, Y. (2003). IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. Proc Natl Acad Sci U S A *100*, 5986-5990.

Nakagawa, K., Brusic, V., McColl, G., and Harrison, L. C. (1997). Direct evidence for the expression of multiple endogenous retroviruses in the synovial compartment in rheumatoid arthritis. Arthritis Rheum *40*, 627-638.

Nakagawa, T., Roth, W., Wong, P., Nelson, A., Farr, A., Deussing, J., Villadangos, J. A., Ploegh, H., Peters, C., and Rudensky, A. Y. (1998). Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. Science *280*, 450-453.

Neel, N. F., Schutyser, E., Sai, J., Fan, G. H., and Richmond, A. (2005). Chemokine receptor internalization and intracellular trafficking. Cytokine Growth Factor Rev *16*, 637-658.

Nerlov, C., and Graf, T. (1998). PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. Genes Dev *12*, 2403-2412.

Nerlov, C., McNagny, K. M., Doderlein, G., Kowenz-Leutz, E., and Graf, T. (1998). Distinct C/EBP functions are required for eosinophil lineage commitment and maturation. Genes Dev *12*, 2413-2423.

Nishi, T., and Forgac, M. (2002). The vacuolar (H+)-ATPases--nature's most versatile proton pumps. Nat Rev Mol Cell Biol *3*, 94-103.

Noben-Trauth, N., Lira, R., Nagase, H., Paul, W. E., and Sacks, D. L. (2003). The relative contribution of IL-4 receptor signaling and IL-10 to susceptibility to Leishmania major. J Immunol *170*, 5152-5158.

Noben-Trauth, N., Paul, W. E., and Sacks, D. L. (1999). IL-4- and IL-4 receptor-deficient BALB/c mice reveal differences in susceptibility to Leishmania major parasite substrains. J Immunol *162*, 6132-6140.

Nore, B. F., Vargas, L., Mohamed, A. J., Branden, L. J., Backesjo, C. M., Islam, T. C., Mattsson, P. T., Hultenby, K., Christensson, B., and Smith, C. I. (2000). Redistribution of Bruton's tyrosine kinase by activation of phosphatidylinositol 3-kinase and Rho-family GTPases. Eur J Immunol *30*, 145-154.

Oka, Y., Rolink, A. G., Andersson, J., Kamanaka, M., Uchida, J., Yasui, T., Kishimoto, T., Kikutani, H., and Melchers, F. (1996). Profound reduction of mature B cell numbers, reactivities and serum Ig levels in mice which simultaneously carry the XID and CD40 deficiency genes. Int Immunol *8*, 1675-1685.

Olivier, M., Brownsey, R. W., and Reiner, N. E. (1992). Defective stimulus-response coupling in human monocytes infected with Leishmania donovani is associated with altered activation and translocation of protein kinase C. Proc Natl Acad Sci U S A *89*, 7481-7485.

Olsen, N. J., and Kovacs, W. J. (2001). Effects of androgens on T and B lymphocyte development. Immunol Res *23*, 281-288.

Otterness, I. G., and Moore, P. F. (1988). Carrageenan foot edema test. Methods Enzymol *162*, 320-327.

Ouyang, W., Jacobson, N. G., Bhattacharya, D., Gorham, J. D., Fenoglio, D., Sha, W. C., Murphy, T. L., and Murphy, K. M. (1999). The Ets transcription factor ERM is Th1-specific and induced by IL-12 through a Stat4-dependent pathway. Proc Natl Acad Sci U S A *96*, 3888-3893.

Ouyang, W., Lohning, M., Gao, Z., Assenmacher, M., Ranganath, S., Radbruch, A., and Murphy, K. M. (2000). Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity *12*, 27-37.

Ouyang, W., Ranganath, S. H., Weindel, K., Bhattacharya, D., Murphy, T. L., Sha, W. C., and Murphy, K. M. (1998). Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity *9*, 745-755.

Pan, H., Yan, B. S., Rojas, M., Shebzukhov, Y. V., Zhou, H., Kobzik, L., Higgins, D. E., Daly, M. J., Bloom, B. R., and Kramnik, I. (2005). Ipr1 gene mediates innate immunity to tuberculosis. Nature *434*, 767-772.

Park, A. Y., Hondowicz, B. D., and Scott, P. (2000). IL-12 is required to maintain a Th1 response during Leishmania major infection. J Immunol *165*, 896-902.

Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., and Dong, C. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol *6*, 1133-1141.

Pashine, A., John, B., Rath, S., George, A., and Bal, V. (1999). Th1 dominance in the immune response to live Salmonella typhimurium requires bacterial invasiveness but not persistence. Int Immunol *11*, 481-489.

Perou, C. M., Leslie, J. D., Green, W., Li, L., Ward, D. M., and Kaplan, J. (1997). The Beige/Chediak-Higashi syndrome gene encodes a widely expressed cytosolic protein. J Biol Chem *272*, 29790-29794.

Perskvist, N., Roberg, K., Kulyte, A., and Stendahl, O. (2002). Rab5a GTPase regulates fusion between pathogen-containing phagosomes and cytoplasmic organelles in human neutrophils. J Cell Sci *115*, 1321-1330.

Peters, G., Placzek, M., Brookes, S., Kozak, C., Smith, R., and Dickson, C. (1986). Characterization, chromosome assignment, and segregation analysis of endogenous proviral units of mouse mammary tumor virus. J Virol *59*, 535-544.

Pharoah, P. D., Antoniou, A., Bobrow, M., Zimmern, R. L., Easton, D. F., and Ponder, B. A. (2002). Polygenic susceptibility to breast cancer and implications for prevention. Nat Genet *31*, 33-36.

Piedrafita, D., Proudfoot, L., Nikolaev, A. V., Xu, D., Sands, W., Feng, G. J., Thomas, E., Brewer, J., Ferguson, M. A., Alexander, J., and Liew, F. Y. (1999). Regulation of macrophage IL-12 synthesis by Leishmania phosphoglycans. Eur J Immunol *29*, 235-244.

Piliponsky, A. M., Gleich, G. J., Bar, I., and Levi-Schaffer, F. (2002). Effects of eosinophils on mast cells: a new pathway for the perpetuation of allergic inflammation. Mol Immunol *38*, 1369.

Piliponsky, A. M., Pickholtz, D., Gleich, G. J., and Levi-Schaffer, F. (2001). Human eosinophils induce histamine release from antigen-activated rat peritoneal mast cells: a possible role for mast cells in late-phase allergic reactions. J Allergy Clin Immunol *107*, 993-1000.

Pitt, A., Mayorga, L. S., Stahl, P. D., and Schwartz, A. L. (1992). Alterations in the protein composition of maturing phagosomes. J Clin Invest *90*, 1978-1983.

Premack, B. A., and Schall, T. J. (1996). Chemokine receptors: gateways to inflammation and infection. Nat Med *2*, 1174-1178.

Proudfoot, L., Nikolaev, A. V., Feng, G. J., Wei, W. Q., Ferguson, M. A., Brimacombe, J. S., and Liew, F. Y. (1996). Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of Leishmania lipophosphoglycan in murine macrophages. Proc Natl Acad Sci U S A *93*, 10984-10989.

Proudfoot, L., O'Donnell, C. A., and Liew, F. Y. (1995). Glycoinositolphospholipids of Leishmania major inhibit nitric oxide synthesis and reduce leishmanicidal activity in murine macrophages. Eur J Immunol *25*, 745-750.

Redecke, V., Hacker, H., Datta, S. K., Fermin, A., Pitha, P. M., Broide, D. H., and Raz, E. (2004). Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. J Immunol *172*, 2739-2743.

Reiner, S. L., Zheng, S., Wang, Z. E., Stowring, L., and Locksley, R. M. (1994). Leishmania promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4+ T cells during initiation of infection. J Exp Med *179*, 447-456.

Ridderstad, A., Nossal, G. J., and Tarlinton, D. M. (1996). The xid mutation diminishes memory B cell generation but does not affect somatic hypermutation and selection. J Immunol *157*, 3357-3365.

Ridderstad, A., and Tarlinton, D. M. (1997). B cell memory in xid mice is long-lived despite reduced memory B cell frequency. Scand J Immunol *45*, 655-659.

Rigley, K. P., Harnett, M. M., Phillips, R. J., and Klaus, G. G. (1989). Analysis of signaling via surface immunoglobulin receptors on B cells from CBA/N mice. Eur J Immunol *19*, 2081-2086.

Roder, J. C., Haliotis, T., Klein, M., Korec, S., Jett, J. R., Ortaldo, J., Heberman, R. B., Katz, P., and Fauci, A. S. (1980). A new immunodeficiency disorder in humans involving NK cells. Nature *284*, 553-555.

Rodriguez, R., Matsuda, M., Perisic, O., Bravo, J., Paul, A., Jones, N. P., Light, Y., Swann, K., Williams, R. L., and Katan, M. (2001). Tyrosine residues in phospholipase Cgamma 2 essential for the enzyme function in B-cell signaling. J Biol Chem *276*, 47982-47992.

Rodriguez-Palmero, M., Hara, T., Thumbs, A., and Hunig, T. (1999). Triggering of T cell proliferation through CD28 induces GATA-3 and promotes T helper type 2 differentiation in vitro and in vivo. Eur J Immunol *29*, 3914-3924.

Rohrer, J., and Conley, M. E. (1999). Correction of X-linked immunodeficient mice by competitive reconstitution with limiting numbers of normal bone marrow cells. Blood *94*, 3358-3365.

Rothenberg, M. E., and Hogan, S. P. (2006). The eosinophil. Annu Rev Immunol *24*, 147-174.

Sacks, D., and Noben-Trauth, N. (2002). The immunology of susceptibility and resistance to Leishmania major in mice. Nat Rev Immunol *2*, 845-858.

Saha, B., Das, G., Vohra, H., Ganguly, N. K., and Mishra, G. C. (1995). Macrophage-T cell interaction in experimental visceral leishmaniasis: failure to express costimulatory

molecules on Leishmania-infected macrophages and its implication in the suppression of cell-mediated immunity. Eur J Immunol *25*, 2492-2498.

Sanderson, C. J. (1992). Interleukin-5, eosinophils, and disease. Blood *79*, 3101-3109.

Sanderson, C. J., and de Souza, W. (1979). A morphological study of the interaction between Trypanosoma cruzi and rat eosinophils, neutrophils and macrophages in vitro. J Cell Sci *37*, 275-286.

Sanjabi, S., Hoffmann, A., Liou, H. C., Baltimore, D., and Smale, S. T. (2000). Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. Proc Natl Acad Sci U S A *97*, 12705-12710.

Santos-Argumedo, L., Lund, F. E., Heath, A. W., Solvason, N., Wu, W. W., Grimaldi, J. C., Parkhouse, R. M., and Howard, M. (1995). CD38 unresponsiveness of xid B cells implicates Bruton's tyrosine kinase (btk) as a regular of CD38 induced signal transduction. Int Immunol *7*, 163-170.

Satoskar, A. R., Stamm, L. M., Zhang, X., Satoskar, A. A., Okano, M., Terhorst, C., David, J. R., and Wang, B. (1999). Mice lacking NK cells develop an efficient Th1 response and control cutaneous Leishmania major infection. J Immunol *162*, 6747-6754.

Satterthwaite, A. B., and Witte, O. N. (2000). The role of Bruton's tyrosine kinase in B-cell development and function: a genetic perspective. Immunol Rev *175*, 120-127.

Scher, I., Ahmed, A., Strong, D. M., Steinberg, A. D., and Paul, W. E. (1975a). X-linked B-lymphocyte immune defect in CBA/HN mice. I. Studies of the function and composition of spleen cells. J Exp Med *141*, 788-803.

Scher, I., Steinberg, A. D., Berning, A. K., and Paul, W. E. (1975b). X-linked B-lymphocyte immune defect in CBA/N mice. II. Studies of the mechanisms underlying the immune defect. J Exp Med *142*, 637-650.

Schirrmacher, V., Beutner, U., Bucur, M., Umansky, V., Rocha, M., and von Hoegen, P. (1998). Loss of endogenous mouse mammary tumor virus superantigen increases tumor resistance. J Immunol *161*, 563-570.

Schluger, N. W., and Rom, W. N. (1997). Early responses to infection: chemokines as mediators of inflammation. Curr Opin Immunol *9*, 504-508.

Schmidt, U., Boucheron, N., Unger, B., and Ellmeier, W. (2004). The role of Tec family kinases in myeloid cells. Int Arch Allergy Immunol *134*, 65-78.

Scianimanico, S., Desrosiers, M., Dermine, J. F., Meresse, S., Descoteaux, A., and Desjardins, M. (1999). Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by Leishmania donovani promastigotes. Cell Microbiol *1*, 19-32.

Seder, R. A., Gazzinelli, R., Sher, A., and Paul, W. E. (1993). Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. Proc Natl Acad Sci U S A *90*, 10188-10192.

Segal, A. W. (2005). How neutrophils kill microbes. Annu Rev Immunol *23*, 197-223.

Shao, D., Segal, A. W., and Dekker, L. V. (2003). Lipid rafts determine efficiency of NADPH oxidase activation in neutrophils. FEBS Lett *550*, 101-106.

Shi, H. Z., Humbles, A., Gerard, C., Jin, Z., and Weller, P. F. (2000). Lymph node trafficking and antigen presentation by endobronchial eosinophils. J Clin Invest *105*, 945-953.

Singh, B., Prange, S., and Jevnikar, A. M. (1998a). Protective and destructive effects of microbial infection in insulin-dependent diabetes mellitus. Semin Immunol *10*, 79-86.

Singh, N., Bhatia, S., Abraham, R., Basu, S. K., George, A., Bal, V., and Rath, S. (1998b). Modulation of T cell cytokine profiles and peptide-MHC complex availability in vivo by delivery to scavenger receptors via antigen maleylation. J Immunol *160*, 4869-4880.

Smith, C. I., Baskin, B., Humire-Greiff, P., Zhou, J. N., Olsson, P. G., Maniar, H. S., Kjellen, P., Lambris, J. D., Christensson, B., Hammarstrom, L., and et al. (1994). Expression of Bruton's agammaglobulinemia tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells. J Immunol *152*, 557-565.

Smith, C. I., Islam, T. C., Mattsson, P. T., Mohamed, A. J., Nore, B. F., and Vihinen, M. (2001). The Tec family of cytoplasmic tyrosine kinases: mammalian Btk, Bmx, Itk, Tec, Txk and homologs in other species. Bioessays *23*, 436-446.

Sochorova, K., Horvath, R., Rozkova, D., Litzman, J., Bartunkova, J., Sediva, A., and Spisek, R. (2007). Impaired Toll-like receptor 8-mediated IL-6 and TNF-alpha production in antigen-presenting cells from patients with X-linked agammaglobulinemia. Blood *109*, 2553-2556.

Soong, L., Xu, J. C., Grewal, I. S., Kima, P., Sun, J., Longley, B. J., Jr., Ruddle, N. H., McMahon-Pratt, D., and Flavell, R. A. (1996). Disruption of CD40-CD40 ligand interactions results in an enhanced susceptibility to Leishmania amazonensis infection. Immunity *4*, 263-273.

Spath, G. F., Epstein, L., Leader, B., Singer, S. M., Avila, H. A., Turco, S. J., and Beverley, S. M. (2000). Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite Leishmania major. Proc Natl Acad Sci U S A *97*, 9258-9263.

Sprent, J., and Bruce, J. (1984). Physiology of B cells in mice with X-linked immunodeficiency. II. Influence of the thymus and mature T cells on B cell differentiation. J Exp Med *160*, 335-340.

Stamm, L. M., Satoskar, A. A., Ghosh, S. K., David, J. R., and Satoskar, A. R. (1999). STAT-4 mediated IL-12 signaling pathway is critical for the development of protective immunity in cutaneous leishmaniasis. Eur J Immunol *29*, 2524-2529.

Stauffer, Y., Marguerat, S., Meylan, F., Ucla, C., Sutkowski, N., Huber, B., Pelet, T., and Conrad, B. (2001). Interferon-alpha-induced endogenous superantigen. a model linking environment and autoimmunity. Immunity *15*, 591-601.

Stetson, D. B., Mohrs, M., Mallet-Designe, V., Teyton, L., and Locksley, R. M. (2002). Rapid expansion and IL-4 expression by Leishmania-specific naive helper T cells in vivo. Immunity *17*, 191-200.

Stinchcombe, J. C., Page, L. J., and Griffiths, G. M. (2000). Secretory lysosome biogenesis in cytotoxic T lymphocytes from normal and Chediak Higashi syndrome patients. Traffic *1*, 435-444.

Stoye, J. P., and Coffin, J. M. (2000). A provirus put to work. Nature *403*, 715, 717.

Strieter, R. M., Standiford, T. J., Huffnagle, G. B., Colletti, L. M., Lukacs, N. W., and Kunkel, S. L. (1996). "The good, the bad, and the ugly." The role of chemokines in models of human disease. J Immunol *156*, 3583-3586.

Stumptner-Cuvelette, P., Morchoisne, S., Dugast, M., Le Gall, S., Raposo, G., Schwartz, O., and Benaroch, P. (2001). HIV-1 Nef impairs MHC class II antigen presentation and surface expression. Proc Natl Acad Sci U S A *98*, 12144-12149.

Su, D. M., Navarre, S., Oh, W. J., Condie, B. G., and Manley, N. R. (2003). A domain of Foxn1 required for crosstalk-dependent thymic epithelial cell differentiation. Nat Immunol *4*, 1128-1135.

Su, Y. W., and Jumaa, H. (2003). LAT links the pre-BCR to calcium signaling. Immunity *19*, 295-305.

Sutkowski, N., Conrad, B., Thorley-Lawson, D. A., and Huber, B. T. (2001). Epstein-Barr virus transactivates the human endogenous retrovirus HERV-K18 that encodes a superantigen. Immunity *15*, 579-589.

Swihart, K., Fruth, U., Messmer, N., Hug, K., Behin, R., Huang, S., Del Giudice, G., Aguet, M., and Louis, J. A. (1995). Mice from a genetically resistant background lacking the interferon gamma receptor are susceptible to infection with Leishmania major but mount a polarized T helper cell 1-type CD4+ T cell response. J Exp Med *181*, 961-971.

Swingler, S., Mann, A., Jacque, J., Brichacek, B., Sasseville, V. G., Williams, K., Lackner, A. A., Janoff, E. N., Wang, R., Fisher, D., and Stevenson, M. (1999). HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. Nat Med *5*, 997-103.

Szabo, S. J., Sullivan, B. M., Stemmann, C., Satoskar, A. R., Sleckman, B. P., and Glimcher, L. H. (2002). Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. Science *295*, 338-342.

Takahashi-Tezuka, M., Hibi, M., Fujitani, Y., Fukada, T., Yamaguchi, T., and Hirano, T. (1997). Tec tyrosine kinase links the cytokine receptors to PI-3 kinase probably through JAK. Oncogene *14*, 2273-2282.

Takeuchi, K., Wood, H., and Swank, R. T. (1986). Lysosomal elastase and cathepsin G in beige mice. Neutrophils of beige (Chediak-Higashi) mice selectively lack lysosomal elastase and cathepsin G. J Exp Med *163*, 665-677.

Tang, B., Mano, H., Yi, T., and Ihle, J. N. (1994). Tec kinase associates with c-kit and is tyrosine phosphorylated and activated following stem cell factor binding. Mol Cell Biol *14*, 8432-8437.

Taub, D. D., Turcovski-Corrales, S. M., Key, M. L., Longo, D. L., and Murphy, W. J. (1996). Chemokines and T lymphocyte activation: I. Beta chemokines costimulate human T lymphocyte activation in vitro. J Immunol *156*, 2095-2103.

Tavares, D., da Conceicao Ribeiro, R., and Carlos da Silva, A. (2006). Inflammatory lesion and parasite load are inversely associated in Leishmania amazonensis infected mice genetically selected according to oral tolerance susceptibility. Microbes Infect *8*, 957-964.

Taylor, P. R., Martinez-Pomares, L., Stacey, M., Lin, H. H., Brown, G. D., and Gordon, S. (2005). Macrophage receptors and immune recognition. Annu Rev Immunol *23*, 901-944.

Theilgaard-Monch, K., Knudsen, S., Follin, P., and Borregaard, N. (2004). The transcriptional activation program of human neutrophils in skin lesions supports their important role in wound healing. J Immunol *172*, 7684-7693.

Theilgaard-Monch, K., Porse, B. T., and Borregaard, N. (2006). Systems biology of neutrophil differentiation and immune response. Curr Opin Immunol *18*, 54-60.

Thomas, C. A., Li, Y., Kodama, T., Suzuki, H., Silverstein, S. C., and El Khoury, J. (2000). Protection from lethal gram-positive infection by macrophage scavenger receptor-dependent phagocytosis. J Exp Med *191*, 147-156.

Tisch, R., and McDevitt, H. (1996). Insulin-dependent diabetes mellitus. Cell *85*, 291-297.

Titus, M. A. (1999). A class VII unconventional myosin is required for phagocytosis. Curr Biol *9*, 1297-1303.

Tominaga, A., Takaki, S., Koyama, N., Katoh, S., Matsumoto, R., Migita, M., Hitoshi, Y., Hosoya, Y., Yamauchi, S., Kanai, Y., and et al. (1991). Transgenic mice expressing a B cell growth and differentiation factor gene (interleukin 5) develop eosinophilia and autoantibody production. J Exp Med *173*, 429-437.

Tomonari, K., Fairchild, S., and Rosenwasser, O. A. (1993). Influence of viral superantigens on V beta- and V alpha-specific positive and negative selection. Immunol Rev *131*, 131-168.

Tran, E. H., Prince, E. N., and Owens, T. (2000). IFN-gamma shapes immune invasion of the central nervous system via regulation of chemokines. J Immunol *164*, 2759-2768.

Tsan, M.-F., Clark, R. N., Goyert, S. M., and White, J. E. (2001). Induction of TNF-{alpha} and MnSOD by endotoxin: role of membrane CD14 and Toll-like receptor-4. Am J Physiol Cell Physiol *280*, C1422-1430.

van Rijt, L. S., Vos, N., Hijdra, D., de Vries, V. C., Hoogsteden, H. C., and Lambrecht, B. N. (2003). Airway eosinophils accumulate in the mediastinal lymph nodes but lack antigen-presenting potential for naive T cells. J Immunol *171*, 3372-3378.

Varkila, K., Chatelain, R., Leal, L. M., and Coffman, R. L. (1993). Reconstitution of C.B-17 scid mice with BALB/c T cells initiates a T helper type-1 response and renders them capable of healing Leishmania major infection. Eur J Immunol *23*, 262-268.

Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., and Stockinger, B. (2006). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity *24*, 179-189.

Venge, P., Bystrom, J., Carlson, M., Hakansson, L., Karawacjzyk, M., Peterson, C., Seveus, L., and Trulson, A. (1999). Eosinophil cationic protein (ECP): molecular and biological properties and the use of ECP as a marker of eosinophil activation in disease. Clin Exp Allergy *29*, 1172-1186.

Vieira, O. V., Botelho, R. J., and Grinstein, S. (2002). Phagosome maturation: aging gracefully. Biochem J *366*, 689-704.

Vig, M., Srivastava, S., Kandpal, U., Sade, H., Lewis, V., Sarin, A., George, A., Bal, V., Durdik, J. M., and Rath, S. (2004). Inducible nitric oxide synthase in T cells regulates T cell death and immune memory. J Clin Invest *113*, 1734-1742.

Vilhardt, F., and van Deurs, B. (2004). The phagocyte NADPH oxidase depends on cholesterol-enriched membrane microdomains for assembly. Embo J *23*, 739-748.

von Stebut, E., Belkaid, Y., Jakob, T., Sacks, D. L., and Udey, M. C. (1998). Uptake of Leishmania major amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-Leishmania immunity. J Exp Med *188*, 1547-1552.

von Stebut, E., Belkaid, Y., Nguyen, B. V., Cushing, M., Sacks, D. L., and Udey, M. C. (2000). Leishmania major-infected murine langerhans cell-like dendritic cells from susceptible mice release IL-12 after infection and vaccinate against experimental cutaneous Leishmaniasis. Eur J Immunol *30*, 3498-3506.

Wakil, A. E., Wang, Z. E., Ryan, J. C., Fowell, D. J., and Locksley, R. M. (1998). Interferon gamma derived from CD4(+) T cells is sufficient to mediate T helper cell type 1 development. J Exp Med *188*, 1651-1656.

Wang, Z. E., Reiner, S. L., Hatam, F., Heinzel, F. P., Bouvier, J., Turck, C. W., and Locksley, R. M. (1993). Targeted activation of CD8 cells and infection of beta 2-

microglobulin-deficient mice fail to confirm a primary protective role for CD8 cells in experimental leishmaniasis. J Immunol *151*, 2077-2086.

Wang, Z. E., Reiner, S. L., Zheng, S., Dalton, D. K., and Locksley, R. M. (1994). CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with Leishmania major. J Exp Med *179*, 1367-1371.

Ward, D. M., Griffiths, G. M., Stinchcombe, J. C., and Kaplan, J. (2000). Analysis of the lysosomal storage disease Chediak-Higashi syndrome. Traffic *1*, 816-822.

Wei, X. Q., Charles, I. G., Smith, A., Ure, J., Feng, G. J., Huang, F. P., Xu, D., Muller, W., Moncada, S., and Liew, F. Y. (1995). Altered immune responses in mice lacking inducible nitric oxide synthase. Nature *375*, 408-411.

Weiner, H. L., Friedman, A., Miller, A., Khoury, S. J., al-Sabbagh, A., Santos, L., Sayegh, M., Nussenblatt, R. B., Trentham, D. E., and Hafler, D. A. (1994). Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. Annu Rev Immunol *12*, 809-837.

Weinheber, N., Wolfram, M., Harbecke, D., and Aebischer, T. (1998). Phagocytosis of Leishmania mexicana amastigotes by macrophages leads to a sustained suppression of IL-12 production. Eur J Immunol *28*, 2467-2477.

West, M. A., Wallin, R. P., Matthews, S. P., Svensson, H. G., Zaru, R., Ljunggren, H. G., Prescott, A. R., and Watts, C. (2004). Enhanced dendritic cell antigen capture via toll-like receptor-induced actin remodeling. Science *305*, 1153-1157.

Willenborg, D. O., Fordham, S., Bernard, C. C., Cowden, W. B., and Ramshaw, I. A. (1996). IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. J Immunol *157*, 3223-3227.

Wortis, H. H., Burkly, L., Hughes, D., Roschelle, S., and Waneck, G. (1982). Lack of mature B cells in nude mice with X-linked immune deficiency. J Exp Med *155*, 903-913.

Wu, W., Chen, Y., and Hazen, S. L. (1999). Eosinophil peroxidase nitrates protein tyrosyl residues. Implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. J Biol Chem *274*, 25933-25944.

Yamada, Y., Cancelas, J. A., Williams, D. A., and Rothenberg, M. E. (2007). IL-5 and the FIP1L1/PDGFRA Fusion Gene Cooperatively Promote Eosinophil Development, Activation, and Signal Transduction. Journal of Allergy and Clinical Immunology Program and Abstracts of Papers to be Presented During Scientific Sessions - 2007 AAAAI Annual Meeting, 2007 AAAAI Annual Meeting *119*, S214.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640-643.

Yang, J., Murphy, T. L., Ouyang, W., and Murphy, K. M. (1999). Induction of interferon-gamma production in Th1 CD4+ T cells: evidence for two distinct pathways for promoter activation. Eur J Immunol *29*, 548-555.

Yang, W. C., Ghiotto, M., Castellano, R., Collette, Y., Auphan, N., Nunes, J. A., and Olive, D. (2000). Role of Tec kinase in nuclear factor of activated T cells signaling. Int Immunol *12*, 1547-1552.

Yao, L., Kawakami, Y., and Kawakami, T. (1994). The Pleckstrin Homology Domain of Bruton Tyrosine Kinase Interacts with Protein Kinase C 10.1073/pnas.91.19.9175. PNAS *91*, 9175-9179.

Yu, C., Cantor, A. B., Yang, H., Browne, C., Wells, R. A., Fujiwara, Y., and Orkin, S. H. (2002). Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. J Exp Med *195*, 1387-1395.

Yu, C. Y., and Whitacre, C. C. (2004). Sex, MHC and complement C4 in autoimmune diseases. Trends Immunol *25*, 694-699.

Zaffran, Y., Zhang, L., and Ellner, J. J. (1998). Role of CR4 in Mycobacterium tuberculosis-human macrophages binding and signal transduction in the absence of serum. Infect Immun *66*, 4541-4544.

Zhang, Q., Calafat, J., Janssen, H., and Greenberg, S. (1999). ARF6 is required for growth factor- and rac-mediated membrane ruffling in macrophages at a stage distal to rac membrane targeting. Mol Cell Biol *19*, 8158-8168.

Zheng, L., Zomerdijk, T. P., Aarnoudse, C., van Furth, R., and Nibbering, P. H. (1995). Role of protein kinase C isozymes in Fc gamma receptor-mediated intracellular killing of Staphylococcus aureus by human monocytes. J Immunol *155*, 776-784.

Zheutlin, L. M., Ackerman, S. J., Gleich, G. J., and Thomas, L. L. (1984). Stimulation of basophil and rat mast cell histamine release by eosinophil granule-derived cationic proteins. J Immunol *133*, 2180-2185.

Zimmermann, N., Daugherty, B. L., Kavanaugh, J. L., El-Awar, F. Y., Moulton, E. A., and Rothenberg, M. E. (2000). Analysis of the CC chemokine receptor 3 gene reveals a complex 5' exon organization, a functional role for untranslated exon 1, and a broadly active promoter with eosinophil-selective elements. Blood *96*, 2346-2354.

Zimmermann, N., Hershey, G. K., Foster, P. S., and Rothenberg, M. E. (2003). Chemokines in asthma: cooperative interaction between chemokines and IL-13. J Allergy Clin Immunol *111*, 227-242.