Discussion
**Discussion**

- **Bruton's tyrosine kinase (BTK) in myeloid cells: Role in eosinophil lineage** 89-93
- **Role of the BTK-targeted transcription factor c-Rel in macrophage effector functions** 93-96
- **A role for BTK in T-B cell developmental cross-talk** 96-101
- **The role of lysosomal trafficking regulator LYST in myeloid cell functions** 101-107
- **The role of endogenous retroviral loci in controlling susceptibility to Lm infection** 107-115
Disease phenotypes are a manifestation of the eventual outcome of multiple interactions between 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
Discussion

observed. Macrophages from male and female mtce 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 erythematosus (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
Discussion

possible role for c-Rel in controlling myeloid cell effector functions was investigated using the c-Rel deficient (c-Rel<sup>−/−</sup>) mouse strain.

c-Rel<sup>−/−</sup> mice had normal differential and total leukocytes counts in peripheral blood (Fig. 3A&B). c-Rel<sup>−/−</sup> 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<sup>−/−</sup> macrophages was also seen to be normal as evidenced by similar levels of RNIs generated by c-Rel<sup>−/−</sup> and WT macrophages (Fig. 4A). Phagocytic ability of myeloid cells, monocytes and granulocytes from c-Rel<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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
Discussion

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-κB 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 isotypes (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 decrease 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
Discussion

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<sub>low</sub>IgG<sub>low</sub>) and the remaining (IgM<sub>low</sub>IgG<sub>high</sub>) showing IgG levels similar to that observed in XID and wild-
Discussion

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<sup>low</sup>IgG<sup>high</sup> 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<sup>low</sup>IgG<sup>high</sup> XID/nu mice had two-fold higher total splenic T cell numbers (Fig. 8B) as compared to the IgM<sup>low</sup>IgG<sup>low</sup> 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<sup>low</sup>IgG<sup>low</sup> XID/nu mice displayed very low splenic T and B cell frequency and cell yields, with a complete absence of mature IgD<sup>+</sup> B cells (Fig. 8A-lower panel). The B cell phenotype in the IgM<sup>low</sup>IgG<sup>high</sup> XID/nu mice was similar to that seen in CBA/N mice with an apparent immature-mature developmental block resulting in accumulation of IgM<sup>+</sup> B cells and reduced frequencies of IgD<sup>+</sup> 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 β⁻/⁺ 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 β⁻/⁻ mice analyzed. Remarkably low total splenic and lymph node yields at were observed in the XID/TCR β⁻/⁻ 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 β⁻/⁻ 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 possess 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 β−/− mice.

The data from the XID/TCR-β−/− 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
Discussion

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
Discussion

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 engulf 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
Discussion

to analyze the effect of such a defect on the clinical course of a leishmanial infection in 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 \textit{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 \textit{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-$\gamma$ 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-$\gamma$ induced nitrite generation, producing NO levels similar to that induced in wild-type BL/6 macrophages (Fig. 17A). IFN-$\gamma$ 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-$\gamma$ 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-$\gamma$ has also been reported to effect phagosome maturation. Virulent strains of intracellular pathogens like Lm and \textit{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-$\gamma$ treatment of cells before \textit{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
Discussion

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 (mtvs) 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 \textit{mtv-6} and \textit{mtv-7} in the CBA/J. On genetic intercross analysis, the (CBA/J x CBA/CaJ) F1 mice, which inherit both \textit{mtv-6} & \textit{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 \textit{mtv-7} integration, either alone or along with \textit{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 \textit{mtv-7} made lower levels of IFN-γ, as seen in parental CBA/J mice (Fig. 29B). These data implicate the \textit{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 \textit{mtv-null} mouse strain has been shown to be specifically more sensitive to infection by \textit{Vibrio cholerae} but not other pathogens (Bhadra et al., 2006). More particularly, the \textit{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 \textit{mtv-7} locus (Schirrmacher et al., 1998). Inheritance of \textit{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)
Discussion

and to chronic graft-versus-host disease (Gorham et al., 1996). In Lm infection, a subset of T cells expressing TCRβ4 TCRαβ 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 TCRβ4-deficient by MMTV (SIM) but not littermates rendered TCRβ6-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-SAgs-7-reactive TCRβ-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 TCRβ subset representation, since all of the TCRβ subsets tested, irrespective of v-SAgs reactivity, showed comparable frequencies of Lm-specific CD4 T cells (Fig. 30A).

It was also possible that the v-SAgs-7-reactive TCRβ 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 TCRβ-using CD4 T cell subpopulations in a given strain, irrespective of
Discussion

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.
Discussion

These data suggested that the presence of \textit{mtv-7} leads to a modulation of APC function during T cell activation, resulting in poor signals for IFN-$\gamma$ commitment during priming, and/or that the presence of \textit{mtv-7} during T cell development conditions them for poor IFN-$\gamma$ 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-$\gamma$ component of the T cell response, when immunized with a protein antigen in adjuvant (Fig. 34A&B). Thus, \textit{mtv-7} appears to provide a specific alteration in the commitment of Lm-specific T cells to IFN-$\gamma$ in the CBA mouse strain during Lm infection, probably in \textit{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-$\gamma$-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-$\gamma$ 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.