# Toll-like receptors and control of mycobacterial infection in mice

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*Abstract.* Microbial products including mycobacterial antigens are recognized by distinct Toll-like receptors (TLRs) resulting in activation of cells of the innate immune system. Ablation of most of the TLR signalling in mice deficient for the common adaptor protein MyD88 revealed that TLRs are crucial for the activation of an innate immune response as MyD88-deficient mice are highly sensitive to infection with *Mycobacterium tuberculosis*. Despite the profound defect of the innate immune response, MyD88 deficiency allows the emergence of an adaptive immunity. These data demonstrate that activation of multiple TLRs contributes to an efficient innate response to mycobacteria, while MyD88-dependent signalling is dispensable to generate adaptive immunity.

*2006 Innate immunity to pulmonary infection. Wiley, Chichester (Novartis Foundation Symposium 279) p 127–141*

Infectious tuberculosis due to *Mycobacterium tuberculosis* (*Mtb*), an intracellular pathogen capable of surviving and persisting within host mononuclear cells, represents a major global health challenge. A coordinated response of cells of the innate and adaptive immune system is required to control infection (Flynn & Chan 2001a, North & Jung 2004). Pathogen sequestration in macrophages within granulomas, dynamic structures containing activated lymphocytes and macrophages, contains the spread of infection. Gene targeted mice and neutralizing antibodies demonstrated critical mediators controlling *Mtb* infection, including interferon (IFN)γ, interleukin (IL)12, IL23, tumour necrosis factor (TNF), lymphotoxins, CD40 and nitric oxide (Flynn 2004, Cooper et al 2002, Ehlers et al 2003, Holscher et al 2001, Lazarevic et al 2003, Roach et al 2001, 1999, Garcia et al 2000, Jacobs et al 2000b). Mycobacteria have evolved to resist the eradication by macrophages by using elaborate evasion mechanisms (Flynn & Chan 2003). In a healthy host, a subclinical latent or chronic infection may persist (Gomez & McKinney 2004). Neutraliztion of IFNγ or TNF, inhibition of inducible nitric oxide synthase (iNOS), or



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T cell depletion leads to reactivation of latent infection (Chan et al 1995, Flynn et al 1998, Flynn & Chan 2001b, Mohan et al 2001, Scanga et al 1999, 2000, Botha & Ryffel 2003).

Persistent macrophage and T lymphocyte activation control the viable, but sequestered bacilli within phagocytes in the granuloma structure. We hypothesize that mycobacterial products released by the sequestered bacilli, such as glycolipids, lipomannan (LM), phosphatidyl-myo-inositol mannoside (PIM), lipoarabinomannan (LAM), lipoproteins and other mycobacterial factors, may contribute to continued macrophage and dendritic cell activation through pathogen pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and others.

Mammalian TLRs represent a structurally conserved family of membrane receptors, which have homology to the *Drosophila* Toll system (Medzhitov et al 1997, Akira & Takeda 2004). Microbial products activate mammalian TLRs inducing gene transcription regulating the adaptive immune response, resulting in chemokines, cytokines and costimulatory molecules. The TLR family now consists of 11 members, with TLR11 being discovered to be critical in the control of uropathogenic bacteria (Zhang et al 2004, Quesniaux & Ryffel 2004). The greatest variety of TLR mRNAs is found in professional phagocytes, suggesting a key role of TLRs in innate immunity. The main ligands for the individual TLRs are shown in Fig. 1.

TLRs are type I membrane proteins containing an extracellular domain with 19–25 tandem copies of leucine-rich repeats (LRRs) and a cytoplasmic Toll/IL1 receptor (TIR) domain similar to that of the IL1 receptor family (Takeda & Akira 2004, Akira & Takeda 2004). TLR signal transduction is mediated by binding of the adaptor protein MyD88 to the TIR domain of TLRs, followed by the recruitment of IL1 receptor associated kinases (IRAKs), TNF receptor associated factor (TRAF) 6, TGFβ-activated protein kinase 1 (TAK1), mitogen-activated protein (MAP) kinase and NF-κB activation (Akira et al 2003, Takeda & Akira 2004). Mal/TIRAP participates in signalling of TLR2 and TLR4, together with MyD88 (Horng et al 2002, Yamamoto et al 2002a, O'Neill et al 2003). TIR domain-containing adaptor inducing IFNβ (TRIF), also known as TIR-containing adaptor molecule 1 (TICAM1) or Lps2, has been identified (Yamamoto et al 2003, 2002b, Oshiumi et al 2003). TRIF is particularly important for interferon regulatory factor 3 (IRF3) activation mediated by viral-induced TLR3 engagement, but it is also involved in the TLR4 MyD88-independent activation of costimulatory molecules CD40 and CD86, through an IFNβ autocrine/paracrine loop (Hoebe et al 2003, Yamamoto et al 2003, 2002b). Pathogen binding to specific TLRs or to combinations of TLRs may recruit different adaptor proteins allowing a specific signalling cascade and gene activation programmes. The serine/threonine kinase family consists of two active kinases, IRAK and IRAK4, and two inactive or inhibitory kinases, IRAK2 and IRAKM (also named IRAK3).

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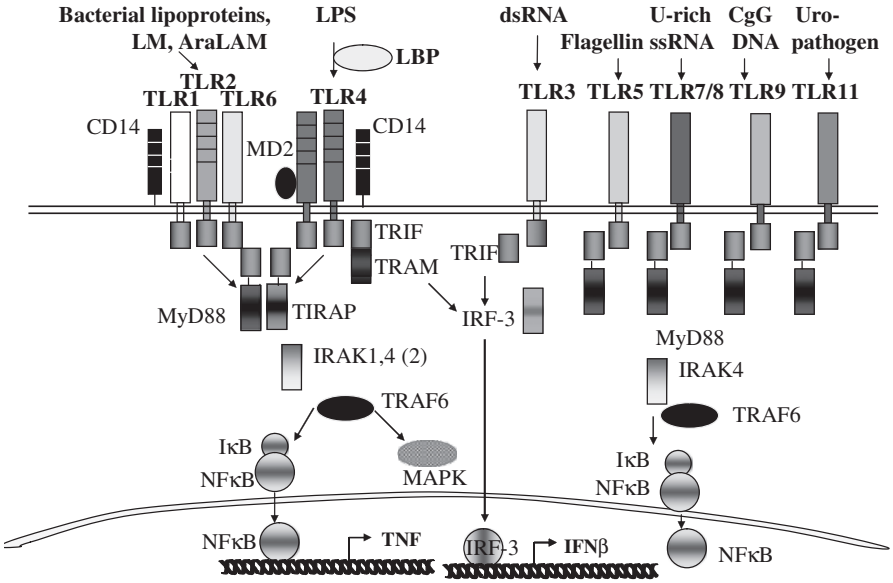


FIG. 1. Microbial ligands and association with known TLRs and adaptor molecules. Schematic representation of the structure of TLRs and the major TLR ligands. Most TLRs form homodimers, while TLR2 associates with either TLR1 or TLR6. TLR signalling is mediated through adaptors such as MyD88, TIRAP, TRIF or TRAM (Reproduced from Ryffel et al 2005, with permission).

This review is aimed at discussing current knowledge about the interaction of mycobacterial ligands or mycobacteria such as *Mtb* with TLRs and their role in controlling mycobacterial infection in gene deficient mice.

## Mycobacterial TLR ligands and responses *in vitro*

The mycobacteria cell wall is composed of different glyocolipids such as LAM, mycolic acid, lipopeptides and phosphoinositol, which may be recognized by the immune system (Chatterjee & Khoo 1998, Daffe & Draper 1998, Nigou et al 2002). So far, TLR2, TLR4 and TLR1/TLR6 that heterodimerize with TLR2, have been implicated in the recognition of mycobacterial antigens (Bulut et al 2001, Hajjar et al 2001).

TLR2-dependent cell activation has been described for LAM from rapidly growing mycobacteria, lipomannan (LM), PIM (phosphatidyl-*myo*-inositol mannoside), or the 19 kDa mycobacterial lipoprotein (Aliprantis et al 1999, Brightbill et al 1999, Means et al 1999a, Jones et al 2001b, Gilleron et al 2003, Barnes et al 1992, Vignal et al 2003, Quesniaux et al 2004b). LMs, the biosynthetic precursors

of LAMs, represent another class of abundant pro-inflammatory molecules of the mycobacterial cell wall. We showed that LMs from various mycobacterial origins are potent activators of proinflammatory cytokines in macrophages requiring TLR2 signalling (Quesniaux et al 2004b). LM induces CD40 and CD86 cell surface expression, abundant cytokine expression and nitric oxide production (Vignal et al 2003, Quesniaux et al 2004b). PIMs, the anchor motifs of LM and LAM, have pro-inflammatory activities (Jones et al 2001a). Dimannoside ($PIM_2$) and hexamannoside ($PIM_6$), the two most abundant classes of PIM found in *M. bovis* BCG and *Mtb* H37Rv, were recently shown to activate macrophages to secrete TNF through TLR2, irrespective of their acylation pattern, and to signal through MyD88 (Gilleron et al 2003). Based on the present knowledge the balance between PIM, LM and LAM synthesis by pathogenic mycobacteria might provide pro- or anti-inflammatory immunomodulatory signals during primary infection, but also during latent infection.

Mycobacterial lipoproteins were shown to activate antigen-presenting cells (APC) through TLR2 signalling (Underhill et al 1999, Brightbill et al 1999). Recent reports further suggest that the 19 kDa lipoprotein, LpqH (Rv3763) has also TLR2-dependent inhibitory functions on IFNγ regulated responses, including MHC class II antigen processing in macrophages (Noss et al 2000, 2001, Pai et al 2003, Gehring et al 2003). Further, the 24 kDa lipoprotein, LprG (Rv1411c) also appears to inhibit in MHC-II antigen processing and hence $CD4^+$ T cell activation, although short-exposure induces TLR2-dependent TNF production (Gehring et al 2004). Furthermore, a phenolic glycolipid (PGL) from a virulent *Mtb* strain has been shown to inhibit innate immune responses (Reed et al 2004). A more detailed discussion of mycobacterial ligands, their receptor specificity and biological properties is given elsewhere (Quesniaux et al 2004b, 2004a).

Viable and killed *Mtb* bacilli (virulent and attenuated) activate CHO cells and murine macrophages that express either TLR2 or TLR4 (Means et al 1999b). Macrophages expressing a dominant-negative mutant for MyD88 failed to react to mycobacteria, underlining the requirement of TLRs mediating the downstream signalling cascade responsible for the transcription of TNF. Using bone marrow-derived macrophages derived from TLR2 and/or TLR4 deficient mice, we showed TLR2- and to a lesser extent TLR4-dependent activation of TNF and IL12 production after infection with live *M. bovis* BCG (Fremond et al 2003, Nicolle et al 2004b), which is completely abrogated in MyD88 deficient macrophages (Nicolle et al 2004a). However, recognition of heat-killed *M. bovis* BCG, extensive-freeze-dried *M. bovis* BCG, a soluble fraction of *M. bovis* BCG culture supernatant, or a 'well dispersed' live *M. bovis* BCG cultivated in the presence of detergent, was predominantly mediated through TLR2, as essentially no response remained in TLR2-deficient macrophages or dendritic cells (Nicolle et al 2004b). Neither TLR1 nor TLR6 signalling on their own are critical for mycobacteria macrophage

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activation induced by these mycobacterial preparations as TLR1- or TLR6-deficient macrophages respond normally suggesting a potential compensation of TLR1/6 (unpublished). Further, mycobacteria induced nitric oxide production, a potent antimycobacterial effector molecule, in primary macrophages in a TLR2-dependent way, in contrast with previous reports (Means et al 2001). Recent work demonstrated that MyD88 is crucial for macrophages to acquire a normal IFNγ response (Ehrt et al 2001). Interestingly, the expression of CD40, CD80 and CD86 on macrophages and dendritic cells was not affected by the absence of single TLRs or the TLR signalling adaptor protein MyD88, suggesting a normal costimulation of T cells (Nicolle et al 2004a, Fremond et al 2004).

In summary, these results suggest that purified mycobacterial antigens and whole bacilli preferentially interact with TLR2 and TLR4, possibly in combination with additional TLRs and PRRs, leading to MyD88-dependent activation of antibacterial effector pathways.

## Role of TLR *in vivo* infection with *Mycobacterium bovis* BCG

To test the role of TLR signalling *in vivo* in controlling mycobacterial infection we have performed studies in TLR gene deficient mice.

Infection with *Mycobacterium bovis* (BCG) caused persistent inflammation in C3H/HeJ TLR4 mutant mice, and therefore TLR4 is not required to control acute BCG infection, but may have a function for the fine tuning of inflammation in chronic infection (Fremond et al 2003). At high infectious dose bacterial clearance and reduced IFNγ secretion was reported in TLR deficient mice (Heldwein et al 2003). Further, BCG infection resolved in the chronic phase in TLR2-deficient mice (Nicolle et al 2004b). Interestingly, the adaptive response of TLR2- and/or TLR4-deficient mice seemed essentially normal on day 14 or 56 after infection, as T cells responded normally to soluble BCG antigens unlike previously reported (Heldwein et al 2003). In conclusion, our data demonstrate that TLR2, TLR4 or TLR6 are redundant for the control of *M. bovis* BCG mycobacterial infection.

To assess the role of a global TLR signalling in host response to mycobacterial infection, we infected mice deficient in the TLR adaptor molecule myeloid differentiation factor 88 (MyD88) with the vaccine strain *M. bovis* BCG, and the immune response and bacterial burden were investigated. BCG ($2 \times 10^6$ CFU i.v.) infected MyD88-deficient mice had increased lung weights at 8 months with confluent chronic pneumonia and two log higher CFU in the lung than wild-type mice (Nicolle et al 2004a), while the infection was controlled in liver and spleen and there was efficient systemic T cell priming with high IFNγ production by $CD4^+$ splenic T cells in MyD88-deficient mice. Lung infiltrating cells showed IFNγ production by pulmonary $CD4^+$ T cells upon specific restimulation, and a reduced capacity to produce nitric oxide and IL10. In summary, despite the dramatic

reduction of the innate immune response, MyD88-deficient mice were able to mount an efficient T cell response to mycobacterial antigens, which was however insufficient to control infection in the lung, resulting in chronic pneumonia in MyD88 deficient mice (Nicolle et al 2004b).

These results are surprising as BCG was cleared from all organs, but was capable of surviving in the lung in the absence of TLR–MyD88 signalling. However, as the absence of single TLRs had only a minor effect *in vivo* in response to the attenuated *M. bovis* BCG vaccine strain, we tested a virulent *Mtb* strain.

## Critical role of TLR signalling in *Mtb* infection

Using single TLR-deficient mice we investigated their susceptibility to low dose aerosol infection with virulent *Mtb* H37Rv. TLR4 deficient mice displayed reduced bacterial clearance during a long-term infection protocol and developed a chronic pneumonia and died within 15 weeks (Abel et al 2002). The data were confirmed recently by an independent group (Branger et al 2004). In short-term infections, no significant differences in the inflammatory response or the bacterial burden in infected organs during the first 50 days of infection and long-term were found (Reiling et al 2002, Shim et al 2003, Kamath et al 2003). CD14, a coreceptor of TLR4, appears not to be involved in host resistance, as CD14-deficient mice clear the infection normally (Reiling et al 2002) (M. Jacobs, unpublished data).

Then we investigated the role of TLR2 in the host response and infected TLR2-deficient mice by aerosol using 500 CFU. TLR2-deficient mice initially control an aerosol infection with signs of T cell activation, but develop increased bacterial burden and chronic pneumonia with death in 5 months (Drennan et al 2004). Although inflammatory cells such as macrophages and activated T cells are recruited, no distinct granulomas are formed in TLR2-deficient mice. Inflammation in the presence of a high bacterial load is associated with increased TNFα, IL12p40 and IFNγ production in the lung (Drennan et al 2004). Therefore, the data suggest that TLR2 may function as a regulator of inflammation, and in its absence an exaggerated immune-inflammatory response develops. By contrast, others found only a minor role for TLR2 in the control of *Mtb* infection (Reiling et al 2002, Sugawara et al 2003).

Since TLR2 forms heterodimers with either TLR6 or TLR1, we asked whether the co-receptors are involved in mycobacterial responses. TLR6-deficient mice are resistant to high *Mtb* aerosol infection (Gomes et al 1999), and no data are so far available on the role of TLR1 signalling in the *in vivo* host response. Since at least TLR2 and TLR4 are modulating host resistance to mycobacterial infection, we asked whether inactivation of both TLR2 and TLR4 augment the susceptibility to *Mtb* infection, which is indeed the case (Fremond et al unpublished results 2006).

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Finally, we investigated the role of the common TLR adaptor molecule MyD88. We infected MyD88-deficient mice with *Mtb* (Fremond et al 2004). Aerogenic infection of MyD88-deficient mice with *Mtb* was lethal within 4 weeks as shown for TNF-deficient mice. Mice succumbed to acute necrotic pneumonia with 2 log higher CFU in the lung (Fig. 2). This was associated with high pulmonary levels of cytokines and chemokines, and acute, necrotic pneumonia, despite a normal T cell response with IFNγ production to mycobacterial antigens upon *ex vivo* restimulation. The phenotype resembles that of TNF deficiency (Flynn et al 1995, Jacobs et al 2000a). Fatal infection in MyD88-deficient mice is not surprising in view of their profound defect of innate immunity (Shi et al 2003, Scanga et al 2004).

We then asked whether MyD88 deficient mice are able to develop an adaptive immune response. In view of the fact that MyD88 deficient macrophages upregulated costimulatory molecules such as CD40 and CD86 (Nicolle et al 2004a), we investigated the response to BCG vaccination. We found comparable antigen

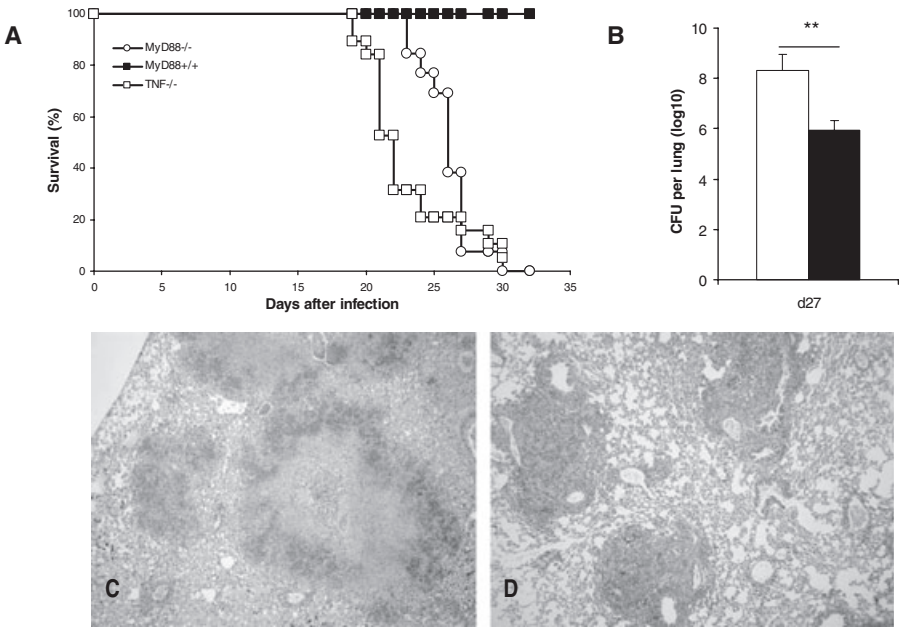


FIG. 2. MyD88-deficient mice are unable to clear virulent *M. tuberculosis* H37Rv and succumb to infection with acute necrotic pneumonia. Survival of MyD88- and TNF-deficient mice as compared with controls. (A) Mycobacterial burden (CFO) in the lung (B) and lung pathology: Acute necrotic pneumonia MyD88 deficient mice (C) and typical granulomas in wild-type mice (D). Wild-type, MyD88- and TNF-deficient mice were infected with *M. tuberculosis* H37Rv (200 CFUin. and sacrificed at 27 days (modified from Fremond et al 2004).

specific responses with IFNγ production in the absence of MyD88 as in wild-type mice.

Furthermore, prior BCG vaccination conferred a substantial protection in vaccinated MyD88$^{-/-}$ mice from acute *Mtb* infection with 2 log reduced mycobacterial load (CFU) in the lung as compared to non-vaccinated mice (Fremond et al 2004). These data demonstrate that MyD88 signalling is dispensable to raise an acquired immune response, which however is not sufficient to compensate the profound innate immune defect of MyD88-deficient mice to control *Mtb* infection.

In conclusion, the present *in vivo* evidence suggests that signalling through single TLRs has only a modest effect in acute mycobacterial infection, while abrogation of most of TLR signalling as found in MyD88-deficient mice results in profound deficiency of innate immunity with preserved adaptive immunity.

## Role of other pattern recognition receptors in the control of mycobacterial infection

Recognition of pathogens is however not limited to TLRs. TLR-unrelated receptors such as the CD14, scavenger and complement receptors, pulmonary surfactant protein A, DC-SIGN (DC-specific intercellular adhesion molecule-3 grabbing nonintegrin), CD40 and CD44 have been implicated in recognition of mycobacterial antigens and coupling a cellular response. The evidence will be discussed briefly.

Nucleotide-binding oligomerization domain proteins (NOD) belong to a TLR related protein family with leucine-rich repeats which likely have a role in the intracellular recognition of pathogen ligands such as peptidoclycans, muramyl dipeptides and diaminopimelate-containing *N*-acetylglucosamine-*N*-acetylmuramic acid (GlcNAc-MurNAc) tripeptide (Inohara & Nunez 2003, Chamaillard et al 2003, Girardin et al 2003a). NOD2 has been linked to the inflammatory bowel disorder Crohn's disease (Girardin et al 2003b). *Mycobacterium avium* subspecies paratuberculosis (MAP) is presently the most favourite pathogen linked to Crohn's disease (Greenstein 2003). Except for the circumstantial evidence for MAP, the role of NOD proteins in response to mycobacterial antigen is so far unknown.

## Conclusion

The present results support the emerging paradigm that at least TLR2 and TLR4 play a role in sensing mycobacteria and mounting an antimycobacterial immune response *in vitro* and *in vivo*. In future, the repertoire of identified mycobacterial antigens which mediate TLR signalling is likely to continue to increase. The combinatorial recognition of pathogen-associated molecular patterns by more than one TLR, and cross-talk between TLRs and other PRRs opens a new dimension

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(Underhill 2003). A more detailed knowledge of the stimulatory and inhibitory ligands of TLRs and PRRs might allow (Janeway & Medzhitov 2002) modulation of the immune response to mycobacteria. In summary, the common IL1R TLR adaptor molecule MyD88 is critical to develop a robust host response to *Mtb* infection, and the profound defect of innate immunity is not compensated by other molecules. Interestingly, MyD88 deficiency allows the emergence of an adaptive immunity. Therefore IL1R TLRs contribute to an efficient innate response to mycobacteria, while MyD88-dependent signalling is dispensable for adaptive immunity.

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## DISCUSSION

*Speert:* A similar thing has been found with *Pseudomonas* in cystic fibrosis. The state of acylation of LPS influences the extent of inflammation in human cells but not in murine cells. Does this hold for both mouse and human? Second, if you follow the organism during the course of infection from acute to chronic phase, does the degree of acylation change?

*Quesniaux:* It's difficult to say in the course of the infection, to ask the biochemists to purify the LM/LAM forms from murine tissues in well controlled mouse infections. We hope to do this in bovine models.

*Speert:* Were your data purely from murine studies?

*Quesinaux:* Yes.

*Latgé:* This reminds me of the antigenic variation in parasites.

*Wilkinson:* I am interested to see you describe the pneumonia that occurs in both MyD88- and TNF-deficient mice as necrotic. Is it truly necrotic, or an alveolitis?

*Ryffel:* It is clearly an acute pneumonia with confluent necrosis reminding of caseation, which we find in MyD88, TNF and lymphotoxin knockout mice which develop uncontrolled infection, with extensive and necrotic pneumonia (Jacobs et al 2002, Fremond et al 2004, 2005).

*Wilkinson:* Necrosis in TB has been ascribed classically to TNF. So what drives the necrosis in TNF-deficient mice?

*Ryffel:* That's a difficult question. If we look at macrophage *in vitro* there is no proinflammatory response. TNF and other cytokines have alternative pathways. At 4 weeks of infection cytokine responses are correlated with bacterial load. It is possible that there is a combination of interferon, TNF and IL12 which may cause necrosis. I don't think we can pinpoint TNF only in this process.

*Gordon:* Is there apoptosis in the mouse model?

*Ryffel:* Yes, along with necrosis there is apoptosis as shown by caspase staining.

*Steinman:* What do you know about the resistance of the MyD88 knockout mice that are vaccinated with BCG? Do you know that this is $CD4^+$ T cell dependent? Have you looked if innate lymphocytes are responsible?

*Ryffel:* That is an important question. We haven't done any transfer experiments, but we should do. The fact that we find $CD4^+$ cells expressing IFNγ *ex vivo* is not sufficient.

*Steinman:* People are looking for a role for the CD1-dependent presentation of glycolipids. Perhaps this will be seen in a MyD88-deficient background.

*Gordon:* What do those people more interested in the human disease think about this? Is this relevant to anything? Has anyone looked at Toll-like receptors (TLR) polymorphisms in susceptibility, and what is the outcome?

*Wilkinson:* There are some data but the associations are moderate.

*Quesniaux:* There are some published data on TLR2 polymorphisms: R753Q is associated with higher predisposition for *Mtb* and Staphylococci infections while R667W seems associated with increased *Mtb* and *M. leprae* infections (Cook et al 2004).

*Segal:* There is a linkage of TLR4 to Crohn's disease, particularly of the large bowel (Franchimont et al 2004). I think it is a secondary involvement.

*Gordon:* Ultimately, there will be quite a lot of these claims. What does one make of them?

*Speert:* The issue of looking at human cells as regards their response to the degree of acylation of *Pseudomonas aeruginosa* is interesting, as inflammation is dramatically different between human and murine cells. During chronic infection in cystic fibrosis the degree of acylation increases, and there is no change on the effects on the mouse, but the effect on human cells is dramatic.

*Quesniaux:* The proinflammatory effect has also been looked at in a human monocytic THP1 cell line.

*Didierlaurent:* In the MyD88 knockout mouse there are some defects in cell proliferation in the gut. Have you looked at the basic function of the lungs in these mice?

*Ryffel:* I don't see a defect, but any proinflammatory response is dramatically reduced. In the gut a critical role of MyD88 in controlling commensal bacteria entry has been demonstrated (Rakoff-Nahoum et al 2004). The lung function in the absence of MyD88 appears normal, and the response to inhaled endotoxin is completely ablated (Noulin et al 2005).

*Gordon:* So the idea is coming out that the TLRs are necessary for integrity of epithelia in the gut and perhaps the lung also. There's a recent paper in *Nature Medicine* on this (Jiang et al 2005). I don't know how you assay for this easily.

*Didierlaurent:* The infection is long-term so the absence of TLR signalling may have some effect on lung repair mechanisms occurring after the initial inflammatory response.

*Ryffel:* The way we plan to address this is by using Cre-Lox for MyD88 to inactivate it in the lung.

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