Toll-like receptors contribute to host responses against mycobacterial infection

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ABSTRACT

Mammalian toll-like receptor (TLR) proteins are pattern recognition receptors that mediate cellular activation by a wide variety of bacterial products. TLR activation leads to the expression of numerous mediators of innate immunity. We used the nonpathogenic mycobacterium *M. bovis* BCG (BCG) to define the roles of TLR proteins in the normal development of an immune response against the bacilli. We found that TLR2$^{-/-}$ mice were unable to effectively control the growth of BCG *in vivo*, compared with normal and TLR4$^{-/-}$ mice. Furthermore, splenic T cells isolated from infected TLR4$^{-/-}$ mice could proliferate *in vitro* following antigen challenge, but were unable to generate a strong Th1-type response. In contrast, splenic T cells isolated from infected TLR2$^{-/-}$ mice could neither proliferate *in vitro* following antigen challenge nor generate a strong Th1-type response. Together, these studies indicate that TLR proteins participate in the development of both innate and adaptive immune responses.

INTRODUCTION

Infection of macrophages with mycobacteria is accompanied by activation of the transcription factor NF-κB, secretion of inflammatory mediators (e.g. TNF-α, IL-1b), release of the reactive nitrogen intermediate nitric oxide (NO), and secretion of several chemokines.[1] Until recently, the signalling pathways that elicit the production of these mediators have remained unknown. Members of the mammalian Toll-like receptor (TLR) family have been implicated in the activation of macrophages by a variety of chemically diverse bacterial products.[2,3] We previously demonstrated that viable *M. tuberculosis* (Mtb) bacilli contain distinct ligands that activate cells via TLR2 and TLR4, whereas heat-killed Mtb failed to activate cells via TLR4.[4] In contrast, *M. avium* appears to lack any TLR4 agonists.[5] Several purified mycobacterial products have now been identified as TLR2 agonists, including arabinose-capped lipoarabinomannan,[6] dimannosylated phosphatidylinosito,[7] and the 19 kDa lipoprotein antigen.[8] The identity of the Mtb TLR4 agonist remains unknown. Interestingly, TLR2 agonists activate macrophages to express only some of target genes activated by TLR4 agonists.[7,9] This difference is due, at least in part, to the capacity of TLR4 agonists, but not TLR2 agonists, to induce β-interferon (IFN-β) expression. IFN-β acts in an autocrine/paracrine manner to activate the transcription factor STAT1.[10] TLR4-dependent STAT1 activation, in combination with NF-κB, results in the expression of several genes that mediate host responses against bacterial pathogens. These include iNOS, IL-12p40, IL-6, and several chemokines.[10]

Given the spread of drug-resistant Mtb strains, there is a pressing need to develop treatments that augment host innate immunity rather than to rely on new antibiotics. One novel approach would be to develop therapeutics that antagonise TLR proteins. This has been accomplished for one TLR4 agonist, Gram-negative bacterial lipopolysaccharide (LPS) and its pharmacophore lipid A. Three lipid A analogues, lipid IVA, *Rhodobacter sphaeroides* lipid A (RSLA) and E5531 have all been reported to function as LPS antagonists when tested both *in vitro* and *in vivo*.[11-13] We subsequently demonstrated that RSLA could also block signalling by a TLR2 agonist, the mycobacterial glycolipid lipoarabinomannan.[14] Together, these data suggest that certain lipid A structural antagonists are capable of blocking TLR-dependent activation by molecules that are chemically dissimilar to LPS. We later showed that the synthetic lipid

A-like antagonist E5531 could block TLR4-dependent signalling induced by Mtb. E5531 could inhibit selected Mtb-induced macrophage responses, namely apoptosis and TNF-α secretion, but it did not block Mtb-induced NO production.[15] Subsequent studies revealed that induction of NO production by Mtb was mediated by a TLR-independent mechanism[15] (and unpublished observations). This underscores the concept that while purified bacterial TLR agonists can activate macrophages to express a variety of proinflammatory mediators *in vitro*, this is not predictive of TLR-dependent macrophage activation by intact bacteria. Here we have sought to determine the relative contributions of both TLR2 and TLR4 to innate and adaptive immune responses. In these studies we have used the nonpathogenic mycobacterium BCG to investigate the role of TLR proteins in host immune defence *in vivo*. We reasoned that the use of BCG would allow us to examine the contributions of TLR proteins to the normal development of host immunity in the absence of mycobacterial virulence factors that corrupt the normal development of these host responses.

METHODS AND MATERIALS

Reagents

M. bovis BCG (ATCC 35734) was purchased from the ATCC. Bacteria were grown in LPS-free Middlebrook 7H9 liquid medium, supplemented with AODC, Tween 80, and glycerol. Cultures were grown to a density of 0.5 to 0.6 (OD_{620}). Numbers of BCG per ml of culture were determined by colony counting.

Animals and cells

$TLR2^{-/-}$ and $TLR4^{-/-}$ mice (female, 5 to 7 weeks old) were provided by Dr Shuzio Akira (Osaka University), and have been previously described.[16] These mice had been previously backbred onto a C57Bl/6 background for four generations prior to their use in these studies. Inbred C57Bl/6 mice were used as control animals. Thioglycollate-elicited peritoneal exudate macrophages were isolated from uninfected mice, and cultured *in vitro*, as we have previously described.[6] Each mouse was infected with 10^6 CFU of *M. bovis* BCG by intraperitoneal injection, and sacrificed 14 days later. Spleens were removed from the infected mice, and total splenocytes were prepared for culture *in vitro*, as previously described. Contaminating LPS levels in all media components were <10 pg/ml final concentration as measured by *Limulus* amebocyte lysate kit (BioWhittaker).

Measurements of cytokine production by macrophages *in vitro*

Peritoneal macrophages from $TLR2^{-/-}$, $TLR4^{-/-}$ and normal mice were infected *in vitro* (5 BCG per macrophage) with live BCG for 18 hours. The culture supernatants were removed, filtered to remove any BCG, and then cytokine levels were measured using specific ELISA kits, as recommended by the manufacturer (R&D Systems and Pharmingen).

Measurement of bacterial loads in the lung

Lungs removed from infected mice were homogenised in a sterile blender, using a lysis buffer consisting of sterile water containing 0.025% SDS. Homogenates were diluted in the lysis buffer, and 1 ml aliquots were cultured on Middlebrook 7H11 agar plates supplemented with glycerol, L-arginine and cycloheximide (10 mg/ml). Colonies were counted 14 days later.

Splenic T cell restimulation assay

Spleens were recovered from uninfected and BCG-infected mice 14 days after inoculation. Total splenocytes were obtained by tissue disruption between sterile frosted glass slides, and erythrocytes were removed by lysis in Tris-buffered ammonium chloride (Sigma). Splenocytes were then cultured in 96 well plates (5×10^5 cells/well) in the presence or absence of heat-killed BCG (10^3 CFU/well). For proliferation measurements, splenocytes were cultured for two additional days and pulsed with ^3H thymidine (1 mCi/well) eight hours prior to harvesting. Cells were harvested using an automated cell harvester (Skatron Instruments) and incorporation of radiolabelled thymidine was measured by scintillation counting. For cytokine secretion, splenocytes were cultured for three additional days. Culture supernatants were then recovered and specific cytokine levels measured by ELISA.

RESULTS AND DISCUSSION

TLR proteins are necessary for specific macrophage responses to BCG *in vitro*

Peritoneal macrophages from $TLR2^{-/-}$, $TLR4^{-/-}$ and normal mice were infected *in vitro* with live BCG for 18 hours. Cytokine levels in the culture supernatants were measured using ELISA and the Greiss assay respectively. As shown in *figure 1*, BCG-induced TNF-α and IL-6 secretion was substantially lower in the $TLR2^{-/-}$ macrophages compared with both $TLR4^{-/-}$ and normal macrophages. In contrast, secretion of IL-12p70 was similar in BCG-stimulated macrophages from all three mouse strains examined. This suggests that TLR2, but not TLR4, is necessary for BCG-induced TNF-α and IL-6 production, whereas IL-12 production does not depend on these TLR proteins. In these experiments, TNF-α production was strongly dependent on the presence of TLR2. Numerous investigators have shown that TNF-α expression in macrophages can be induced using purified TLR2 agonists.[6,17-19] Intact bacteria,

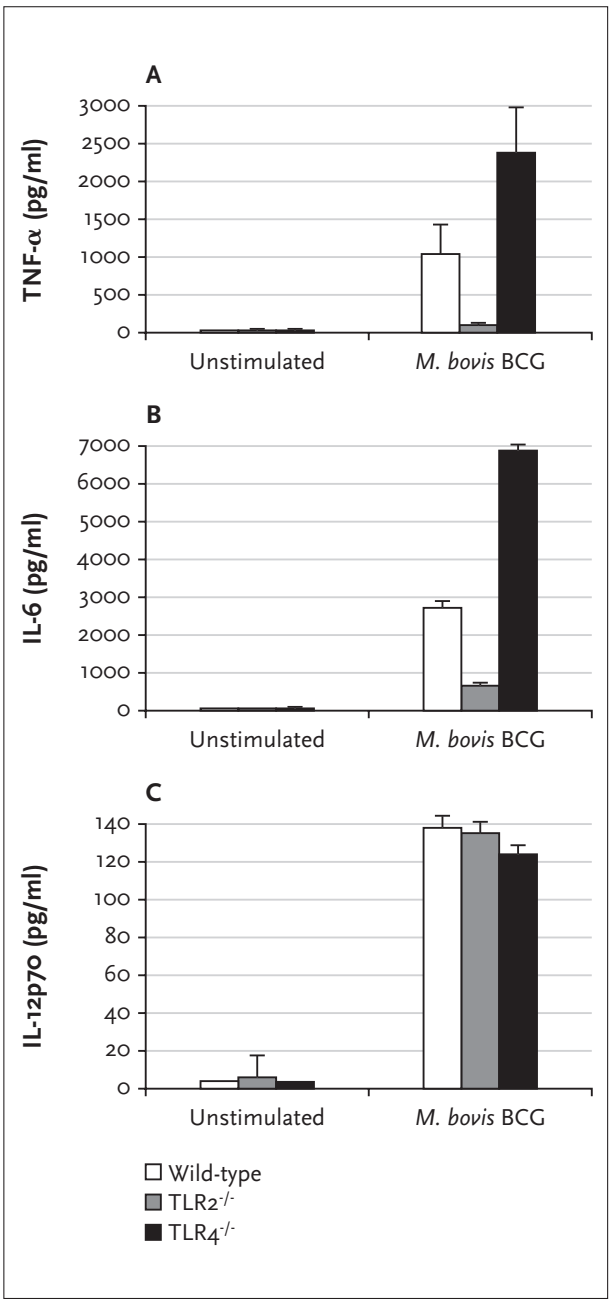

Figure 1
Cytokine production by normal and TLR-deficient macrophages stimulated with BCG

Thioglycollate-elicited peritoneal macrophages (5 x 10^5 cells/well) from wild-type, $TLR2^{-/-}$ and $TLR4^{-/-}$ mice were stimulated with live BCG (five bacilli/macrophage) for 24 hours, and supernatants assayed for TNF-α (A), IL-6 (B) and IL-12p70 (C) by quantitative ELISA. Data are presented as the mean values of triplicate wells ± SD. Results are representative of duplicate experiments.

such as Gram-negative organisms and Mtb, possess both TLR2 and TLR4 agonists, although it is likely that the relative expression of these agonists can vary greatly. Thus, the relative levels of TLR2 and TLR4 agonists expressed by different mycobacterial species are likely to dictate which TLR protein is most necessary for the induction of TNF-α expression. We had previously shown that Mtb possesses both TLR2 and TLR4 agonists, and that blocking TLR4 signalling with the lipid A-like LPS antagonist E5531 largely blocked TNF-α secretion induced by Mtb.[15] In contrast *M. avium* does not appear to be capable of activating cells via TLR4.[5] It has been reported that BCG possess both TLR2 and TLR4 agonists,[20] but the relative abundance of these agonists had not been determined. We hypothesise that BCG express lower levels (or fewer types) of TLR4 agonists than Mtb. Similarly, BCG may express higher levels (or more types) of TLR2 agonists, compared with Mtb. In either case, this could explain why TLR4 is not necessary for macrophage activation by BCG. In contrast to TNF-α and IL-6, IL-12 secretion by BCG-stimulated macrophages did not depend on the presence of TLR2 or TLR4. This finding was unexpected, but is reminiscent of Mtb-induced NO production, which is independent of TLR proteins.[15] It remains to be formally determined whether BCG-induced IL-12 production, like NO production, is also mediated by a TLR-independent mechanism.

TLR2 is necessary for resistance to mycobacterial infection *in vivo*

$TLR2^{-/-}$, $TLR4^{-/-}$ and normal mice were infected with live BCG for 14 days. The lungs were then removed and the number of bacteria in the tissues was counted. As shown in *figure 2*, the lungs from infected $TLR2^{-/-}$ mice contained more BCG bacilli than lungs from $TLR4^{-/-}$ and control mice.

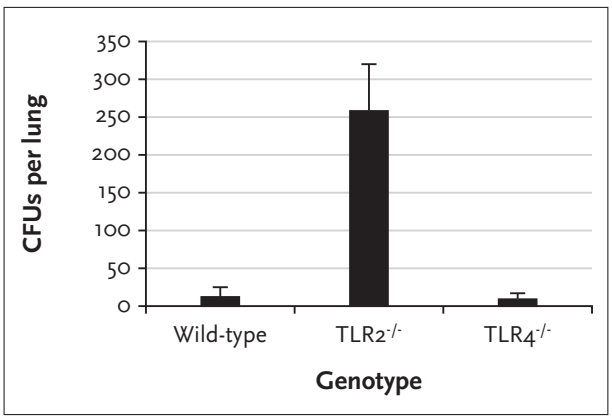

Figure 2
Bacterial loads in the lungs of normal and TLR-deficient mice infected with BCG

Normal and TLR-deficient mice were infected intraperitoneally with 10^6 CFU *M. bovis* BCG, and the lungs were harvested 14 days later. Lung homogenates were then prepared, diluted, and cultured on Middlebrook 7H11 agar plates for colony counting. Data are presented as mean of lung CFUs from three mice per genotype ± SEM. Results are representative of duplicate experiments.

Both normal and TLR4-deficient mice were fully capable of controlling the infection. Thus, the absence of TLR2 led to permissive growth of BCG in the lung. This finding is consistent with the inability of TLR2$^{-/-}$ macrophages to secrete substantial amounts of TNF-α following BCG challenge *in vitro* (*figure 1*). Studies performed using TNF-α$^{-/-}$ mice have demonstrated the importance of TNF-α in controlling mycobacterial growth *in vivo*.[21] It remains to be determined whether diminished production of TNF-α is responsible for the lack of mycobacterial growth control in the TLR2$^{-/-}$ mice. These data do indicate that TLR2 is necessary to generate an effective host response against mycobacterial infection. These findings do not completely exclude a role for TLR4 in the host response against mycobacterial infection. Our studies shown here used a background mouse strain that is genetically resistant to mycobacterial infection (i.e. C57Bl/6), as well as a relatively low inoculum of BCG. Preliminary studies performed using a mouse strain that is genetically sensitive to mycobacterial infection and lacks functional TLR4 (i.e. C3H/HeJ mice) have revealed that TLR4 does indeed contribute to the resistance to BCG infection *in vivo* (SN Vogel and MJF, unpublished observations). Furthermore, this protective role for TLR4 was only observed using a higher inoculum of BCG (>10^7 CFU/mouse).

Splenic T cells from both TLR2$^{-/-}$ and TLR4$^{-/-}$ mice fail to generate a potent Th1-type response *in vitro*

In order to characterise TLR-dependent control of BCG growth *in vivo*, we sought to determine whether TLR-deficient mice failed to elicit an effective innate and/or adaptive immune response following BCG infection. To test these possibilities, we isolated total splenocytes from uninfected and BCG-infected normal and TLR-deficient mice. Splenic T cells were then stimulated *in vitro* with heat-killed BCG as a source of antigen. Activation of the T cells was assessed 48 hours later by measuring both cytokine production and T-cell proliferation (^{3}H thymidine incorporation). As shown in *figure 3*, splenocytes isolated from uninfected mice did not respond to antigenic stimulus, as measured by either cytokine production or T-cell proliferation. In contrast, normal splenocytes from infected normal mice secreted IFN-γ following antigenic stimulation. This IFN-γ production was not observed using splenocytes from infected TLR2$^{-/-}$ or TLR4$^{-/-}$ mice. These findings suggest that TLR-deficient splenocytes fail to develop a potent Th1-type response *in vitro*. We do not believe that this failure to secrete IFN-γ represents a skewing of Th responses, as antigen-stimulated splenocytes from infected normal and TLR-deficient mice did not secrete measurable amounts of IL-4 or IL-5 *in vitro* (data not shown). In subsequent studies, we examined T-cell proliferation *in vitro* and observed that cells from normal and TLR4$^{-/-}$ mice proliferated in response to antigenic stimulation, whereas cells from TLR2$^{-/-}$ mice did not (*figure 3B*). Taken together, these data suggest that TLR2 and TLR4 contribute in a fundamentally distinct manner to the development of an effective Th1 response. Antigen-specific T cells are generated in BCG-infected TLR4$^{-/-}$ mice, as indicated by the proliferation of these cells *in vitro* following antigenic challenge. Nevertheless, these T cells fail to develop into effective Th1 responder cells, as indicated by the absence

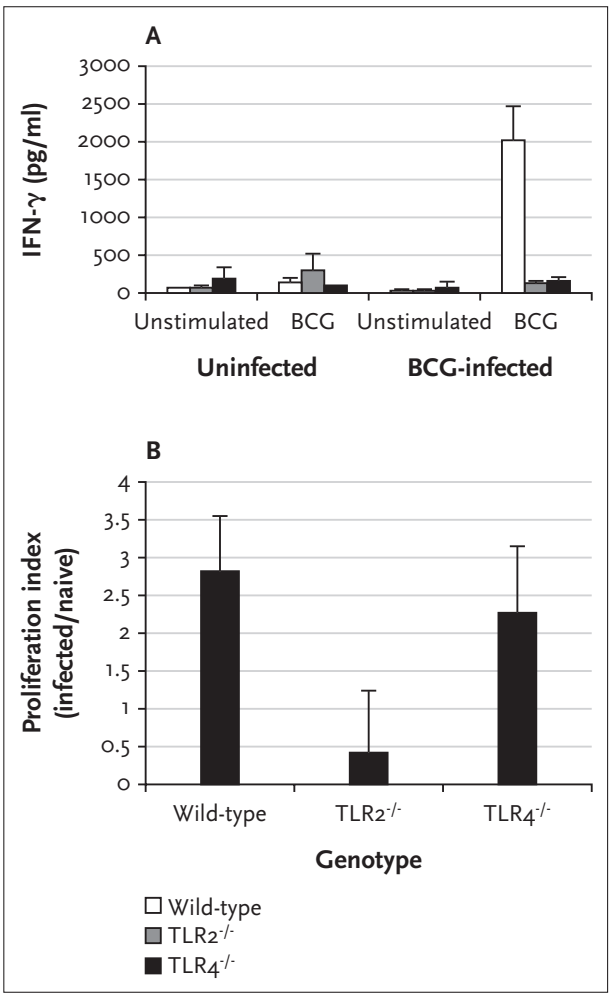

Figure 3

Antigen-dependent activation of splenocytes from BCG-infected normal and TLR-deficient mice

Splenocytes isolated from uninfected and *M. bovis* BCG-infected mice were cultured (5 x 10^5 splenocytes/well) in the presence or absence of heat-killed BCG (10^3 CFU/well). (A) After three days, culture supernatants were harvested and IFN-γ levels were measured by specific ELISA. (B) After two days in culture, splenocytes were pulsed with ^{3}H-thymidine for eight hours. Background values of isotope incorporation in unstimulated cells were subtracted from values of stimulated cells, and data are expressed as a ratio of specific isotope incorporation in stimulated cells from infected mice over uninfected mice. Triplicate wells of cells from each mouse were assayed. Data are presented as the mean of values from three uninfected or three infected mice per genotype ± SEM. Results are representative of duplicate experiments.

of IFN-γ secretion. This is not a consequence of skewing towards a Th2 phenotype, as there is no increase in IL-4 and IL-5 production by the antigen-stimulated $TLR4^{-/-}$ splenocytes. Thus, the immune defect in these cells appears to come from the inability of Th0 cells to commit to a Th1 phenotype. It should be noted that the absence of a strong Th1 response did not detract from the ability of $TLR4^{-/-}$ mice to control BCG infection *in vivo* (*figure 2*). This may be due, in part, to effective innate immunity (as evidenced by normal proinflammatory cytokine production, *figure 1*) additional to Th1-independent adaptive immunity. Furthermore, Th1 responses may not be absent in these mice, only diminished relative to control animals.

The finding that $TLR2^{-/-}$ splenocytes failed to both proliferate and secrete IFN-γ following antigenic challenge *in vitro* contrasts with the phenotype of the $TLR4^{-/-}$ cells. The BCG-infected $TLR2^{-/-}$ mice appear to possess a defect in the development of antigen-specific T cells. The inability of TLR2-deficient cells to secrete IFN-γ would then simply be a consequence of the lack of antigen-responsive T cells, rather than (or in addition to) a defect in Th1 commitment. This possibility is consistent with our finding that antigen-independent activation of $TLR2^{-/-}$ splenocytes, using cross-linked anti-TCR antibodies, induced both T-cell proliferation and IFN-γ secretion (data not shown). We do believe that $TLR2^{-/-}$ mice are capable of developing antigen-specific T cells, and published data report that these mice contain normal numbers and types of immune cells.[16] Taken together, our data suggest that the absence of TLR2 renders the mice incapable of responding to the adjuvant activity manifested by the mycobacteria themselves. This adjuvant activity is critical for the development of T cells that recognise mycobacterial antigens, and for the ability of the host to mount an effective adaptive immune response. Because $TLR2^{-/-}$ macrophages fail to secrete critical proinflammatory cytokines in response to mycobacterial infection *in vitro*, it is likely that these cells also fail to express critical cytokines and co-stimulatory molecules *in vivo* during the course of infection. Dendritic cell maturation and function may also be defective in $TLR2^{-/-}$ mice due to the inability of these cells to respond to mycobacterial TLR2 agonists. Several laboratories have previously shown that TLR agonists induce dendritic cell maturation and activation.[22-24] Furthermore, those dendritic cells that are incapable of responding to TLR agonists fail to mature and become functional *in vitro*.[25] Studies are currently underway in the laboratory to characterise the immune defects observed in $TLR2^{-/-}$ and $TLR4^{-/-}$ mice.

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Discussion following lecture by M.J. Fenton

Van der Meer: I enjoyed that very much. Just to go back to the concept of the Toll-like receptor (TLR) as first line of response, can you tell us a bit more about the molecular interaction between bacterial components and TLR? Do they actually bind, and if so, how?

Fenton: Some of you may have noticed that I tried to stick to the word 'agonist' rather than to the word 'ligand'. That is not a mere coincidence. With the exception of Gram-negative bacterial lipopolysaccharide (LPS) there has really been no clear biochemical demonstration of any of these molecules serving as classic ligands and we have little sense of what the affinity of ligands for these receptors is. In the matter of LPS, Richard Ulevitch's lab and a few others have sought to demonstrate a physical interaction in using cross-linking approaches. They have been able to show a physical association between Gram-negative LPS and TLR4 in the presence of the coreceptors CD14 and MD2. If you transfect the cell with an expression plasmid encoding TLR4 in the absence of the coreceptors CD14 and MD2 you basically have to throw in milligram amounts of LPS to get an activation. So from the point of view of ligand-receptor interaction all these Toll-receptor agonists are very poor ligands by themselves. They probably operate in the context of a larger receptor complex that includes coreceptors such as CD14. I think it is reasonable to assume that there are a variety of co-receptors that may help TLR2 agonists or TLR9 agonists to also recognise the receptors and engage a signal. The other part of this paradigm relates to how strongly the receptor is engaged and what the kinetics of this interaction are. Several studies that have been published using FACS analysis to look at receptor complex interaction have shown that, if you use FITC-labelled LPS as your classic ligand, you can demonstrate a rapid and transient interaction between LPS, TLR4 and CD14. But interestingly, a few seconds or minutes after this initial interaction and activation the receptor components seem to move to different places on the cell and in some cases some of the components are internalised. Many of you here are familiar with Sam Wright's work and the story of LPS internalisation. I think that if you take this together for LPS it suggests that there is a multireceptor component that is not assembled and only engaged on the cell surface for a very short period of time to allow a signal to be generated. The receptor components then disassemble and go elsewhere following the activation. There does not seem to be a strong high-affinity interaction between TLR proteins and any of their agonists in the absence of coreceptors.

Hermans: I have a question about the polymorphisms that are known to occur both in TLR4 and TLR2. In TLR4 there are amino acid substitutions known to lead to hyporesponsiveness. But as far as I am aware they do not show much of a link with the severity of or susceptibility to infectious diseases.

Fenton: As far as I am aware there is not a strong correlation in this case between TLR4 mutations and susceptibility to disease. In experimental models with normal human volunteers looking at the susceptibility to LPS by inhalation, David Schwartz has been able to show a correlation between certain polymorphisms in TLR4 and hyporesponsiveness of humans to LPS.[1] But at least so far there does not seem to be an increased incidence of disease. The very few studies that have been published so far looked at the extracellular domains of the Toll-like receptor proteins. Most of the work today is focused on the intracellular domains looking at intracellular signalling. Some new studies in the Journal of Immunology have shown that the extracellular domain of TLR4 possesses a hypervariable domain and most of the polymorphisms seen in different human populations or amongst different species seem to cluster in this hypervariable region. It does seem to have an impact on the ability of TLR4 to recognise LPS or different forms of LPS as well. So we are beginning to get a sense of where within the molecule recognition really occurs, but so far the correlation with disease has been minimal.

Ottenhoff: I have a question about the IL-12. You said that TLR2 knock-out mice produce normal IL-12 upon BCG

stimulation. *In vitro* there is little interferon-α production. So my question actually is, what is the mechanism to explain that?

Fenton: That is a good point. Dr van der Meer said last night at dinner that we were supposed to expose all of our Achilles heels to the panel today. So it turns out to be a very complicated story. You really need to approach it from three different angles. One is to look at the question of how purified TLR agonists induce specific responses in macrophages *in vitro*. The next would be then to look at the whole bacterium as well, because those responses are going to be very different. A great example of this is if you look at nitric oxide (NO) production induced by mycobacteria. I am a bit slow maybe in getting to the answer to your question, but you can add purified LPS for example as a classic TLR4 agonist and show that you can activate nitric oxide production and that occurs through the TLR4 pathway. If you then use live mycobacteria to induce NO it turns out that in that setting it is a TLR-independent process that drives the NO production. We published those results a few years ago. So you have got to try to keep the two pathways apart. With the data I showed for IL-12, we have not yet determined whether the IL-12 production is dependent on TLRs or not in that setting. It may be in the setting of whole live mycobacteria that the macrophages do not need TLRs to make IL-12. Experiments are going on right now in the laboratory to determine that. Now *in vivo* you would have expected that the presence of IL-12 would have correlated with a fine ability to produce γ-interferon. So I think that is going to be the biggest Achilles heel of the data I showed you today, which are all unpublished and very preliminary, which is why do we see good IL-12 production and not good γ-interferon production? I think the reasons for that can differ for the two kinds of mice. In the case of the TLR2 knock-out mice, I think we are failing to get γ-interferon production because we failed to develop a large panel of antigen-specific T cells. The T cells are just not there. You can give them all the IL-12 you want and they will not do anything with it. The TLR4 knock-out animal is a bigger mystery because we – presumably – have antigen-specific T cells there but they are not making γ-interferon. I think part of that answer comes from the process of a Th0 cell maturing into a Th1 cell. So one possibility is that in the absence of TLR4 we are unable to fully activate the development of a commitment to the Th1 phenotype. I should point out that we do not have Th skewing here. We are not skewing the animals towards Th2. There is no production of IL-4 and IL-5 in these TLR4 knock-out animals. We therefore think that there is a defect in Th1 commitment and we are testing that possibility now. The other issue may be a threshold issue. We have not looked at IL-18 or at IL-23 as other components of these three cytokines leading to α-interferon production. It may be that again we have plenty of IL-12 but not sufficient IL-18 and IL-23 to lead to a threshold of γ-interferon production. Lastly, in the C3H/HeJ model, where we now have a higher infective dose and we do see disseminated bacterial growth when you look at IL-12 levels in the serum of these mice, you do find in this case that the IL-12 levels in the animal are lower. I think that under those conditions we are seeing a correlation and γ-interferon levels are way down in these animals as well. So under the right conditions we can see it. All I can say to answer your question directly is that the splenocyte assay gives us an answer with regard to T-cell responses in the dish, but I do not think that it will really surprise anyone here that that does not necessarily correlate with what happens *in vivo*.

Netea: I have two questions. One is going back to the coreceptors for TLRs. When you mentioned all the TLRs and the agonists that are known, you did not include TLR6 and TLR1. They are described as possibly serving as coreceptors. Do you think that there are two classes of TLRs? TRLs which are really signalling and TLRs which are coreceptors? That is my first question, and a second one relates to the Toll reacting to Spätzle, an endogenous substance in *Drosophila*. In humans most of the data are done in the context of pathogens and we get more or less the same signals as in the context of IL-1 stimulation. My question is, why do you think that we also need an IL-1 system? We do have more or less the same intracellular signalling going through TLRs. Do you think that we in fact developed a kind of intention to amplify our TLR system by using IL-1?

Fenton: The TLR2 and coreceptor question is of course an important one. Those of you who are familiar enough with the data will probably agree that TLR2 probably does not work alone. TLR proteins probably function as dimers. In the case of TLR2 it probably functions only as a heterodimer in association with either TLR1 or TLR6. We do not have any data so far to answer the question whether TLR2-dependent responses also utilise TLR6 *vs* TLR1, so I cannot answer that question. In terms of whether they are coreceptors or actually contribute differently to the signalling in the intracellular responses, it is quite likely that they will. I think that if you look at responses mediated through TLR2.1 *vs* 2.6 you are likely to find some differences. Most people who have looked at TLR-dependent responses have focused on a similar set of cellular responses such as IL-6 or TNF production, IL-1 production. Those seem to be shared in common with all of the TLR receptors. It is only when you begin looking at different responses that you start to see differences: for example our findings that TLR4 engagement leads to type-1 interferon production, but engagement of TLR2

does not. I think you are likely to see differences between TLR2.6 and TLR2.1, but the data have not been published in that regard. It is also – just before I leave the topic of coreceptors – quite likely that non-TLR coreceptors are going to be involved as well. I think there is indirect evidence to support complement receptors as being TLR coreceptors, perhaps the macrophage mannose receptor or the scavenger receptor, maybe even certain Fc receptors as well.
To answer your second question, I think it ties in a bit to what I have just said, which is that there are certain common responses that seem to occur when you activate all of these receptors, not only all of the TLR receptors but also the IL-1 and IL-18 receptors. But clearly the functional responses of the cells are different. You can induce for example apoptosis in some cells triggered through TLR4, TLR2, but certainly not in cells stimulated through IL-1 or IL-18. So as usual the devil is in the details. There are certain responses that seem to be Toll dependent and certain responses that are specific for Tolls and not for IL-1 or IL-18 receptors. So what does that mean in the big picture of the biology? I think that like the IL-1 and IL-18 receptors, Toll receptors play an important role in the inflammatory response. The inflammatory response as a result of a pathogen invasion, but perhaps an inflammatory response mediated by endogenous factors as well. There certainly is an amplification loop going on here. IL-1 and IL-18 production is certainly going to be important in maintaining the inflammatory response and the duration of the response, and there is also the likely possibility that endogenous factors are being made that feed back into the Toll receptors themselves.
You pointed out the ligand for the *Drosophila* Toll, the Spätzle protein. Spätzle is produced and cleaved and feeds back into the Toll itself, but just as in the mammalian Toll agonist, no-one has actually demonstrated Spätzle binds as a ligand to Toll. It is all indirect evidence that supports that hypothesis. There are some studies suggesting that mammalian proteins can feed back into Toll receptors, heat-shock proteins being an important example.
Cells that are damaged in the course of bacterial invasion may release these heat-shock proteins, they may be an endogenous danger signal as well. There is no evidence that these proteins can recognise IL-1 or IL-18 receptors. So I think we can draw a series of circles some of which will overlap. I think the IL-1 and IL-18 receptors and the Toll receptors play similar roles in the development and maintenance of inflammation. But I think they also play highly specific roles depending on the type of pathogen and the type of response and whether it is an organism possessing TLR4 antigens or not. I think it is almost as if we can say that the innate immune response is adaptive in that it can recognise a variety of different motives through this large variety of different TLR proteins that are available.

Verbrugh: I have three more discussants who want to ask a question.

Appelmelk: What is your explanation that a knock-out in TLR2 completely blocks all T-cell development? I can understand it for lipoarabinomannan within the CD1 environment but not for Toll-independent proteins in the CD4 environment.

Fenton: Hopefully it will be the simple answer that in the absence of TLR2 you fail to get the expression of a basic set of cytokines and costimulatory molecules that are necessary to clonally activate T-cell populations. Let's hope it is just as simple as that. We have tested the antigen presentation function in the TLR2-deficient cells and antigen presentation appears normal. So we think it is a defect in either costimulus or cytokines.

Kuijpers: My question is related to the fact that a moderate dose of BCG could be coped with in the TLR4 knock-out mice. Is it due to the fact that there is an increased level of other cytokines available (thinking about TNF), or has that been dealt with experimentally?

Fenton: We have only looked at a few cytokines in that model. The circulating TNF levels appeared to be normal in those animals. So far, we have just seen the deficiency in α-interferon and IL-12.

McAdam: I have a question similar to the last one. Did you see any granulomatosis inflammation in the TLR2-deficient mice?

Fenton: We have not looked into the model long enough to test that out. The other problem is, we cannot do that with the BCG model, because the mice clear the infection, even the TLR2-deficient mice at that initial inoculum of bacteria that we would use. So where I would normally want to look at granuloma formation 40 days after infection even the TLR2 knock-out mice have cleared the infection by then. In the case of using higher inocula, we see a disseminated infection, as you saw on the one slide. We did not see anything that looked like a true granuloma. We did not see any organisation in either the TLR4 or the TLR2 knock-out mice. So I did not want to jump to any quick conclusions, but from looking at the initial data we have, there seems to be very poor granuloma formation.

REFERENCE

1. Arbour NC, Lorenz E, Schutte BC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000;25:187-91.