COMMENTARY

Toll-like receptor signalling pathways as key targets for mediating the anti-inflammatory and immunosuppressive effects of glucocorticoids

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Abstract

Toll-like receptors (TLRs) play crucial roles in the induction of innate immune responses by recognising pathogen-associated molecular patterns. The engagement of TLRs by pathogens results in induction of co-stimulatory molecules that facilitate a specific immune response and also in the induction of pro-inflammatory proteins that will promote the elimination of pathogens from the body. TLRs employ many of the same signalling components as the type I interleukin (IL)-1 receptor (IL-1R). This is hardly surprising since the intracellular regions of TLRs and the IL-1R share a conserved Toll/IL-1R homology domain (TIR) that allows the receptors to recruit the

intracellular TIR-containing adaptor protein Myd88. The latter then activates IL-1R-associated kinases that in turn recruit well-characterised downstream effectors culminating in activation of MAP kinases and transcription factors such as NF κ B and AP-1. Since glucocorticoids are known to target the latter transcription factors and the MAP kinase cascades, this commentary highlights the likely crucial importance of Toll-like receptor signalling pathways as key targets for mediating the anti-inflammatory and immunosuppressive effects of steroids.

Journal of Endocrinology (2003) **179**, 139–144

Toll-like receptors (TLRs) and immunity

Human TLRs are key components in innate immune responses due to their ability to recognise pathogen-associated molecules (reviewed by Krutzik *et al.* 2001). Many of the TLRs have defined functions in the host defence system. As examples, TLR2 recognises peptidoglycan and bacterial lipoprotein from Gram-positive bacteria (Aliprantis *et al.* 1999, Brightbill *et al.* 1999, Takeuchi *et al.* 1999), TLR3 mediates responses to double-stranded RNA (Alexopoulou *et al.* 2001), TLR4 is involved in recognition of Gram-negative lipopolysaccharide (LPS) (Poltorak *et al.* 1998, Chow *et al.* 1999, Hoshino *et al.* 1999, Qureshi *et al.* 1999, Takeuchi *et al.* 1999), TLR-5 recognises bacterial flagellin (Hayashi *et al.* 2001) and TLR9 functions as a receptor for bacterial DNA containing CpG motifs (Hemmi *et al.* 2000). Some TLRs, such as TLR2 and -6 also show functional co-operativity (Ozinsky *et al.* 2000). The engagement of TLRs by pathogenic components results in induction of co-stimulatory molecules that facilitate T-cell activation and pro-inflammatory proteins that effect elimination of the pathogen from the body (Medzhitov *et al.* 1997).

TLR/interleukin-1 (IL-1) signalling and NF κ B

TLRs employ many of the same signalling components as the type I IL-1 receptor (IL-1RI) (O'Neill & Greene 1998) (Fig. 1) by virtue of a conserved cytoplasmic Toll/IL-1R homology domain (TIR). The latter is important in initiating various signalling pathways, especially that regulating the transcription factor NF κ B. The latter exists in the cytosol of resting cells as a homo- or heterodimer of proteins of the Rel family of transcription factors (Ghosh *et al.* 1998). The transcriptional activity of the Rel proteins is tightly regulated by their association with members of the inhibitory I κ B family (e.g. I κ B- α , I κ B- β and I κ B- ϵ) that sequester nuclear factor kappaB (NF κ B) in the cytosol. TLRs and IL-1R cause phosphorylation of I κ B on two specific N-terminal serines by the I κ B kinases (IKKs), IKK α and IKK β , which form a large multiprotein complex with a scaffold protein called NEMO (IKK γ). The phosphorylation of I κ B proteins represents a signal for polyubiquitination followed by their degradation via the 26S proteasome (May & Ghosh 1998, Karin & Ben-Neriah 2000). This allows for translocation of NF κ B to the nucleus, where it activates genes encoding

inflammatory proteins and co-stimulatory molecules. Thus the IKKs are crucial regulators of NFκB and much effort has probed the upstream signalling components employed by TLRs and IL-1R in regulating IKK activity.

The TIR domain plays a crucial role in transducing signals from TLRs and IL-1RI (Fig. 1). Thus the binding of IL-1 to IL-1RI causes association with another TIR domain-containing protein, IL-1R-accessory protein (Greenfeder *et al.* 1995, Wesche *et al.* 1997a). This receptor complex then recruits the intracellular TIR-containing adaptor protein Myd88 (Burns *et al.* 1998). TLRs can directly associate with Myd88 (Medzhitov *et al.* 1998) and other TIR domain-containing adapter proteins such as MyD88 adapter-like/TIR domain-containing adapter protein (Fitzgerald *et al.* 2001, Horng *et al.* 2001) and TIR domain-containing adapter inducing IFN-β/TIR-containing adaptor molecule (Yamamoto *et al.* 2002, Oshiumi *et al.* 2003). These adapter proteins subsequently recruit and activate members of the IL-1R-associated kinase (IRAK) family (Muzio *et al.* 1997, Wesche *et al.* 1997b, Kobayashi *et al.* 2002, Li *et al.* 2002, Suzuki *et al.* 2002). IRAK is recruited to Myd88 in association with another protein termed Toll-interacting protein (Tollip) (Burns *et al.* 2000). IRAK associates with Myd88 via the

homophilic interaction of their death domains. IRAK also contains a kinase domain but the kinase activity is not required for NFκB activation but may be required for other pathways such as the p38 MAP kinase (MAPK) cascade (Knop & Martin 1999, Schmidt *et al.* 2001). The IRAK–Myd88 association triggers hyperphosphorylation of IRAK by itself (Cao *et al.* 1996) and/or by other additional kinases (Li *et al.* 1999), leading to its dissociation from Myd88 and Tollip and its interaction with the downstream adaptor tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF-6) (Burns *et al.* 2000). The phosphorylation of IRAK also ultimately leads to its degradation by proteasomes and this may be a regulatory mechanism by which cells become desensitised after prolonged activation of IL-1RI and TLRs (Yamin & Miller 1997, Li *et al.* 2000). The interaction of IRAK with TRAF-6 leads to activation of transforming growth factor-β-activating kinase (TAK1) (Ninomiya-Tsuji *et al.* 1999). IRAK is essential in this activation process since it promotes the translocation of TAK1 binding protein 2 (TAB2) from the membrane to the cytosol where TAB2 interacts with TRAF-6 and bridges the association of TRAF-6 with TAK1 (Takaesu *et al.* 2000, 2001). The latter, with the help of another TAK1-binding protein,

TAB1, becomes activated and in turn activates NF κ B-inducing kinase (NIK) (Ninomiya-Tsuji *et al.* 1999). NIK can then activate NF κ B through phosphorylation and activation of the IKKs (Ling *et al.* 1998, Nakano *et al.* 1998). However, the role of NIK has recently been equivocated with analysis of NIK knockout mice and alymphoplasia mice that contain a point mutation in NIK showing no defect in activation of NF κ B by TNF, IL-1 and LPS (Shinkura *et al.* 1999, Yin *et al.* 2001). Interestingly IKKs may also be activated by MAPK/ERK kinase 1 (MEKK-1) that is activated by TRAF-6 via a novel adaptor protein termed ECSIT (Kopp *et al.* 1999). Thus TRAF-6 appears to act as a bifurcation point to activate the TAK1 and MEKK-1 pathways, both of which activate NF κ B.

TLR/IL-1 signalling and MAPKs

In addition to NF κ B activation, IL-1RI and TLRs can also initiate MAPK signalling cascades and activate multiple transcription factors, including AP-1 and Elk-1. Thus IL-1 and LPS induce phosphorylation of p38, ERK1/2 and JNK (Freshney *et al.* 1994, Rouse *et al.* 1994, Derijard *et al.* 1995, Lin *et al.* 1995, Ulevitch & Tobias 1995). Whilst the mechanisms by which the MAPKs are activated by IL-1RI and TLRs are incompletely understood, several upstream regulators have been identified. Interestingly, as described above, such regulators also play integral roles in mediating activation of NF κ B (Fig. 1). Thus TAK1/TAB1 can activate the MAPKs MKK3/6 and MKK4, which in turn activate p38 and JNK respectively (Ninomiya-Tsuji *et al.* 1999). In addition MEKK-1 can also activate the JNK pathway by phosphorylating MKK4 (Xia *et al.* 1998).

Transcription factors as targets for glucocorticoids

Whilst the molecular mechanisms underlying the anti-inflammatory and immunosuppressive effects of glucocorticoids are complex and incompletely understood, the signalling pathways described above for TLRs are likely to emerge as lead targets for mediating such effects. A succinct overview of the known molecular effects of glucocorticoids gives significant credence to this proposal.

The primary target for glucocorticoids is a specific intracellular glucocorticoid receptor (GR) (Beato *et al.* 1995). The engagement of GR by glucocorticoids promotes its dissociation from the 90 kDa heat shock protein and translocation into the nucleus. The liganded GR binds as a dimer to DNA by recognising specific palindromic sequences known as glucocorticoid response elements (GREs) (Luisi *et al.* 1991). The binding of GR to GREs can induce the transcription of genes whose promoters are in close proximity to GREs, by facilitating recruitment of

co-activator complexes (such as CREB-binding protein (CBP)/p300) and RNA polymerase II. By this process glucocorticoids can directly promote the expression of anti-inflammatory proteins such as lipocortin, IL-1R antagonist and IL-10 (Adcock & Caramori 2001). It is also interesting to note that glucocorticoids can directly induce the expression of I κ B- α , the inhibitor of NF κ B (Almawi & Melemedjian 2002). The author has shown this phenomenon to be cell-type specific (Bourke & Moynagh 1999), but it is still worth emphasising that in responsive cells the induction of I κ B- α by glucocorticoids is likely to have a negative influence on TLR signalling. Additional mechanisms by which glucocorticoids regulate NF κ B are discussed below.

Whilst the direct induction of anti-inflammatory proteins by glucocorticoids is a significant contributory mechanism to their clinical anti-inflammatory effects, the pleiotropic ability of glucocorticoids to inhibit the expression of a plethora of pro-inflammatory proteins is likely to be of crucial importance (Barnes 1995). Thus glucocorticoids inhibit the expression of an array of cytokines (including IL-1 and TNF), chemokines and leukocyte adhesion molecules. In general the promoters of the genes encoding these proteins lack GREs and thus gene repression is not dependent on GR-GRE interaction. Instead repression is mediated by inhibitory effects of activated GR on transcription factors that are known to promote the expression of the pro-inflammatory proteins. Two of the best characterised transcription factors that are known targets for activated GR are NF κ B and AP-1. In addition to the positive regulation of I κ B- α expression, as described above, activated GR is also known to directly associate with AP-1 and the p65 subunit of NF κ B (Adcock & Caramori 2001). Such interactions lead to reduced association of AP-1 and NF κ B with the co-activator CBP resulting in inhibition of their transactivation potential. A recent report has suggested a related but novel mechanism underlying GR repression of pro-inflammatory gene expression in which GR inhibits histone acetylation by directly inhibiting CBP-associated histone acetyltransferase activity and by actively recruiting a histone deacetylase complex (Ito *et al.* 2000). Such histone deacetylation will prevent unwinding of DNA from histone complexes and so limit access of transcription factors such as NF κ B and AP-1 to their binding sites, resulting in inhibition of expression of pro-inflammatory genes. Furthermore, another report suggests an additional specific mechanism for the inhibitory effects of GR on NF κ B. Thus GR has been shown to inhibit NF κ B by interfering with serine-2 phosphorylation of the C-terminal domain of RNA polymerase II, resulting in reduced recruitment of the latter to NF κ B-regulated promoter regions (Nissen & Yamamoto 2000). A complex picture thus emerges of the regulatory effects of activated GR on the transcription factors NF κ B and AP-1. However, it is intuitively obvious that glucocorticoids may target TLR signalling since TLR

signalling culminates in activation of NF κ B and AP-1 (Fig. 1).

MAPKs as targets for glucocorticoids

Whilst NF κ B and AP-1 are key targets for GR, it is also apparent that regulation of these transcription factors is not the exclusive basis for GR repression of inflammatory genes. The MAPK signalling pathways play important roles in promoting inflammatory gene expression and it has emerged that these pathways are also prone to regulation by glucocorticoids. Dexamethasone has been shown to inhibit activation of ERK1/2, JNK and p38 MAPKs (Caelles *et al.* 1997, Swantek *et al.* 1997, Gonzalez *et al.* 1999, Lasa *et al.* 2001) and repress inflammatory gene expression. As an example GR blocks the JNK signalling pathway resulting in lack of phosphorylation of c-Jun on serine-63 and -73 and ultimately inhibition of AP-1 and repression of gene expression (Caelles *et al.* 1997). This represents another mechanism by which GR can regulate AP-1 activity and is distinct from the direct GR–AP-1 association described above. Furthermore since JNK is required for LPS stimulation of TNF translation, the inhibition of JNK by GR results in reduced TNF translation in response to LPS (Swantek *et al.* 1997). Since the latter is recognised by TLR-4 it is apparent that JNK in the TLR-4 signalling pathway is a key target for glucocorticoids. However, the other MAPKs are also likely to play roles in mediating anti-inflammatory effects of glucocorticoids on TLR signalling. Thus LPS is well known to induce cyclooxygenase 2 and dexamethasone has been shown to destabilise cyclooxygenase 2 mRNA by inhibiting the phosphorylation and activity of p38 MAPK (Lasa *et al.* 2001). The inhibition of the MAPKs by glucocorticoids is mediated by an increased expression and decreased degradation of the MAPK phosphatase-1 (Kassel *et al.* 2001). Overall a convincing picture emerges of MAPK pathways playing key roles as targets for glucocorticoids. The employment of MAPK pathways by TLRs again emphasises the obvious relevance of TLR signalling pathways as targets for glucocorticoids (Fig. 1).

Likely consequences of regulation of TLR signalling by glucocorticoids

The above discussion highlights TLR signalling pathways as lead targets for glucocorticoids. The global use of the transcription factors NF κ B and AP-1 and the MAPK pathways by the various TLRs strongly hints that all of the TLR signalling pathways will be subject to interference by glucocorticoids. This has important functional consequence in terms of widespread suppression of the innate immune response to a variety of pathogens. TLRs are strategically located at the host–pathogen interface and

serve to recognise a number of molecules that are expressed by pathogens and not the host. This recognition triggers a complex cascade of intracellular signalling pathways, as described above, that will ultimately promote the expression of pro-inflammatory proteins such as IL-1 and TNF. The latter orchestrate the inflammatory response and thus the inhibition of TLR signalling by glucocorticoids will repress IL-1 and TNF expression and this is likely to make a major contribution to dampening the inflammatory response. Since the latter is a crucial component of the innate immunity, it is clear that the regulation of TLR signalling by glucocorticoids lies at the heart of their immunosuppressive and anti-inflammatory properties. The targeting of TLR signalling by steroids probably contributes to the compromised immune status of individuals on long-term use of steroids for the treatment of inflammatory diseases. The blunting of TLR signalling by steroids will interfere with the initial recognition phase of the immune response and will dampen the innate response to pathogens. However, the fallout of inhibition of TLR signalling is not restricted to innate immunity. The triggering of TLRs, such as TLR-4 by LPS, also induces the expression of co-stimulatory molecules on antigen-presenting cells and this is a key process in initiating a specific immune response (Medzhitov *et al.* 1997). The signalling pathways employed by TLRs in promoting the expression of co-stimulatory molecules are common to the above transduction systems used in inducing pro-inflammatory gene expression. Thus the regulation of these pathways by glucocorticoids will repress the expression of co-stimulatory molecules and suppress the specific immune response.

In summary, glucocorticoids have the potential to inhibit the intracellular signalling pathways employed by TLRs. The latter act at the crossroads of innate and adaptive immunity and thus the paralysis of TLR signalling is likely to be central in manifesting the remarkable immunosuppressive effects of glucocorticoids.

Funding

I would like to acknowledge research funding from the European Biotechnology 5th Framework programme (Contract Nos QLG1-CT-1999-00549 and QLK3-CT-2000-00270), Health Research Board of Ireland, Enterprise Ireland and Higher Education Authority of Ireland.

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Received 28 March 2003

Accepted 21 July 2003