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REVIEW

Are toll-like receptors potential drug targets for atherosclerosis? Evidence from genetic studies to date

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Abstract

Low-density lipoprotein cholesterol lowering, most notably via statin therapy, has successfully reduced the burden of coronary artery disease (CAD) in recent decades. However, the residual risk remaining even after aggressive lipid lowering has renewed interest in alternative targets. Anti-inflammatory drugs are thought to have much potential in this context, but side effects associated with long-term use of conventional anti-inflammatories, such as NSAIDs and glucocorticoids, preclude their use as preventive agents for CAD. Evidence from epidemiological studies and murine models of atherosclerosis suggests that toll-like receptors (TLRs) may have utility as targets for more focused anti-inflammatories, but it remains unclear if this pathway is causally related to CAD in man. Here, we review recent insight into this question gained from genetic studies of cardiovascular risk and innate immune function, focussing on the potential of Mendelian randomisation approaches based on intracellular-signalling pathways to identify and prioritise targets for drug development.

Keywords Toll-like receptors · Cardiovascular disease · Genetics · Mendelian randomisation · Drug target discovery

Background

Coronary artery disease (CAD) remains a leading cause of premature death globally, despite recent progress in risk reduction through the lowering of serum low-density lipoprotein cholesterol (LDL-C) levels by statins and other strategies (Nabel and Braunwald 2012). More aggressive lipid lowering, through the use of proprotein convertase subtilisin-kexin type 9 (PCSK9)-targeting antibodies, has shown that LDL-C can be safely reduced to levels far below those currently achieved by statin therapy (Sabatine et al. 2017). However, the residual risk remains large even when LDL-C levels are reduced by up to 60% compared to statin-only therapy (Sabatine et al. 2017), highlighting the pressing need to identify alternative targets for the reduction of cardiovascular risk.

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Inflammatory signalling pathways are increasingly viewed with optimism in this context. Evidence from epidemiological studies and murine models of atherosclerosis supports the view that inflammatory signalling is not merely associated with the disease, but rather that it underpins atherogenesis (Rocha and Libby 2009). This emerging consensus was recently strengthened by the results of the CANTOS trial of the interleukin-1 (IL-1)-β neutralising antibody canakinumab, which showed that this anti-inflammatory drug reduces risk of cardiovascular events significantly compared to placebo, despite having no impact on serum lipids (Ridker et al. 2017). This success, combined with existing support for the hypothesis, has led to much current interest in the potential of other anti-inflammatory drugs to reduce cardiovascular risk.

However, the two most widely used forms of anti-inflammatory therapy, glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs), are unsuitable for use as preventive agents due to the relatively high incidence of side effects associated with their long-term use. These issues are thought to arise largely because these drugs target components of the inflammatory mechanism that are shared by numerous other physiological processes, such as cyclo-oxygenase-dependent maintenance of intestinal barrier function (in the case of NSAIDs) and nuclear receptor-dependent regulation of diverse intracellular-signalling pathways (in the case of

glucocorticoids) (Kearney et al. 2006). Thus, there is a need to develop more focused anti-inflammatory drugs, targeting mediators which are more specifically related to inflammation, and which ideally lack significant overlap with other cellular mechanisms.

In this review, we summarise the growing evidence from genetic studies of CAD risk and innate immune function which suggest that toll-like receptors (TLRs) may represent useful therapeutic targets for CAD. The potential utility of Mendelian randomisation-based approaches to test whether these and other intracellular-signalling pathways may be causally related to disease is also highlighted.

Experimental evidence of the roles for TLRs in atherosclerosis

Diverse forms of experimental and observational evidence have solidified the now widely held view that inflammatory processes underpin atherogenesis (Rocha and Libby 2009). Among recently suggested candidate inflammatory stimuli, conserved microbe-derived molecules, termed pathogen associated molecular patterns (PAMPs), are receiving much interest, since they are both stimulants of inflammatory signalling, and associated with established environmental cardiovascular risk factors, such as infection, tobacco use and diet (Elsenberg et al. 2013; Erridge 2008; Malik et al. 2010).

PAMPs trigger inflammatory signalling via their detection by pattern recognition receptors (PRRs) of the innate immune system, of which the toll-like receptors represent a major family. Broadly speaking, the ten human TLRs can be subdivided into two major categories: those which recognise bacterial cell wall components and signal mainly from the cell surface to promote inflammatory cytokine production (TLRs 1, 2, 4, 5, 6 and 10) and those which recognise nucleic acid motifs and signal from the endosome to trigger anti-viral responses (e.g. production of type I interferons, TLRs 3, 7, 8 and 9) (Gay et al. 2014).

Mounting evidence supports roles for both TLR2 and TLR4 in atherogenesis. For example, the experimental administration of ligands of TLR2 (bacterial lipopeptides) or TLR4 (bacterial lipopolysaccharides, LPS) accelerates atherosclerosis in murine models of the disease (Malik et al. 2010; Mullick et al. 2005), and mice deficient in TLR2 or TLR4 develop significantly less plaque (Michelson et al. 2004; Mullick et al. 2005). However, the role of endosomal TLR-signalling is less clear, as both pro- and anti-atherogenic properties of endosomal TLRs or their stimulants have been reported (Cole et al. 2011; Goossens et al. 2010; Koulis et al. 2014; Levy et al. 2003; Salagianni et al. 2012; Zhang et al. 2008).

Epidemiological studies also support the notion that TLRs may contribute to atherogenesis in man. Cardiovascular risk is increased, for example, in subjects with chronic or recurrent bacterial infections (Kiechl et al. 2001), elevated concentrations of LPS in plasma (Wiedermann et al. 1999), increased expression of TLR2 and TLR4 on monocytes (Ashida et al. 2005), or responsiveness of leukocytes to TLR2 or TLR4 ligands (Elsenberg et al. 2013; Methe et al. 2005; Van Den Biggelaar et al. 2004; Versteeg et al. 2008). Together, these findings suggest that TLRs may contribute to the inflammatory processes that underpin atherosclerosis, and could represent useful new drug targets for CAD.

Evidence from genetic studies of involvement of TLRs in CAD

Genetic studies, particularly those based on genome-wide association (GWAS), offer useful insight into the potential involvement of specific mediators in disease. To date, meta-analyses of GWAS for CAD have revealed 66 loci associated with CAD risk reaching genome-wide significance ($P < 5 \times 10^{-8}$), and at least 243 that meet the less stringent threshold of false discovery rate (FDR) $q < 0.05$ (Nelson et al. 2017). Although many of these loci are proximal to genes with expected roles in lipid regulation and inflammatory signalling, none have so far been identified in or near any of the ten TLR genes.

Nevertheless, recent analyses suggest that the absence of core genes from a pathway in GWAS results should not rule out the involvement of that pathway in disease. It is increasingly recognised that GWAS SNPs are rarely located near genes coding for central mediators of key biological networks, particularly if the functions of those genes are likely to be under strong selective pressure (Goh et al. 2007). Instead, the emerging picture is that GWAS SNPs tend to be discovered more frequently in the peripheral genes of a network, namely those that regulate the signalling or function of central mediators. In these instances, network reconstruction algorithms can help to identify patterns in these genes, based on data from transcriptional co-regulation or experimental delineation of pathways, which may then highlight networks or cellular functions that are likely to be involved in disease. For example, GWAS for systemic lupus erythematosus (SLE), which is well established to be driven by inherited propensity to excessive IFN-α production, have so far identified no SNPs in any of the 13 IFN-α genes, or the shared type I IFN receptor (IFNAR) (Bronson et al. 2012). Nevertheless, > 50% of SLE SNPs are proximal to genes that regulate type I IFN production or signalling, and network analyses of these genes are in close agreement with the results of experimental interventions which identify this pathway as a core mediator of SLE risk (Bronson et al. 2012). Likewise, despite a significant contribution of IL-1β signalling to cardiovascular risk in man as revealed by the CANTOS trial, (Ridker et al. 2017) neither this gene nor its immediate regulators have emerged as

GWAS hits for CAD to date. Together, these findings support the notion that pathways which contribute substantially to disease risk may not be immediately apparent in current GWAS results.

Two major studies have recently attempted to reconstruct the key pathways involved in the pathogenesis of CAD from GWAS results, each taking different approaches to identify the most likely effector genes of CAD GWAS SNPs (Ghosh et al. 2015; Mäkinen et al. 2014). Mäkinen et al. (2014) assigned genes to GWAS SNPs based on expression quantitative trait loci (eQTL) data from studies of CAD-related tissues. Six CAD-related gene networks emerged from this analysis, of which two were based mainly on lipid metabolism and coagulation, two had unknown function and two comprised genes mainly involved in immunity. Notably, TLR2 was identified as a well-connected member of one of the immunity networks (Mäkinen et al. 2014). By contrast, Ghosh et al. (2015) assigned clusters of genes based on proximity to GWAS SNPs, followed by Reactome-based gene-set enrichment analysis to identify CAD-associated networks. The core biological pathways identified through this approach included extracellular matrix degradation, various elements of lipid metabolism and TLR-signalling. Notably, confirming earlier observations (Goh et al. 2007), the driver genes at the centre of the networks resolved by these studies were rarely the same as the GWAS signal genes. Of course, some caution is required in the interpretation of such pathway-driven analyses, since there may be bias towards specific pathways (such as inflammation or lipids), in the literature. Nevertheless, these two different approaches to network reconstruction from GWAS results show that although individual TLRs do not emerge as hits in GWAS directly, the TLR-signalling pathway is identified as a potential contributor to CAD risk.

Genetic variants regulating endosomal TLR-signalling

Although emerging GWAS data suggest that TLRs may contribute to CAD risk, these studies do not distinguish whether the effect is mediated by inflammatory or anti-viral TLR-signalling. One possible approach to address this question is Mendelian randomisation (MR), a technique that depends on the identification of one or more genetic variants which serve as proxies for levels of the biomarker of interest. The effects of these variants on disease risk, as determined by GWAS, are then used to determine whether the biomarker is causally related to disease risk (Lewis 2010). Because genetic variants are assigned randomly at conception, MR studies are generally less susceptible to issues such as confounding, reverse causation and bias, which can hinder the interpretation of conventional observational studies (Lewis 2010).

To date, most MR studies for CAD have focussed on circulating biomarkers, or phenotypic data such as height or BMI, since these variables are commonly measured in cardiovascular GWAS protocols. These studies have been instrumental for our understanding of how these markers affect CAD risk, showing that LDL-C and triglycerides are causally related to atherosclerosis (Do et al. 2013; Linsel-Nitschke et al. 2008), and that HDL-C, CRP, fibrinogen, homocysteine and sPLA2-IIa are most likely not causal for CAD (Dehghan et al. 2011; Elliott et al. 2009; Holmes et al. 2013; Sabater-Lleal et al. 2013; van Meurs et al. 2013).

However, because many potential therapeutic targets are likely to be intracellular, cell-type specific, or produced only transiently in response to a specific stimulus, it may be more informative to query whether a specific cellular signalling pathway, rather than a circulating biomarker, is causal for disease. We recently suggested that the systematic screening of cultured cells of genotyped donors challenged with defined stimuli could be used to identify SNPs which regulate candidate signalling pathways of interest, and that these can in turn be used as genetic instruments in MR to test the causality of specific signalling pathways with respect to disease (Nelson et al. 2015). The feasibility of this approach was demonstrated through our discovery of three SNPs which explain 28% of the inter-individual variation in TLR9-dependent type I IFN production (Nelson et al. 2015). Meta-analysis using these variants provided useful evidence that leukocyte type I IFN production is not likely to be a causal mediator of atherosclerosis in man. Notably, these SNPs also regulated type I IFN responses to stimulants of other endosomal TLRs (TLR3, TLR7, TLR8), but were not associated with production of inflammatory cytokines induced by TLR4 (Nelson et al. 2015). Thus, screens of cultured leukocytes can be used to discover SNPs which regulate protein-level gene expression downstream of specific cellular signalling pathways.

Genetic variants regulating TLR4-signalling

Formal MR analyses require quantitative evidence of the association between levels of the biomarker of interest and risk of disease. As leukocyte IFN-I production has not yet been measured in a case-control or longitudinal study of CAD, we were unable to complete a conventional MR analysis in our IFN study. However, several studies have shown that TLR2- or TLR4-dependent leukocyte responses are associated with cardiovascular risk. For example, TLR2- and TLR4-induced pro-inflammatory cytokine production by monocytes or whole blood was shown to be associated with increased cardiovascular mortality in elderly women (Van Den Biggelaar et al. 2004), presence of unstable angina (Methe et al. 2005) or angiographically proven CAD relative to healthy controls (Elsenberg et al. 2013), and, in percutaneous coronary

intervention patients, degree of stenosis and number of diseased vessels (Versteeg et al. 2008). Results such as these could potentially be used to support MR analyses of the TLR2 and TLR4 pathways.

It is also well established that innate immune function is highly heritable. Twin and sibling studies have yielded heritability estimates of > 50% for LPS induced IL-6, IL-1RA, IL-10, IL-1β and TNF-α from whole blood cultured ex vivo (de Craen et al. 2005). Nevertheless, the identification of genetic variants regulating these pathways has not been straightforward. For example, early candidate gene studies suggested that two common co-segregating coding variants in the TLR4 gene, Asp299Gly and Thr399Ile, were associated with hyporesponsiveness to endotoxin and reduced cardiovascular risk (Arbour et al. 2000; Kiechl et al. 2002), but these findings could not be replicated by other groups (Erridge et al. 2003; Ferwerda et al. 2008).

More recently, genome-wide approaches have been applied to the identification of such variants. Lee et al. (2014) measured the induction of 415 genes in dendritic cells (DC) of 534 volunteers stimulated with LPS, IFN-β or influenza virus (Lee et al. 2014). Many of the QTLs identified in this study were detectable only under condition of a specific stimulus, and are therefore referred to as response QTLs (reQTLs), or induced QTLs (iQTLs), to differentiate them from expression QTLs (eQTLs) which are detected in the unstimulated state. Taking a broader approach, Fairfax et al. (2014) measured genome-wide transcript expression in monocytes of 432 volunteers stimulated with LPS or IFN-γ. QTLs were identified for almost every gene studied (with FDR < 0.05), and most of these were detected only in the stimulated state (i.e. reQTLs). Following a similar protocol, Kim et al. (2014) studied mRNA responses of monocytes of 137 healthy volunteers treated with LPS, reporting discovery of *cis*- and *trans*-QTLs for 476 genes, many of which were detected only in the stimulated condition. Interestingly, while the reQTLs identified by both monocyte studies were similar (~ 50% overlap), there was little overlap with the reQTLs identified in DCs, suggesting that reQTLs can be highly cell-type specific (Fairfax et al. 2014; Kim et al. 2014; Lee et al. 2014). Another key finding raised by all three studies is that reQTLs are enriched in the results of GWAS for diseases, supporting the widely held view that gene by environment (G × E) interactions are likely to be key regulators of disease risk. Of particular interest, Kim et al. (2014) reported that of their LPS reQTL SNPs which overlap with GWAS entries, 32% were associated with serum lipid levels, suggesting that lipoprotein metabolism may be more deeply connected to innate immune signalling than previously appreciated.

However, a key limitation of these studies is that the identified reQTLs were defined on transcriptional, rather than protein-level, data. Protein reQTLs will likely be of more utility for MR studies, since proteins are the ultimate effectors of most pathologies, and their abundance does not always track mRNA levels (Vogel and Marcotte 2012). This is particularly true of cytokines, which are heavily regulated by post-translational mechanisms. Two major studies have so far attempted to identify SNPs regulating LPS-induced proteins through genome-wide approaches. Using discovery and replication cohorts of 130 and 186 volunteers, respectively, Larsen et al. (2013) failed to identify reQTLs for LPS-induced cytokine responses from whole blood cultures. However, Li et al. (2016) were able to identify one reQTL associated with LPS-dependent IL-10 cytokine production (rs76753986), and five other reQTLS for cytokine production in response to *Mycobacterium tuberculosis* or *Candida albicans*, in a screen of peripheral blood mononuclear cells (PBMC) from only 79 volunteers. The relative success of this study over the former despite the smaller cohort size may relate to the heterogeneity of whole blood compared to PBMC or the inability to correct for confounders, such as age or BMI, in the anonymised samples used in the earlier study (Larsen et al. 2013).

Nevertheless, the outcome of these studies is that only one protein reQTL has been identified for LPS-signalling to date that reaches genome-wide significance, and this hit was not associated with production of pro-inflammatory cytokines. However, when using a loose definition of significance ($P < 0.05$ without Bonferroni correction), it was noted that ~ 60% of CAD GWAS SNPs are also reQTLs for TLR-dependent cytokine production (Li et al. 2016). This surprising degree of overlap is similar in extent to those of SNPs for risk of infectious disease, in which TLR-signalling would be expected to be heavily involved (also ~ 60%). Taken together, these findings therefore support both the feasibility of systematic approaches to the identification of protein-level reQTLs and a potential contribution of TLR-signalling to CAD risk.

Genetic variants regulating TLR2-signalling

Although TLR4 has received the most attention in studies of TLRs in CAD, TLR2 has emerged as a more significant driver of atherosclerosis in mice (Michelson et al. 2004; Mullick et al. 2005). Mikacenic et al. (2013) recently identified 19 SNPs reaching genome-wide significance for TLR2/TLR1 ligand (Pam_3CSK_4)-induced IL-6 from a screen of whole blood from 360 volunteers. All clustered within the TLR1/10/6 locus on chromosome 4, and together explained > 35% of the variance in cytokine responses to TLR2/1 stimulation. Crucially, it was found that these SNPs also regulated production of two other inflammatory cytokines (IL-1β and TNF-α) induced by Pam_3CSK_4, but not by the other tested stimulants (peptidoglycan, a ligand of NOD1/2, and FSL-1, a di-acyl ligand of the TLR2/6 heterodimer). This adds further weight to the notion that cell-based assays can be used to identify reQTLS which regulate specific signalling pathways.

Association plots of the TLR2 response locus identified in this study reveal no individual SNPs approaching genome-wide significance for CAD risk (Fig. 1a). However, meta-analysis of the four lead SNPs in distinct LD blocks ($R^2 < 0.2$) shows that each allele associated with increased lipopeptide-induced cytokine production is associated with reduced cardiovascular risk, and their combined effect is significant (OR −0.10, CI −0.18 to −0.01, Fig. 1b). Thus, although this locus would likely not emerge as a risk factor for CAD in conventional GWAS, examining reQTLs in this way suggests a potential involvement of TLR2-signalling in the pathogenesis of CAD.

The direction of this effect is surprising. Data from knock-out mouse experiments suggest that deficiency in TLR2 signalling should be associated with reduced risk of CAD (Michelson et al. 2004; Mullick et al. 2005). However, the reQTL meta-analysis suggests that impaired TLR2 signalling may be associated with increased CAD risk in man. This raises the unexpected notion that drug-mediated TLR2 inhibition could in fact increase cardiovascular risk.

Should we target TLRs for atherosclerosis?

One possible explanation for this surprising finding may lie in the observation that the same TLR2 response limiting variants at this locus are also associated with significantly increased risk of chronic bacterial infections, including *Helicobacter pylori* (Mayerle et al. 2013), recurrent urinary tract (UTI) (Hawn et al. 2009) and *Mycobacterium tuberculosis* infections (Schurz et al. 2015). Notably, chronic bacterial infections accelerate plaque development in mice and are strongly

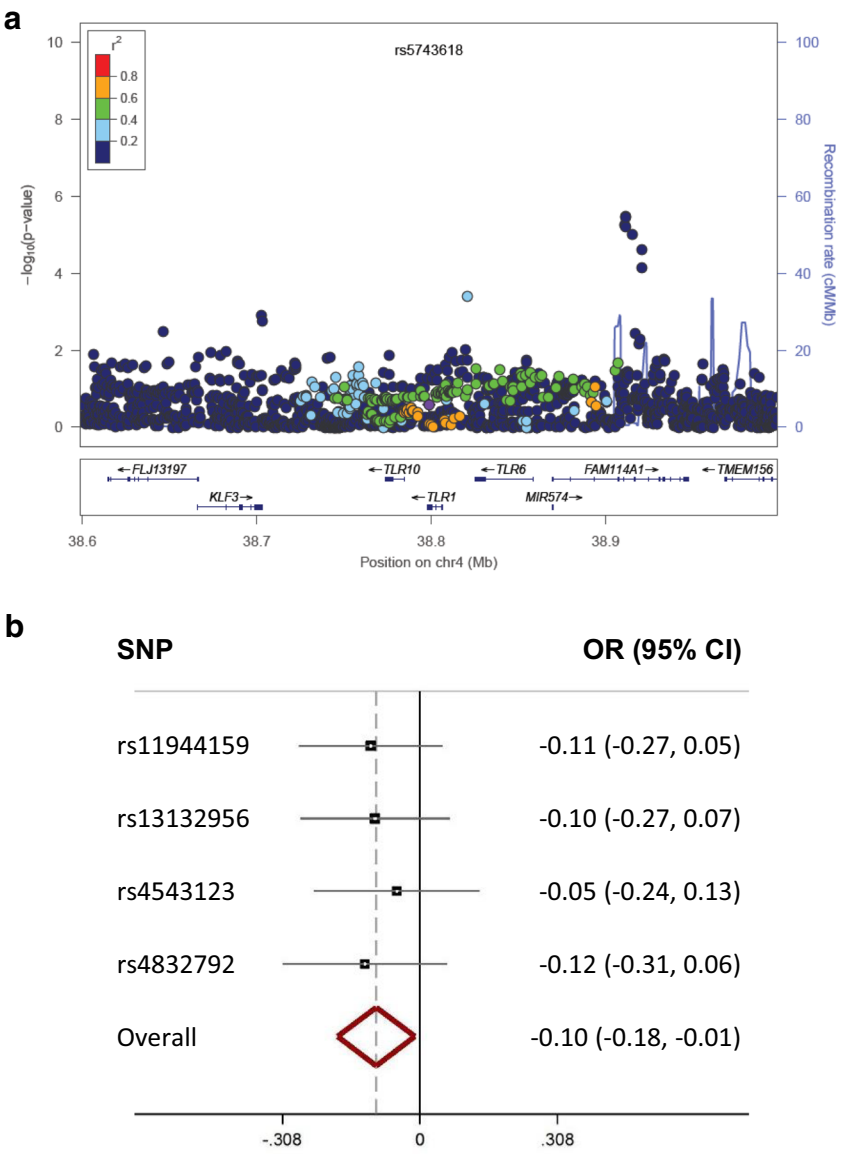

Fig. 1 Meta-analysis of the effects of TLR2-signalling reQTLs on risk of coronary artery disease. **a** Association plot of the TLR1/6/10 locus centred on the TLR2-signalling reQTL rs5743618. **b** Meta-analysis of the effects of the four lead TLR2-signalling reQTLs in distinct LD blocks ($R^2 < 0.2$) on CAD risk, using the CARDIoGRAM dataset (Mäkinen et al. 2014) as reference

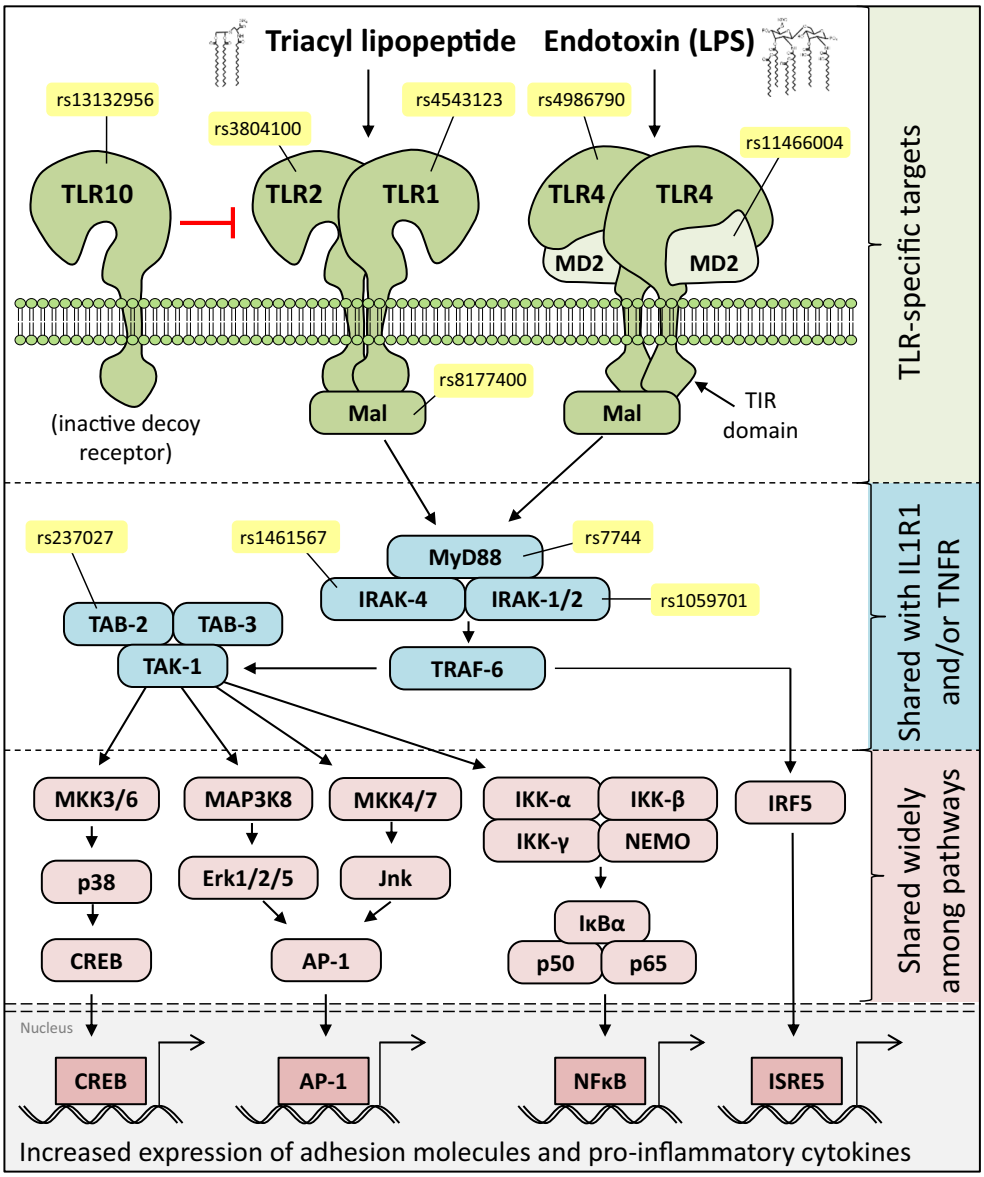

Fig. 2 Hierarchy of shared utilisation of signalling intermediates by the TLR2/TLR1 and TLR4/MD2 signalling pathways. The avoidance of interference from pleiotropic effects of SNPs in MR analyses of specific signalling pathways will likely require the prioritisation of variants regulating genes most proximal to the receptors of interest or their unique upstream mediators. Candidate common variants in or near genes coding for specific (upper panel) or relatively specific (second panel) mediators of the TLR2 and TLR4 signalling pathways are highlighted

associated with risk of incident atherosclerosis in man (Kiechl et al. 2001). Taken together, these observations therefore raise the question of whether it would be safe to target TLRs in the context of CAD risk management?

It has long been thought that targeting TLRs for the purpose of limiting inflammatory disease should be quite safe, since rare individuals lacking pro-inflammatory TLR-signalling completely due to genetic deficiency in IRAK-4 or MyD88, although at high risk of bacterial infection in childhood, show no apparent clinical phenotype in adulthood (Netea et al. 2011). Moreover, human trials of drugs in development to target TLR2 (e.g. OPN-305) or TLR4 (e.g. Eritoran) have also shown these agents to be very well tolerated, while conferring near complete inhibition of cytokine production by leukocytes in a stimulus-specific manner (Reilly et al. 2013).

However, if longer-term use of TLR-inhibitors is shown to increase risk of infection, as suggested by the genetic evidence, this could be mitigated by careful monitoring of patients and the prompt halting of therapy in those contracting infections. This strategy has been very effective for other drugs which increase risk of infection, such as TNF-α inhibitors, which, despite their immunosuppressive effects, have proven to yield excellent clinical benefits in other inflammatory diseases (Pérez-Zafrilla et al. 2012). As CAD is more prevalent than rheumatoid arthritis, the economic case for widespread TLR-inhibition would need to be carefully considered. However, strategies to minimise cost and risk of infection may include focusing on small molecule inhibitors, rather than antibody-based drugs (since the immunosuppressive effects of the former could be reversed more quickly on cessation of therapy), and stratifying prescriptions to those at

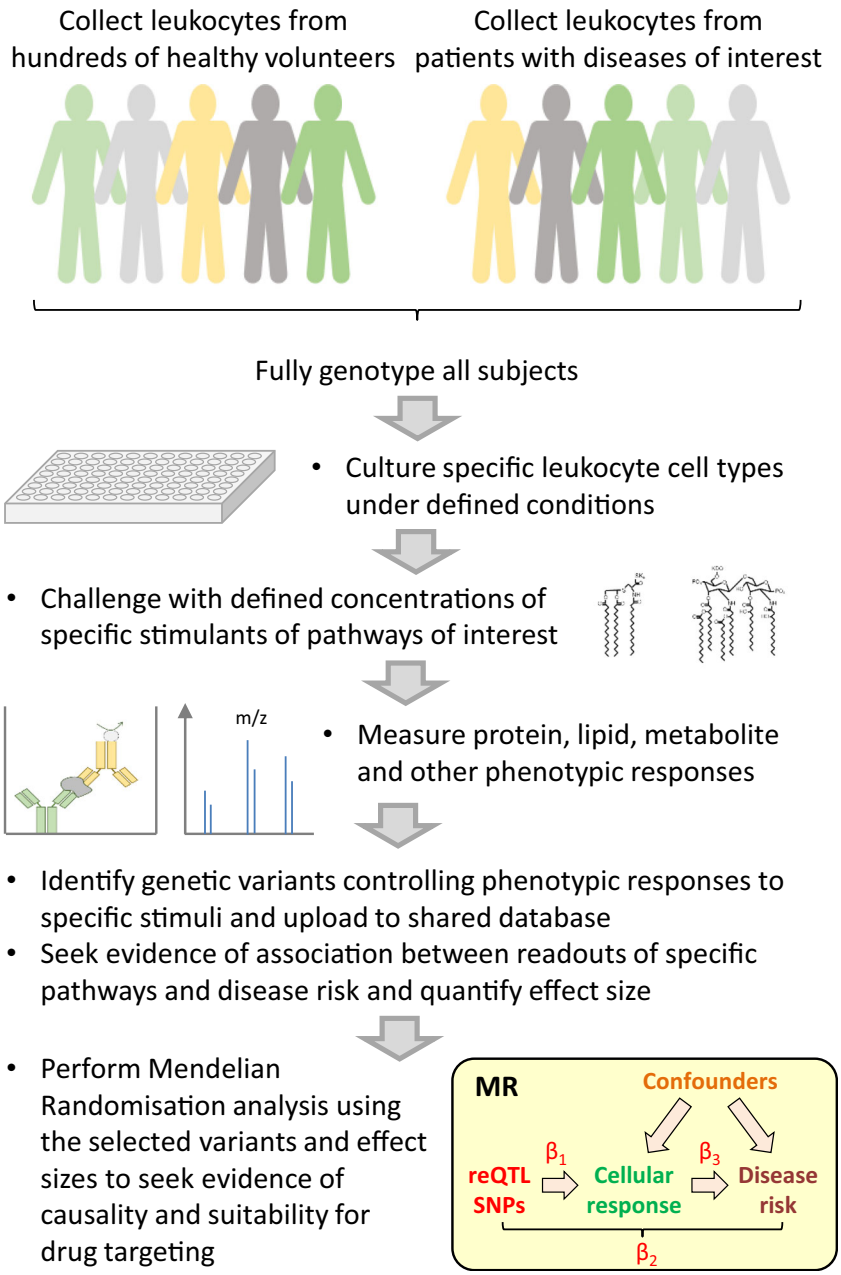

Fig. 3 Scheme for the systematic identification of reQTL SNPs and their use as genetic instruments in Mendelian randomisation studies to prioritise novel targets for drug discovery. The suggested process for the parallel discovery of reQTLs and evidence of response/disease associations is shown schematically. This approach should enable the identification of reQTLs which regulate production of multiple mediators downstream of a specific receptor, and also (different) variants which regulate production of a single mediator downstream of multiple pathways

lower risk of developing infections. Thus, given the application of sensible precautions, TLR2-inhibitors could yet have utility for CAD risk reduction.

Caveats and considerations for the application of cell-signalling MR

A number of caveats should be borne in mind in the design or interpretation of studies based on cell-signalling MR. First, as mentioned above, variants which regulate induced levels of protein, rather than mRNA, will likely be of most utility, since abundance of the former often bears little relation to levels of transcript (Vogel and Marcotte 2012). Experience to date suggests that larger sample sizes will be required to identify protein reQTLs, compared to transcript reQTLs (Fairfax et al. 2014; Kim et al. 2014; Larsen et al. 2013; Lee et al. 2014; Li et al. 2016; Vogel and Marcotte 2012), presumably because protein levels are subject to additional layers of complexity in postranscriptional regulation. Likewise, SNPs regulating stimulus-dependent effects on cellular phenotypes, such as metabolism, proliferation, migration or differentiation could offer yet greater insight, but will likely be harder to find. Responsiveness to stimuli,

rather than absolute levels of elicited mediators, may also be more useful predictors of disease risk, since many conditions are caused or exacerbated by responses to environmental triggers.

A second key issue is the requirement to choose genetic instruments which do not exhibit pleiotropy with other pathways. In the context of reQTLs, this may be best achieved by focussing on SNPs which modify the function of receptors directly, their most proximal downstream effectors, or the ultimate response mediators, and avoiding those which regulate mediators shared by numerous signalling pathways in between (as shown schematically in Fig. 2). Such targeting should enable the identification of genetic variants which regulate production of multiple mediators via a specific receptor, and also (different) variants which regulate production of a single mediator downstream of multiple pathways. Both will likely offer useful insight into disease mechanisms.

MR analyses of reQTLs will also need to draw upon a large database of measurements of stimulus-dependent readouts from cells of both healthy individuals and patients affected by diseases of interest, in order to quantify the effects of propensity to increased activity of specific pathways on disease risk (Fig. 3). Just as in previous major collaborations of this type, such as the ENCODE study (Gerstein et al. 2012), the development of this database will require a priori agreement on standard procedures for cell isolation, culture conditions, ligand concentrations, timepoints and methods for measurements of readouts. To date, very little such data are available, particularly in large cohorts. However, as protein reQTL-focused initiatives, such as the Human Functional Genomics Project (HFGP), are extended to recruit well-defined patient cohorts, this data will likely become a rich resource to aid the prioritisation of drug targets, particularly with respect to TLR-dependent cytokine production (Netea et al. 2016).

Another key consideration for the analysis of reQTLs in disease is that these variants typically exert their effects on cellular functions only in the presence of a particular stimulus, to which not everyone in a given population may be exposed. Successful reQTL MR may therefore require analysis of defined subgroups of individuals deemed to be at highest risk of exposure to the stimulus of interest. In the context of exposure to TLR2 or TLR4 stimulants, this might include, for example, subgroups based on history of bacterial infections, tobacco use or dietary habits. For the same reason, it is worth bearing in mind that reQTLs with potentially large effect sizes may not have emerged in GWAS analyses to date due to lack of power through dilution by subjects not exposed to the stimulus. Indeed, it remains possible that lipid SNPs dominate the results of CAD GWAS to date because all subjects in the populations examined are exposed to serum lipids, so maximising the power for detection of these SNPs.

Conclusions

Increasing genetic and experimental evidence suggests that TLR-signalling, particularly via TLR2 and/or TLR4, is likely to modify CAD risk in man. However, testing whether these or other candidate intracellular-signalling pathways may be causally related to disease is not feasible using eQTLs for circulating biomarkers in MR analyses. Instead, protein-level reQTLs are emerging as more effective genetic instruments for use in MR studies to test causality of specific intracellular-signalling pathways, with respect to disease risk. Such approaches have potential not only to help prioritise potential targets for drug development but also to give early indication of possible side effects of pathway modulation and insight into those genotypes most likely to benefit from therapy.

Further insight into disease mechanisms using reQTL-based MR analyses will require progress in two key areas. First, the systematic identification of protein-level reQTLs using stimulants of disease-related pathways in large-scale screens of relevant cell-types from genotyped donors is required. Second, epidemiological studies of disease risk will need to be expanded to include measurement of stimulus-dependent cellular readouts, under standardised culture conditions. Initiatives such as the Human Functional Genomics Project look set to make great contributions to meeting these needs in the near future (Netea et al. 2016), and we should encourage their extension to include disease-relevant cohorts and cell stimulants.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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