Human LACC1 increases innate receptor-induced responses and a \textit{LACC1} disease-risk variant modulates these outcomes

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Functional consequences for most inflammatory disease-associated loci are incompletely defined, including in the \textit{LACC1} (C13orf31) region. Here we show that human peripheral and intestinal myeloid-derived cells express laccase domain-containing 1 (LACC1); LACC1 is expressed in both the cytoplasm and mitochondria. Upon NOD2 stimulation of human macrophages, LACC1 associates with the NOD2-signalling complex, and is critical for optimal NOD2-induced signalling, mitochondrial ROS (mtROS) production, cytokine secretion and bacterial clearance. LACC1 constitutively associates with succinate dehydrogenase (SDH) subunit A, and amplifies pattern recognition receptor (PRR)-induced SDH activity, an important contributor to mtROS production. Relative to LACC1 Ile254, cells transfected with Crohn’s disease-risk LACC1 Val254 or LACC1 with mutations of the nearby histidines (249,250) have reduced PRR-induced outcomes. Relative to LACC1 Ile254 carriers, Val254 disease-risk carrier macrophages demonstrate decreased PRR-induced mtROS, signalling, cytokine secretion and bacterial clearance. Therefore, LACC1 is critical for amplifying PRR-induced outcomes, an effect that is attenuated by the \textit{LACC1} disease-risk variant.
Inflammatory bowel disease (IBD) is characterized by dysregulated host–microbial interactions and cytokine production. Microbial recognition and responses are mediated by host pattern recognition receptors (PRRs). Either loss-of-function or gain-of-function in PRR-mediated signalling and downstream outcomes can be associated with intestinal inflammation, highlighting the balance in regulation of PRR-mediated outcomes as critical in intestinal tissues. An important role for host–microbial interactions is further highlighted by Crohn’s disease (CD)-associated loss-of-function polymorphisms in NOD2 (refs 1,2; encoding NOD2, a PRR that recognizes bacterial peptidoglycan) as well as in additional pathways regulating microbial clearance mechanisms, such as autophagy and NADPH complex-mediated generation of reactive oxygen species (ROS) (refs 3,4). Despite the success in identification of IBD-associated loci, altered functions for most of the variants in these loci are unknown or not well defined. One such region is at chromosome 13q14 (C13orf31), encompassing the LACC1 gene.

Polymorphisms in LACC1 are associated with CD, ankylosing spondylitis, leprosy and juvenile idiopathic arthritis (refs 5–11). The rs3764147 G risk allele results in an amino-acid change from isoleucine to valine at position 254 in laccase domain-containing protein 1 (LACC1). LACC1 homologues (for example, cytotoxic necrotizing factor (CNF)) in bacteria, fungi and plants can function as virulence factors and in apoptosis prevention (refs 12,13). Laccase domain-containing proteins exhibit enzymatic activity, including oxidase/reductase activity, against various substrates such as polyphenols, aromatic amines and select inorganic ions (ref 14). Polyphenols mediate a number of host-protective functions (refs 14–16). As such, laccase domain-containing proteins have also been shown to confer protection to various organisms in which they are expressed (ref 17). To date, laccase domain-containing proteins have been a particular focus for industry, including the textile, environmental, pharmaceutical and food industries (ref 14). Given the importance of host–microbial interactions in intestinal immune homeostasis, and the critical role of PRRs in recognizing and responding to microbes, we questioned whether LACC1 regulates PRR-initiated outcomes in human myeloid-derived cells. We further questioned whether the IBD risk polymorphism in LACC1 resulting in an Ile254Val amino-acid change modulates PRR outcomes in a genotype-dependent manner.

We identify that LACC1 is expressed in both peripheral and intestinal human myeloid-derived cells and is required for optimal PRR-induced mitochondrial ROS (mtROS) and ROS production, MAPK and NFκB pathway activation, cytokine secretion and intracellular bacterial clearance in primary human monocyte-derived macrophages (MDMs). We define mechanisms and structural regions in LACC1 regulating these LACC1-dependent functions, as well as a reduction in each of these functions in MDMs from LACC1 Val254 (rs3764147 G) IBD risk, relative to Ile254 non-risk carriers. Taken together, we identify roles for human LACC1, and establish loss-of-function consequences for the LACC1 Val254 IBD risk variant, thereby resulting in decreased amplification of PRR-induced mtROS, signalling, cytokines and bacterial clearance.

**Results**

**LACC1 Val254 risk MDMs show reduced PRR-induced cytokines.**

Given the importance of regulating PRR-initiated outcomes in intestinal immune homeostasis and the dysregulation in PRR pathway outcomes that can be observed in IBD (ref 1), we questioned whether the rs3764147 genotype in human LACC1 modulates PRR-initiated outcomes in human MDMs. As NOD2 is associated to CD, we treated MDMs from 100 healthy individuals with muramyl dipeptide (MDP), the minimal bacterial peptidoglycan component activating NOD2 (ref 18). We examined interleukin (IL)-1β-secreted protein, which amplifies PRR-mediated signalling and cytokine secretion in MDMs (ref 19) and is elevated in tissues from IBD patients (ref 20). We normalized IL-1β protein secretion to untreated cells and log2-transformed the data. LACC1 Val254 disease-risk carriers (rs3764147 G carriers) secreted less IL-1β protein relative to Ile254 carriers (rs3764147 AA; Fig. 1a). This was most pronounced at low MDP doses (Fig. 1a). The pro-inflammatory cytokine tumour-necrosis factor (TNF; Supplementary Fig. 1A) was similarly regulated. Anti-inflammatory cytokines can sometimes be regulated in a reciprocal manner; however, the anti-inflammatory cytokine IL-10 was regulated similarly to the pro-inflammatory cytokines (Supplementary Fig. 1D). Moreover, less IL-1β (Fig. 1b), TNF (Supplementary Fig. 1B) and IL-10 (Supplementary Fig. 1E) secretion was observed in MDMs from LACC1 Val254 carriers upon stimulation with the TLR2 ligand, Pam3Cys.

Microbial products activate multiple PRRs, and in addition to macrophages, can activate PRRs on dendritic cells. In a separate cohort of 98 healthy individuals, LACC1 Val254 risk carrier monocyte-derived dendritic cells (MDDCs) secreted lower levels of IL-1β, TNF and IL-10 protein upon stimulation of NOD2, TLR2, TLR4 and TLR5 (Fig. 1c and Supplementary Fig. 1F). Therefore, myeloid cells from LACC1 Val254 risk carriers secrete less PRR-induced cytokines than LACC1 Ile254 carriers.

**Figure 1 | Myeloid cells from LACC1 Val254 disease-risk carriers demonstrate decreased PRR-induced cytokine secretion.** Human MDMs (n = 100) were treated for 24 h with (a) 1, 10 or 100 μg ml⁻¹ MDP, or (b) 1, 10 or 100 μg ml⁻¹ Pam3Cys. (c) Human MDDCs (n = 98) were treated for 24 h with 1 μg ml⁻¹ MDP (NOD2 ligand), 1 μg ml⁻¹ Pam3Cys (TLR2 ligand), 0.01 μg ml⁻¹ lipid A (TLR4 ligand) or 0.5 ng ml⁻¹ flagellin (TLR5 ligand) for 24 h. Shown is fold IL-1β-secreted protein induction (log₂-transformed) upon PRR stimulation stratified on rs3764147 genotype. Error bars depict s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001; determined by 2-tailed t-test.
LACC1 expression increases with PRR stimulation. We next assessed LACC1 expression in human MDMs. Upon NOD2 stimulation of human MDMs, LACC1 mRNA expression increased within 6 h, with elevated expression persisting over 12 h (Fig. 2a). LACC1 protein expression similarly increased with NOD2 stimulation as assessed by both western blot (Fig. 2b) and flow cytometry (Fig. 2c). To confirm the specificity of the antibody, we utilized short interfering RNA (siRNA) to LACC1 and observed significant attenuation of LACC1 expression (Fig. 2c,e) expression. Finally, we examined human intestinal myeloid-derived cells, given the association of polymorphisms in LACC1 with IBD. LACC1 was expressed in these tissue-relevant cells to an even higher level than in peripheral myeloid-derived cells (Fig. 2f), consistent with the ongoing PRR ligand exposure in this environment and the ability of PRR stimulation to increase LACC1 expression. Therefore, LACC1 is expressed in both human intestinal and peripheral myeloid-derived cells, with expression increasing upon PRR stimulation.

LACC1 contributes to PRR-induced cytokines. We next sought to clearly establish that LACC1 is required for regulating PRR-induced cytokines. Upon effective knockdown of LACC1 (Fig. 2c–e), NOD2-induced pro-inflammatory and anti-inflammatory cytokine secretion was reduced (Fig. 3a). The cells were viable (Supplementary Fig. 2B), and anti-inflammatory cytokine secretion through dectin ligands was not affected with LACC1 knockdown (Supplementary Fig. 2C). We confirmed these results with three additional siRNAs to LACC1 (Supplementary Fig. 2D,E). Importantly, we determined that LACC1 was required for optimal cytokine secretion upon stimulation of multiple PRRs (Fig. 3b). We ensured that with LACC1 knockdown, the expression of each of these PRRs was not altered (Supplementary Fig. 3A). LACC1 region polymorphisms are associated with leprosy9, and we found that LACC1 was also required for optimal cytokine secretion by whole-cell *Mycobacterium leprae* lysate and its cytosolic and membrane fractions (Supplementary Fig. 4A). The dependency on LACC1 extended to additional mycobacteria, in particular to *M. tuberculosis* components (Supplementary Fig. 4B). Of note is that we did not detect mycobacteria (16s rDNA for *M. tuberculosis*, *M. leprae* and *M. avium, subspecies paratuberculosis*) in whole blood from 200 healthy controls (n = 85, 76, 39 Ile254 homozygotes, Ile/Val254 heterozygotes and Val254 homozygotes, respectively) or 40 CD patients (n = 18, 16 and 6 Ile254 homozygotes, Ile/Val254 heterozygotes and Val254 homozygotes, respectively). Therefore, LACC1 is required for optimal cytokine secretion by multiple PRRs and mycobacterial components, thereby highlighting LACC1 as a shared pathway mediating responses to these microbes.
LACC1 increases NOD2-induced mitochondrial-derived ROS. We next questioned the mechanisms through which LACC1 regulates PRR-induced signalling and cytokines. CNF1, a bacterial homologue of LACC1, can prevent apoptosis by altering mitochondrial function in host cells. Mitochondria can contribute to PRR-induced outcomes through various mechanisms, including through production of mtROS\(^2\). We found that LACC1 was expressed in both the mitochondrial and cytosolic cellular fractions (Fig. 4c). The isolated mitochondrial fraction did not demonstrate expression of the ER marker protein disulfide isomerase (PDI), whereas PDI was present in whole lysate (Supplementary Fig. 7A). LACC1 homologues in bacteria and fungi exhibit oxidase/reductase activity against polyphenols; polyphenol end products can modulate ROS production. LACC1 knockdown reduced mtROS upon NOD2 stimulation in MDMs (Fig. 4d). Total cellular ROS was similarly reduced (Fig. 4e). MtROS is known to contribute to PRR-induced cytokines through various mechanisms. We confirmed that mtROS contributed to NOD2-induced cytokine secretion by two independent approaches, through use of the mtROS scavenger Mito-Tempo and knockdown of the voltage-dependent anion channel 3 (VDAC3) (Supplementary Fig. 7C), which regulates mtROS. In both cases, we observed the expected decrease in NOD2-induced mtROS and cellular ROS (Supplementary Fig. 7D,E), which was in turn accompanied by a decrease in NOD2-induced signalling (Supplementary Fig. 7F,G), and cytokines (Supplementary Fig. 7H,I). Cell viability was intact under both these conditions (Supplementary Fig. 7J,K). Furthermore, LACC1 was required for optimal mtROS and ROS induction upon stimulation through a range of PRRs (Supplementary Fig. 8A). We ensured that the cells were viable under these conditions (Supplementary Fig. 8B,C). Therefore, consistent with the LACC1 regulation of broad NOD2-induced signalling pathways, LACC1 modulates a broad range of NOD2-induced transcripts.

LACC1 modulates a broad range of NOD2-induced transcripts. As LACC1 regulates both NOD2-induced MAPK and NFκB pathways, and these signalling pathways in turn regulate a broad range of NOD2-induced transcripts, we postulated that in addition to its regulation of PRR-induced cytokines, LACC1 would regulate a broad spectrum of NOD2-induced transcripts. We focused on the NOD2-upregulated transcripts identified through microarray in ref. 25, and found that the upregulation of these transcripts was impaired with LACC1 knockdown (Supplementary Fig. 6A,C). To demonstrate the MAPK/NFκB signalling dependency of these transcripts, we examined these same transcripts upon NOD2 stimulation while inhibiting MAPK and NFκB pathways and found that NOD2-induced transcript upregulation was similarly impaired (Supplementary Fig. 6A,C). We ensured that the cells were viable under these conditions (Supplementary Fig. 6B). As a control for specificity of LACC1 knockdown effects, we examined dectin-induced transcripts, as we had found that dectin-induced IL-10 did not depend on LACC1 (Supplementary Fig. 2C). A subset of NOD2-induced transcripts was not upregulated with dectin stimulation (Supplementary Fig. 6A). However, we selected transcripts previously reported to be regulated by dectin\(^2\), and found that dectin-induced transcripts were not significantly altered with LACC1 knockdown (Supplementary Fig. 6D), including transcripts that were also induced upon NOD2 stimulation (Supplementary Fig. 6C). Therefore, consistent with the LACC1 regulation of broad NOD2-induced signalling pathways, LACC1 modulates a broad range of NOD2-induced transcripts.

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outcomes observed with MDP treatment were clearly dependent on the NOD2 pathway, as knockdown of both NOD2 (Supplementary Fig. 3) and the NOD2 adaptor molecule RIP2 (Supplementary Fig. 10A) led to a loss of the increased LACC1 expression, mtROS, ROS, signalling and cytokines observed with MDP treatment (Supplementary Fig. 10B–E).

LACC1 Val254 or His249,250Ala reduces PRR-induced outcomes. We next sought to define structural requirements in LACC1 for the LACC1-mediated mechanisms we had identified to contribute to PRR-induced outcomes. Human LACC1 is a 430-amino-acid protein, and has a sequence identity ranging from ~85% in dogs to ~34% in bacteria; the sequence identity of the human laccase domain (also referred to as the Cu-oxidase-4 domain) within LACC1 ranges from ~91% to ~33% over these same species (Supplementary Fig. 11A). Interestingly, the CD risk Val254 variant is highly conserved across species (Supplementary Fig. 11B), and the amino acid at position 254 in LACC1 is in proximity to histidines at positions 249 and 250 (Supplementary Fig. 11B,C). Crystal structures of fungal and bacterial laccase domain proteins have demonstrated a requirement for select histidines in mediating metal binding29–31. Further, the histidine at position 250 in human LACC1 is a conserved histidine (Supplementary Fig. 11B) found to form a metal-binding groove in a homologous bacterial protein through crystal structure

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Figure 4 | LACC1 is required for optimal PRR-induced signalling and mtROS production. (a,b) MDMs (n = 4) were transfected with scrambled or LACC1 siRNA, and then treated for 15 min with 100 μg ml⁻¹ MDP. Left: representative flow cytometry plots with MFI values for (a) phospho-ERK, phospho-p38 and phospho-JNK, and (b) phospho-IkBα. Right: fold phospho-protein induction normalized to untreated cells (represented by the dotted line at 1) ± s.e.m. Similar results were observed for an additional n = 8. In a subset of individuals for a,b we simultaneously confirmed reduced LACC1 expression with LACC1 siRNA by western blot (Supplementary Fig. 5A) and flow cytometry. (c) MDMs were fractionated into cytoplasmic (cyto) and mitochondrial (mito) compartments and assessed for LACC1 expression. Shown is a representative western blot for LACC1 in two of six individuals. Tubulin and MTCO2 were used as cytoplasmic and mitochondrial loading controls, respectively. (d,e) MDMs were transfected with scrambled or LACC1 siRNA, and then treated with 100 μg ml⁻¹ MDP for 6 h and assessed for; (d) mtROS (n = 4) and (e) cellular ROS (n = 4). Shown is mean ± s.e.m. Similar results were seen in an additional n = 8 donors. In a subset of individuals for d,e we simultaneously confirmed reduced LACC1 expression with LACC1 siRNA by western blot (Supplementary Fig. 7B) and flow cytometry. scr, scrambled; Tx, treatment. **P < 0.01; ***P < 0.001; ††P < 1 × 10⁻⁴; †††P < 1 × 10⁻⁵; determined by 2-tailed t-test.
To ensure that it was specifically the change from Ile to Val at the 254 amino-acid position of LACC1 that led to the modulation in PRR-induced cytokines between MDMs from LACC1 Ile254 versus LACC1 Val254 carriers (Fig. 1 and Supplementary Fig. 1), we hypothesized both LACC1 Ile254 and LACC1 Val254 variants. Moreover, to assess the role of the nearby histidines, which we hypothesized to be required for optimal LACC1 function, we generated LACC1 in which the histidines at positions 249 and 250 were mutated to alanines. We transfected the LACC1 Ile254, Val254 and His249,250Ala variants into HEK293 cells, which did not express LACC1 histidines at positions 249 and 250 were mutated to alanines. We transfected the LACC1 Ile254, Val254 and His249,250Ala variants into HEK293 cells, which did not express LACC1 variants into HEK293 cells, along with NOD2, so as to assess their regulation of NOD2-induced outcomes. The LACC1 variants were expressed at equal levels in HEK293 cells (Fig. 5a), indicating that neither the Ile254Val nor the His249,250Ala mutations altered LACC1 expression. LACC1 Ile254 increased NOD2-induced mtROS and cellular ROS generation (Fig. 5b), MAPK and NFκB activation (Fig. 5c), AP-1 and NFκB transcriptional activity (Fig. 5d), and IL-6 secretion (Fig. 5e). Neither the Val254 variant nor the His249,250Ala variant enhanced these NOD2-induced outcomes as effectively (Fig. 5b–e). To confirm that the signalling and transcriptional activity observed upon MDP treatment was mediated by NOD2, we used a NOD2 construct with the loss-of-function LeufsinsC (Cins) mutation associated with CD. As expected, signalling and AP-1 and NFκB transcriptional activity were significantly decreased upon MDP treatment with this NOD2 variant in combination with LACC1 Ile254 (Fig. 5c,d). We further confirmed the AP-1 and NFκB transcriptional results at a lower dose of MDP treatment (Supplementary Fig. 12). Therefore, consistent with the decreased PRR-induced cytokine secretion in primary MDMs from LACC1 Val254 carriers, mutating Ile254 to Val254 in LACC1, as well as mutating the nearby histidines, led to decreased NOD2-induced mtROS, signalling and cytokine secretion in cell lines transfected with these variants.
LACC1 mediates optimal intracellular bacterial clearance. In addition to their contribution to signalling pathways, mtROS and ROS can play an important role in microbial clearance. Given the critical role of microbial clearance in intestinal immune homeostasis, and that mechanisms contributing to impaired bacterial clearance can enhance risk for human IBD⁹–⁵, we questioned whether LACC1 contributes to bacterial clearance. Intestinal macrophages demonstrate enhanced microbial clearance relative to peripheral macrophages. The chronic stimulation through PRRs, including through NOD2, that occurs in the intestinal environment contributes to this enhanced microbial clearance. Therefore, we considered LACC1 contributions to microbial clearance in unstimulated MDMs and in MDMs after prolonged NOD2 stimulation, with a focus on intestinal-relevant bacteria, given the association of LACC1 with CD. We first confirmed that both mtROS and ROS are required for optimal intracellular clearance of bacteria in both unstimulated macrophages and chronic NOD2-stimulated macrophages. The mtROS and ROS requirement was observed for clearance of the intestinal pathogen Salmonella typhimurium, adherent invasive Escherichia coli (AIEC), which are enriched in the ilea of CD patients, and Staphylococcus aureus (Fig. 6a). Knockdown of LACC1 impaired the intracellular clearance of these bacteria in unstimulated macrophages, as well as the enhanced bacterial clearance observed after the chronic NOD2 stimulation that simulates conditions in the intestinal environment (Fig. 6b). We further confirmed the role of NOD2 under these MDP-treated conditions through both NOD2 and RIP2 knockdown (Supplementary Fig. 13B). Therefore, LACC1 is required for the enhanced intracellular bacterial clearance observed in macrophages after chronic NOD2 stimulation.

LACC1 amplifies PRR-induced succinate dehydrogenase activity. We next sought to further define the mechanisms through which LACC1 modulates PRR-induced mtROS, and in turn, subsequent signalling and cytokine secretion. We considered mitochondrial enzymes critical in the generation of mtROS with which LACC1 might interact. SDHA is localized in the inner mitochondrial membrane and is a major subunit of succinate-ubiquinone oxidoreductase, a complex of the mitochondrial respiratory chain important for mtROS generation, such that we considered SDHA as a candidate through which LACC1 could cooperate with to modulate mtROS. Endogenous LACC1 and SDHA associated with each other in human MDMs (Fig. 7a); this association was not modulated by NOD2 stimulation (Fig. 7a). Importantly, succinate dehydrogenase (SDH) activity increased with NOD2 stimulation and this increase was impaired upon LACC1 knockdown (Fig. 7b). Through SDHA knockdown (Supplementary Fig. 14B,C), we confirmed that SDHA was required for optimal NOD2-induced SDH activity (Fig. 7c), mtROS and cellular ROS (Fig. 7d), signalling (Fig. 7e) and cytokine secretion (Fig. 7f). Cells were viable with SDHA knockdown (Supplementary Fig. 14D). Having identified a role for LACC1 in increasing SDH activity in human MDMs, we next questioned whether the generated LACC1 mutants regulated these effects. In HEK293 cells transfected with LACC1 Ile254, LACC1 associated with SDHA constitutively (Fig. 7g). Similar to primary MDMs (Fig. 7a), this association was not altered with NOD2 stimulation (Fig. 7g). However, SDH enzyme activity increased with NOD2 stimulation in the presence of LACC1 Ile254 (Fig. 7h). The LACC1 Val254 and LACC1 His249,250Ala variants did not lead to altered association with SDHA relative to the LACC1 Ile254 variant (Fig. 7g). However, in comparison to the LACC1 Ile254 variant, the LACC1 Val254 disease-risk variant and the LACC1 His249,250Ala mutant demonstrated less effective NOD2-induced SDH activity upon transfection into HEK293 cells (Fig. 7h). Therefore, LACC1 associates with SDHA and cooperates to enhance NOD2-induced SDH activity, with the SDHA pathway leading to increased mtROS and cytokine secretion.

LACC1 contributes to NOD2-induced complex assembly. As the LACC1 association with SDHA is constitutive rather than inducible (Fig. 7), we sought to identify NOD2-induced post-translational modifications (for example, phosphorylation, acetylation) on LACC1 that might be contributing to LACC1 function, or additional upstream protein associations or outcomes that might be mediating its regulation of mtROS, ROS, signalling and cytokine secretion with NOD2 stimulation. Upon NOD2 stimulation we observed tyrosine phosphorylation of a band corresponding to the molecular weight of LACC1 and of proteins associated in a complex with LACC1 (Supplementary Fig. 15A). We identified three tyrosine motifs at positions 52, 89 and 265 associated in a complex with LACC1 (Supplementary Fig. 15A). We next sought to further define the mechanisms through which LACC1 modulates PRR-induced mtROS, and in turn, subsequent signalling and cytokine secretion. We considered mitochondrial enzymes critical in the generation of mtROS with which LACC1 might interact. SDHA is localized in the inner mitochondrial membrane and is a major subunit of succinate-ubiquinone oxidoreductase, a complex of the mitochondrial respiratory chain important for mtROS generation, such that we considered SDHA as a candidate through which LACC1 could cooperate with to modulate mtROS. Endogenous LACC1 and SDHA associated with each other in human MDMs (Fig. 7a); this association was not modulated by NOD2 stimulation (Fig. 7a). Importantly, succinate dehydrogenase (SDH) activity increased with NOD2 stimulation and this increase was impaired upon LACC1 knockdown (Fig. 7b). Through SDHA knockdown (Supplementary Fig. 14B,C), we confirmed that SDHA was required for optimal NOD2-induced SDH activity (Fig. 7c), mtROS and cellular ROS (Fig. 7d), signalling (Fig. 7e) and cytokine secretion (Fig. 7f). Cells were viable with SDHA knockdown (Supplementary Fig. 14D). Having identified a role for LACC1 in increasing SDH activity in human MDMs, we next questioned whether the generated LACC1 mutants regulated these effects. In HEK293 cells transfected with LACC1 Ile254, LACC1 associated with SDHA constitutively (Fig. 7g). Similar to primary MDMs (Fig. 7a), this association was not altered with NOD2 stimulation (Fig. 7g). However, SDH enzyme activity increased with NOD2 stimulation in the presence of LACC1 Ile254 (Fig. 7h). The LACC1 Val254 and LACC1 His249,250Ala variants did not lead to altered association with SDHA relative to the LACC1 Ile254 variant (Fig. 7g). However, in comparison to the LACC1 Ile254 variant, the LACC1 Val254 disease-risk variant and the LACC1 His249,250Ala mutant demonstrated less effective NOD2-induced SDH activity upon transfection into HEK293 cells (Fig. 7h). Therefore, LACC1 associates with SDHA and cooperates to enhance NOD2-induced SDH activity, with the SDHA pathway leading to increased mtROS and cytokine secretion.

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Cells were then treated with 100 μg ml⁻¹ MDP and assessed for SDH activity at 2 h. Similar results were observed in an additional n = 8. In a subset of individuals we simultaneously confirmed reduced LACC1 expression with LACC1 siRNA by western blot (Supplementary Fig. 14A) and flow cytometry. (c-f) MDMs were transfected with scrambled or SDHA siRNA, and then treated with 100 μg ml⁻¹ MDP and assessed for: (c) SDH activity at 2 h (n = 4), (d) mtROS and cellular ROS at 6 h (n = 4; similar results in an additional n = 4), (e) fold phospho-protein induction normalized to untreated cells at 15 min (n = 4) as assessed by flow cytometry, (f) cytokines at 24 h (n = 4). (g-h) LACC1 Ile254, Val254 and His249,250Ala variants were transfected into HEK293 cells and is required for the optimal assembly of NOD2-signalling intermediates using a reciprocal immunoprecipitation approach (Supplementary Fig. 16A). With LACC1 knockdown, the assembly of this complex was impaired (Fig. 8). Each intermediate in this complex was required for optimal NOD2-induced mtROS, ROS, signalling, cytokines and bacterial clearance (Supplementary Figs 10C–E, 13B and 16B–E). Therefore, LACC1 associates with and is required for the optimal assembly of NOD2-signalling intermediates following NOD2 stimulation.

Polyphenol oxidase activity contributes to PRR outcomes.
We sought to further define whether a subset of LACC1-regulated functions observed in human MDMs might be attributable to the enzymatic activity exhibited by this family of proteins. Bacterial and fungal laccase-containing proteins can oxidize polyphenols. Mitochondrial-targeted polyphenols can, in turn, modulate mtROS levels. We were unable to observe polyphenol oxidase activity in vitro with human LACC1 protein purified under a variety of conditions and assessed against multiple different laccase substrates. In contrast, we observed copper-dependent polyphenol oxidase activity in side-by-side studies utilizing E. coli CueO laccase, which we generated as a positive control, as well as activity utilizing a fungal laccase from Rhus vernicifera as has been previously described.
We next postulated that human polyphenol oxidase activity would likely be tightly regulated within the cellular environment in which it is functioning, such that we sought to detect this activity under appropriate stimulation conditions in cells. We therefore examined if polyphenol oxidation was regulated by NOD2 stimulation in human MDMs, and if this, in turn, was modulated by LACC1. We observed increased oxidation of two common laccase substrates, syringaldazine and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), by cellular lysates from NOD2-stimulated MDMs (Fig. 9a). We further observed oxidation of two mitochondrial-associated polyphenols44,45 with NOD2 stimulation, genistein and resveratrol (Fig. 9a). Furthermore, this NOD2-induced polyphenol oxidase activity was impaired with LACC1 knockdown (Fig. 9a). To determine whether polyphenol oxidase activity contributed to the LACC1-dependent outcomes we observed in MDMs, we identified two polyphenol oxidase inhibitors, kojic acid and salicylhydroxamic acid, used in prior studies to inhibit laccase-enzymatic activity. There are likely other mitochondrial- and its ability to regulate cellular polyphenol oxidase activity and its contribution to the optimal assembly of a complex of signalling intermediates induced upon NOD2 stimulation, and its ability to regulate cellular polyphenol oxidase activity and SDH enzymatic activity. There are likely other mitochondrial-dependent and -independent mechanisms through which LACC1 might modulate both PRR-dependent and -independent functions in macrophages, as well as other cell subsets in which LACC1 might regulate distinct outcomes. Increased inflammation associated with loss-of-function in innate pathway outcomes is commonly observed; decreased innate function can result in an induced polyphenol oxidase activity, whereas the LACC1 Val254 disease-risk variant and the LACC1 His249,250Ala mutant demonstrated less effective NOD2-induced polyphenol oxidase activity (Fig. 9f).

**LACC1 Val254 MDMs have lower NOD2-induced outcomes.** We next questioned whether the rs3764147 polymorphism in LACC1 (resulting in an Ile254Val mutation in G risk carriers) led to genotype-dependent regulation of the LACC1 functions we had identified to modulate PRR-initiated outcomes. We first assessed whether MDMs from LACC1 Ile254, Val254 or heterozygote carriers demonstrated differences in LACC1 expression. LACC1 protein expression was equivalent between MDMs from these individuals (Fig. 10a), consistent with our findings that transfection of LACC1 Ile254 and Val254 variants into HEK293 cells expressed at equivalent levels (Fig. 5a). The recruitment of LACC1 to and assembly of the complex of proximal intermediates upon NOD2 stimulation was also equivalent between LACC1 Ile254 and Val254 carrier MDMs (Supplementary Fig. 18). In contrast, and consistent with the decreased NOD2-induced polyphenol oxidase activity, mtROS and ROS production, signalling and cytokines in LACC1 Val254-transfected HEK293 cells (Figs 5 and 9f), MDMs expressing the endogenous LACC1 Val254 variant demonstrated lower NOD2-induced polyphenol oxidase activity (Fig. 10b), mtROS and cellular ROS production (Fig. 10c), and activation of the MAPK (Fig. 10d) and NFκB (Fig. 10e) pathways relative to LACC1 Ile254 MDMs. These results are consistent with the decreased NOD2- and PRR-initiated cytokine secretion observed in MDMs from LACC1 Val254 carriers (Fig. 1 and Supplementary Fig. 1). Heterozygote individuals were intermediate or similar to homozygote Val254 risk individuals in the measures assessed (Fig. 10b–e), also consistent with the PRR-initiated cytokine secretion results in Fig. 1. Finally, consistent with the lower NOD2-induced mtROS and ROS production in MDMs from LACC1 Val254 carriers, MDMs from LACC1 Val254 carriers demonstrated decreased intracellular clearance of *S. typhimurium*, *A. IEC* and *S. aureus* (Fig. 10f). Therefore, relative to LACC1 Ile254 carriers, MDMs from LACC1 Val254 risk carriers demonstrate decreased NOD2-induced polyphenol oxidase activity, mtROS and cellular ROS, downstream signalling, cytokine secretion and intracellular bacterial clearance.

**Discussion** We establish that LACC1 is expressed in human peripheral and intestinal myeloid-derived cells, define roles for human LACC1 in amplifying PRR-induced outcomes in macrophages, dissect mechanisms wherein LACC1 contributes to PRR outcomes and determine that the CD- and leprosy-associated LACC1 Val254 risk variant results in lower PRR-initiated polyphenol oxidase activity, mtROS and cellular ROS, signalling, cytokine secretion, broad transcriptional regulation and intracellular bacterial clearance (Supplementary Fig. 19). We established a number of mechanisms through which LACC1 regulates these outcomes, including its contribution to the optimal assembly of a complex of signalling intermediates induced upon NOD2 stimulation, and its ability to regulate cellular polyphenol oxidase activity and SDH enzymatic activity.
inefficient elimination of microbes, thereby leading to compensatory inflammatory responses. As such, we observe that LACC1 is required for the enhanced microbial clearance observed after chronic NOD2 stimulation, conditions that simulate those in the intestinal environment; intestinal macrophages from NOD2-deficient mice demonstrate impaired microbial clearance. In addition to the attenuated production of mtROS and cellular ROS that we now identify in LACC1 risk carriers, decreased ROS production has been observed with NADPH oxidase complex variants associated with early-onset IBD, and with chronic granulomatous disease, a disease in which a substantial percentage of individuals develop intestinal inflammation. In contrast to the decreased responses observed with CD-associated NOD2, the LACC1 Val254 risk variant decreases responses through a broad range of PRRs. Our findings identify a role for human LACC1, and implicate the LACC1 disease-risk variant as a loss-of-function in...
Figure 10 | LACC1 Val254 risk carrier MDMs show reduced LACC1-dependent outcomes with NOD2 stimulation. MDMs from LACC1 Ile254, Ile/Val254 and Val254 carriers (rs3764147 AA, GA and GG carriers, respectively) were treated with 100 μg ml⁻¹ MDP and assessed for: (a) LACC1 expression at 12 h. Representative western blot and summary of densitometry ± s.e.m. for n = 9 per genotype. (b) Polyphenol oxidase activity at 6 h (n = 15 per genotype). Mean ± s.e.m. (c) mtROS (n = 8 per genotype) and cellular ROS (n = 7 per genotype) at 6 h. Mean ± s.e.m. (d) Phospho-ERK, phospho-p38 and phospho-JNK, and (e) phospho-IκBα at 15 min with representative flow cytometry plots showing MFI values and summarized data represented as fold phospho-protein induction normalized to untreated cells ± s.e.m. (n = 8 per genotype). (f) Intracellular clearance of S. typhimurium, AIEC or S. aureus at 48 h. CFU ± s.e.m. (n = 10 per genotype). Tx, treatment. *P < 0.05; **P < 0.01; ***P < 0.001; ††P < 1 × 10⁻⁴; †††P < 1 × 10⁻⁵; determined by 2-tailed t-test.
**Methods**

**Patient recruitment and genotyping.** Informed consent was obtained as per the protocol approved by the institutional review board at Yale University, and healthy controls were recruited for peripheral blood draw. Given the limitation in peripheral cell numbers and the range of innate responses we sought to examine, two separate cohorts of 100 and 98 individuals were studied for NOD2/TLR2 dose–response studies in MDMs, and PRR-induced cytokine studies in MDDCs respectively. We performed genotyping by TaqMan SNP genotyping (Applied Biosystems, Foster City, CA) or Sequenom platform (Sequenom Inc., San Diego, CA).

**mRNA expression analysis.** 

**Phospho-flow.** Phospho-protein induction in permeabilized cells was determined using Alexa Fluor 647-, phycoerythrin-, Alexa Fluor 488- or biotin-labelled antibodies to phospho-Erk (clone D13.14.4E, diluted 1:1,000), phospho-p38 (clone 3D7, diluted 1:1,000), phospho-JNK (clone 9G, diluted 1:1,000) or phospho-IκBα (clone 14D4, diluted 1:1,000; all Cell Signaling) by flow cytometry.

**Polyphenol oxidation activity.** ABTS and syringaldazine oxidation in cell lysates suspended in PBS was measured at 420 and 530 nm, respectively, as previously described. Oxidation of genistein and resveratrol (ThermoFisher Scientific) was monitored at 400 and 320 nm, respectively.

**SDH activity.** SDH activity was assayed in purified mitochondria resuspended in 35 mM potassium phosphate monobasic buffer (pH 7.3; ref. 53). Dsodium succinate (10 mM; Sigma) and 40 μM M M 2.6-dichlorophenolindophenol sodium salt hydrate (DCPIP; Sigma) was added to the mitochondria at 37°C C, and DCPIP extinction was measured at 610 nM as described in ref. 53.

**Intracellular bacterial killing.** Cells were infected with S. typhimurium (10:1), AIEC strain LF82 (10:1; generous gift from Dr. Emiko Mizoguchi) or S. aureus (1:1) for a total of 2 h, with gentamicin added 1 h after bacterial infection. In some cases, cells were pretreated for 1 h with 50 nM Mito-Tempo (Santa Cruz) or 20 nM N-acetylcysteine (Sigma).

**Statistical analysis.** Significance was assessed using two-tailed Student’s t-test. P<0.05 was considered significant. Error bars are shown as s.e.m.

**Data availability.** The data supporting the findings of this study are available within the article and its Supplementary Information files.

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A.E., M.J., F.Y. and C.A. designed, performed and analysed the experiments. M.H. and C.A. wrote the manuscript.

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