Review Article

Innate Immune Recognition of Mycobacterium tuberculosis

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Tuberculosis (TB), caused by Mycobacterium tuberculosis (MTB), is a major health problem, with 10 million new cases diagnosed each year. Innate immunity plays an important role in the host defense against MTB, and the first step in this process is recognition of MTB by cells of the innate immune system. Several classes of pattern recognition receptors (PRRs) are involved in the recognition of MTB, including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and Nod-like receptors (NLRs). Among the TLR family, TLR2, TLR4, and TLR9 and their adaptor molecule MyD88 play the most prominent roles in the initiation of the immune response against tuberculosis. In addition to TLRs, other PRRs such as NOD2, Dectin-1, Mannose receptor, and DC-SIGN are also involved in the recognition of MTB. Human epidemiological studies revealed that genetic variation in genes encoding for PRRs and downstream signaling products influence disease susceptibility, severity, and outcome. More insight into PRRs and the recognition of mycobacteria, combined with immunogenetic studies in TB patients, does not only lead to a better understanding of the pathogenesis of tuberculosis but also may contribute to the design of novel immunotherapeutic strategies.

1. Introduction

Tuberculosis (TB) is a major public health problem, with 10 million new cases diagnosed each year, causing a death toll of 2 million victims. However, from the estimated 2 billion persons individuals that have been initially infected with Mycobacterium tuberculosis, only 5% to 10% develop symptomatic TB.

The reason why some infected individuals develop active disease while others do not is not yet entirely understood. The role of inborn variability in susceptibility to tuberculosis has been accidentally proven by an episode that occurred almost a century ago, when in 1926 newborn infants from the town of Lübeck in Germany received live Mycobacterium tuberculosis (MTB) instead of the vaccine bacillus Calmette-Guérin (BCG). Some of the children became gravely ill, while others were unaffected [1]. This finding indicates that at least some individuals display an effective immune response to MTB and that this plays an important part in determining the outcome of the infection. In addition, this episode in young infants known to have immature adaptive immunity also suggests that the innate host defense is an important arm of antmycobacterial host defense.

Much has been learned during the last decade on the mechanisms through which the immune response to MTB is initiated. The first step is the recognition of mycobacteria as invading pathogens, followed by activation of innate host defense responses, and the subsequent initiation of adaptive immune responses. Knowledge about these processes is crucial for understanding the pathophysiology of tuberculosis, on the one hand, and for the development of novel strategies of vaccination and treatment such as immunotherapy on the other hand. This paper focuses on the first step of the immune response, which is the recognition of mycobacteria by cells of the innate immune system.

Initiation of the innate immune response starts with pattern recognition of microbial structures called pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs is performed by germline-encoded receptors expressed mainly on immune cells termed pattern recognition receptors (PRRs) [2]. The first step in understanding the mechanisms of recognition of pathogenic bacteria is
Man-LAM, LM, and PIMs all share a conserved mannosyl-glycoproteins [4]. Mannan and arabinomannan are present on the cell surface, is an important virulence factor of MTB [6]. Man-LAM is a heterogeneous lipoglycan with a characteristic tripartite structure of a carbohydrate core, the MPI anchor, and various mannosyl-capping motifs. These mannosyl-capped motifs are characteristic for all pathogenic mycobacteria, and they are not present on fast-growing mycobacterial strains which are significantly less pathogenic. These strains have either uncapped LM or have phospho-my-o-inositol caps (PILAM), which are known to display more robust immunostimulatory effects. PIMs can be divided into two groups dependent on the mannose content, which determines its immunogenic effect [7, 8]. Also present on the cell surface are the mannosylglycoproteins, which can also be secreted during growth.

1.2. Innate Immunity and Host Defense. After the inhalation of infected aerosols into the lungs of the host, the first encounter of mycobacteria is with alveolar resident macrophages. Mycobacteria that escape the initial intracellular destruction can multiply and disrupt the macrophage, after which chemokines are released, attracting monocytes and other inflammatory cells to the lung. Inflammatory monocytes will differentiate into macrophages, which readily ingest but do not destroy the mycobacteria [9]. In this stage of the infection, the mycobacteria grow logarithmically and blood-derived macrophages accumulate, but little tissue damage occurs. Two-to-three weeks after infection, T-cell immunity develops and antigen-specific T lymphocytes arrive, proliferate within the early lesions or tubercles, and release proinflammatory cytokines such as interferon-γ (IFNγ) that will activate macrophages to kill the intracellular mycobacteria. Subsequently, the early logarithmic bacillary growth stops, and central solid necrosis in these primary lesions or granuloma inhibits extracellular growth of mycobacteria. Several scenarios may follow, with infection becoming stationary or dormant in some individuals, or progressive in the lung, or with hematogenous dissemination in a minority of patients. In addition, reactivation can occur months or years afterwards, under conditions of failing immune surveillance [9]. Granuloma often contains central caseous necrotic tissue, which gives rise to cavities and aerogenic spread of mycobacteria.

The macrophage is a pivotal cell in these events, as it is involved in phagocytosis and killing of mycobacteria as well as in the initiation of adaptive T-cell immunity. Phagocytosis of MTB involves different receptors such as the scavenger receptors, the mannose receptor (MR), and complement receptors [10-13]. Phagocytosis can involve both uptake of the bacilli after opsonization with complement factors, or it can be initiated as a nonopsonic event. In vitro experiments have shown that complement receptor 3 (CR3) mediates approximately 80% of complement-opsonized MTB phagocytosis [12]. Nonopsonic phagocytosis is an important process in the primary infection of the lung, because complement factors are largely absent in the alveolar space [14].

Macrophages can eliminate mycobacteria through different mechanisms, such as production of reactive oxygen and nitrogem species, acidification of the phagosome, and phagosome fusion with the lysosomes [9]. The fate of intracellular mycobacteria is also influenced by autophagy, a cellular process though which cytoplasmic components, including organelles and intracellular pathogens, are sequestered in a double-membrane-bound autophagosome and delivered to the lysosome for degradation [15]. Activation of autophagy leads to phagosome maturation, an increased acidification in the phagosome, and killing of mycobacteria in macrophages.
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its stimulatory effects on TNFα production in macrophages [31, 38]. In turn, an important role for TLR2 and TLR6 but not TLR4 or TLR9, was found for the stimulation of IL-1β production [43]. TLR2 is also important for IL-12 release in macrophages, but not in DCs [44]. TLR2-/- mice show defective granuloma formation, and when infected with high doses of MTB, they have a greatly enhanced susceptibility to infection compared to the WT mice [45, 46]. In addition, TLR2-/- mice display defects in controlling chronic infection with MTB [46].

TLR4 is activated by heat shock protein 60/65 [37, 47], a protein that is secreted by a variety of MTB species. Studies with TLR4 transfected CHO cells and murine macrophages showed the importance of TLR4 in recognition of MTB [36, 39]. Macrophages of TLR4-deficient mice showed less, but not completely abolished, TNFα production. In vivo murine studies on the role of TLR4 in the recognition of MTB have shown conflicting results, even when the same mouse strain was used. Reiling et al. showed that TLR4-deficient mice, in contrast to TLR2 deficient mice, showed similar susceptibility to MTB infection compared to wild-type animals [45]. In contrast, Abel et al. reported higher mycobacterial outgrowth in lungs, spleen, and liver and a lower survival following infection compared to wild-type animals [48]. More studies are necessary to elucidate the source of these discrepancies and the role of TLR4 for MTB infection.

**Figure 2:** Pattern recognition receptors in the recognition of mycobacteria and downstream signaling pathways. Mycobacteria can be recognized through different pattern recognition receptors (PRRs) of the host. Both intracellular and extracellular receptors are involved in this process. After recognition of mycobacteria, intracellular signaling cascades are activated which eventually will lead to the activation of transcription of NF-κB. After transcription, the production of pro- and anti-inflammatory cytokines and chemokines is induced. The type of signaling cascade induced depends mainly on the type of PRR that recognizes (components of) MTB.
TLR9 recognizes unmethylated CpG motifs in bacterial DNA. In vitro studies showed that MTB-induced IL-12 release in dendritic cells was TLR9-dependent [38, 44]. In vivo experiments showed that when mice were infected with a high infectious dose of MTB, animals lacking TLR9 succumbed earlier to infection than wild-type animals [38].

TLR8 is able to recognize single-stranded DNA from pathogens such as RNA viruses. Interestingly, Davila et al. demonstrated upregulation of TLR8 protein expression in macrophages after infection with BCG [40]. Until now, this is the only study addressing a potential role of TLR8, but the mechanism through which TLR8 recognizes MTB and signals intracellular remains unknown.

A partially redundant role of TLRs for the host defence against mycobacteria has been suggested, and it has been hypothesized that defects in multiple TLRs are necessary to unveil the role of these receptors for antimycobacterial defense. Indeed, TLR2 and TLR9 double knockout mice display greater defects of IL-12 and IFN-y production in comparison with both single TLR knockout mice, and they succumb earlier to infection even when infected with a low inoculum of MTB [38].

2.2. NOD Like Receptors. The NOD like receptors (NLRs) family of proteins highly resembles the family of plant R (resistance) proteins, which have a crucial role in the defence against plant pathogens. The mammalian NLR family consists of more than twenty members with a conserved structure. The core of the molecule is formed by the nucleotide-binding domain, named NACHT (NAIP, CIITA, HET-E, and TP-1 [49]) or NOD (nucleotide oligomerization domain) domain. The C-terminal part consists of a series of leucine-rich repeats, which are thought to recognize the PAMPs of the pathogen and initiate activation of the molecule. The N-terminal portion of the molecule contains an effector domain of CARD (caspase activation and recruitment domain), PYRIN, or BIR (baculovirus inhibitor of apoptosis repeat domain) [50]. CARD-containing NLRs such as NOD1 and NOD2 are thought to form oligomers and then to recruit receptor-interacting protein 2 (RIP2) (or CARD containing kinase—RICK) through CARD-CARD interactions, which leads to the recruitment of NF-kB [51].

A major signalling pathway for the activation of the antimycobacterial host defense is represented by the inflammasome, that through activation of caspase-1 leads to processing of procytokines of the IL-1 family into the bioactive IL-1β and IL-18. Several PYRIN-domain containing NLRs (NALPs) can form different variants of the inflammasome containing either NLRP1, NLRP3 (cryopyrin), or NLRC4 (Ipaf) [52], as well as the adaptor protein ASC [53, 54]. A fourth type of inflammasome formed by the intracellular protein AIM2 is activated by intracytoplasmic DNA [55]. A recent study has shown that induction of IL-1β production by MTB is mediated by TLR2/TLR6 and NOD2 receptors, while caspase-1 is constitutively activated in human primary monocytes [43]. This is in contrast with the study of Master et al. that suggested that MTB inhibits inflammasome activation and IL-1β production [56]. However, these studies are not completely comparable as the latter study has used murine macrophage cell lines, in contrast to the human primary cells used by the former study. In addition, if MTB would inhibit IL-1β production even in normal hosts, this could not explain the increased susceptibility to infection of IL-1R-deficient mice [22].

NOD2 is an intracellular receptor-mediated stimulation of proinflammatory cytokine production by MTB. NOD2 is a receptor for bacterial peptidoglycans [57], and recently, we demonstrated its role in the recognition of mycobacteria [58, 59]. NOD2-deficient mice showed impaired production of proinflammatory cytokines and nitric oxide when infected with MTB. However, the susceptibility to MTB infection of NOD2-deficient mice is variable [60, 61].

2.3. C-Type Lectins. C-type lectins are a family of PRRs involved in the recognition of polysaccharide structures of pathogens. The mannose receptor (MR, CD206) consists of eight linked carbohydrate recognition domains and one cysteine-rich domain. MR is highly expressed on alveolar macrophages [62]. Mycobacterial stimulation through MR leads to production of the anti-inflammatory cytokines IL-4 and IL-13, inhibition of IL-12 production, and failure to activate oxidative responses [63, 64]. Man-LAM and other major components of the MTB cell wall like PIMs are natural mycobacterial ligands for MR. In addition, binding of MTB to MR induces phagocytosis, but phagosome-lysosome fusion is limited [65–67].

Differences at the level of mannosylation between MTB strains may also contribute to recognition by C-type lectins. Torrelles and Schlesinger showed that differences in virulence between MTB strains could be related to expression of ManLAM on the cell wall [4]. Virulent MTB strains with less surface mannosylation do not use MR for phagocytosis but rely primarily for recognition and phagocytosis on CR3 after opsonisation. These strains are virulent because they display more other cell envelope components (like phenolic glycolipids and triacylglycerols) [68, 69]. These cell components regulate the cytokine response and demonstrate rapid intracellular growth and marked tissue damage [70, 71]. On the contrary, heavily mannosylated MTB strains such as the laboratory strain H37Rv use the MR receptor during invasion of the cell and are associated with a higher survival within the macrophage and an anti-inflammatory cytokine response. It is speculated that this type of recognition might lead to a latent stage of infection [4]. This might not be the case for all mycobacterial species; a mutant Mycobacterium bovis strain, which entirely lacked surface mannose, showed a comparable cytokine profile as the nonmutant did [72].

2.4. DC-SIGN. Dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN, CD209) plays an important role in MTB-DC interaction. This receptor is mainly expressed on DCs and serves as both a PRR and an adhesion receptor, due to its functions in DC migration and DC-T-cell interactions [73, 74]. The carbohydrate recognition domain of DC-SIGN recognizes Man-LAM and lipomannans and the amount of Man-LAM determines the binding strength [64]. Recently, it was shown that α-glucan
2.5. Dectin-1. Dectin-1 is a receptor with an extracellular carbohydrate recognition domain and an intracellular ITAM domain. This receptor is mainly expressed on macrophages, DCs, neutrophils, and a subset of T-cells. Dectin-1 mainly recognizes β-glucans present in fungal pathogens, but it has been suggested to play an important role in MTB recognition as well. The precise PAMP that leads to the recognition through dectin-1 is not known although some species of MTB express α-glucan on the cell surface [77] as a ligand for dectin-1. Murine bone marrow-derived macrophages infected with either virulent or avirulent mycobacteria produce TNF-α and IL-6 in a dectin-1-independent or dectin-1-dependent manner, respectively [78]. A study with DCs isolated from spleens showed that dectin-1 triggers the production of IL-12 [79]. Several studies have shown synergistic effects between TLR2 and dectin-1 for the recognition of fungal pathogens [80, 81], but this remains to be demonstrated in case of mycobacteria. Finally, a recent report showed that dectin-1, independent of TLR2 recognition, is important for the innate immunity recognition of MTB and for inducing Th1 and Th17 responses [82].

3. Recognition of Mycobacterium tuberculosis—Human Genetic Studies

In order to have a complete picture of the role of PRRs for the host defense to MTB, the results of in vitro and animal studies need to be corroborated with studies in patients. The association of host genetic factors with susceptibility or resistance to TB has been studied extensively with candidate gene approaches and genome-wide association studies. These analyses have revealed several important candidate genes for susceptibility to TB [83, 84]. For the scope of this paper this section is limited to PRRs and their signaling pathways only. Table 1 shows an overview of investigated SNPs with or without association with TB.

The TLR2 gene is located on chromosome 4q32 and is composed of two noncoding exons and one coding exon [85]. More than 175 SNPs for the human TLR2 have been reported. In a Turkish cohort, an association between Arg753Gln and susceptibility to TB [86] was reported, while this was not confirmed in two Asian cohorts due to the absence of this particular polymorphism in these populations [87, 88]. Arg753Gln seems to be present only in Caucasian populations, with percentages ranging from 0 to 0.49% in East Asian populations [87, 89–91]. In a Tunisian cohort, Arg677Thr showed an association with susceptibility to TB [92], but these results were put in doubt by the discovery of a pseudogene on which this SNP seems to be located [93].

The TLR2 genotype 597CC has been correlated with susceptibility to TB, especially with disseminated forms of the infection (miliary and meningitis) caused by a particular MTB genotype family (“the Beijing genotype”), in a cohort of patients from Vietnam [107, 108]. A highly polymorphic guanine-thymine repeat, located 100 base pairs upstream of the TLR2 translation start site in intron 2, was correlated with promoter activity and the expression of TLR2 on CD14+ PBMCs (the shorter the repeat, the weaker the promoter activity and the lower the expression of TLR2) for both tuberculosis and nontuberculosis mycobacterial lung infections in a Korean cohort [109, 110]. However, these data could not be reproduced in a Taiwanese population [111]. Another variation in genotype that seems to influence TLR2 expression is −196 to −174 insertion/deletion, with a recent study displaying an association with TB, while another study showed a possible effect on development of systemic symptoms [96, 111]. Many other polymorphisms in human TLR2 are examined for their association with enhanced susceptibility to TB, but this requires further confirmation [96].

Since TLR1 and 6 form heterodimers with TLR2, SNPs in these receptors might influence TLR2 signalling as well. One example is Ile602Ser SNP in TLR1, which leads to aberrant TLR1 cell trafficking, no functional TLR1 on the cell surface, and which might influence the mycobacterial recognition [112]. The 602I variant is overexpressed in African-Americans infected with TB [94]. In addition, an association between the TLR6 SNPs Ser249Pro and Thr361Thr and MTB-induced cytokine production has been shown [100].

Immunogenetic studies have reported in two other TLR genes: TLR4 and TLR8. In these genes, the genetic variation associated with susceptibility to TB seems to be less pronounced.

TLR4 Asp299Gly SNP showed an association with TB in HIV positive Caucasians and Tanzanians, but not in a Gambian population [97–99]. TLR8 has always been linked with recognition of viral PAMPs, but in an immunogenetic study in Indonesia, the TLR8 gene, which is located on the X chromosome, was the only gene showing an association with TB. This finding was confirmed in a second much larger cohort from Russia and supported by functional data, as discussed above [40]. Further studies are needed to confirm these findings.

Besides the PRR receptor polymorphisms, SNPs in the TLR signaling pathways may also influence susceptibility to MTB. Khor et al. proposed that the Ser180Leu SNP in the gene coding for TIR domain-containing adaptor protein (TIRAP) was associated with a higher susceptibility to TB in a cohort from West Africa [101] although the frequency of the mutant allele was very rare. However, this association could not be confirmed in a study involving 9000 individuals from Ghana, Russia and Indonesia [102].

Regarding the other PRRs important for MTB recognition, the 871G and 336A variants located in the promoter region of DC-SIGN were associated with protection against tuberculosis in a South African cohort of patients [103]. This finding was, however, not confirmed in a Tunisian cohort [105], while a later study even showed an association in opposite direction (a protective effect of 336G)
Table 1: SNPs associated with susceptibility to tuberculosis.

<table>
<thead>
<tr>
<th>Receptor/signalling pathway</th>
<th>Gene</th>
<th>Amino acid</th>
<th>Association</th>
<th>No association</th>
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<td>TLR1</td>
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<td>Lle602Ser</td>
<td>[94]</td>
<td>[95]</td>
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<td>[86]</td>
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<td>[96]</td>
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<td>Ser180Leu</td>
<td>[101]</td>
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</table>

* I/D insertion/deletion.

Furthermore, genetic variation of the neck region of DC-SIGN (which supports the carbohydrate recognition domain) failed to show an association with tuberculosis susceptibility [105, 113].

4. Conclusions and Future Research

Pattern recognition of MTB is a complex process in which a multitude of receptors recognize specific PAMPs of the microorganism. Recognition by specific receptors is followed by different intracellular signalling pathways, in order to integrate and induce an efficient activation of the innate host defense mechanisms. While activation through TLRs, NLRs and dectin-1 initiates essentially a proinflammatory response, signalling through the C-type lectins DC-SIGN or MR have mainly a modulatory function. The interplay between these pathways lead to finely tuned response of the immune system during the encounter with MTB.

One has to acknowledge that both in vitro and in vivo studies suffer from specific limitations, which may at least partly explain some discrepancies between experimental and immunogenetic studies in TB patients. In vitro studies use various cell types, murine macrophages (bone-marrow derived or alveolar), DCs or PBMCs. This can influence the outcome due to the preferential expression of specific receptors on different cell types. A second limitation is that in most in vitro studies only a single receptor is examined, isolated from its physiological environment, while the interplay between different pathways is probably one of the most relevant aspects of pathogen recognition. The role of the innate immune receptors involved in MTB recognition has often been studied in transfected cell lines, while animal models deficient of specific receptors show that these receptors can compensate for each other and sometimes display redundant roles [114–116].

In vivo animal studies have the disadvantage that the most commonly used murine models do not represent human TB; granuloma are not formed in these models, which is a crucial step in the latency of this disease. Rabbit and monkey models which are more similar with human TB are rarely used. Even human genetic studies have limitations in terms that these studies often lack the translation at the level of protein function, while in other situations, an important gene is highly conserved and lacks functionally relevant genetic variants that can be assessed.

While pattern recognition is an important component of the host response to infection with MTB, other factors are relevant as well, including the intrinsic capacity of macrophages to kill MTB, the distribution and function of different T-cell subsets, and regenerative and fibrotic tissue responses. These particular aspects were beyond the scope of this paper.

Humans and MTB have coevolved for millennia, and it is likely that a close relationship exist at the genomic level. Indeed, two studies have shown a direct association between the genetic characteristics of patients with tuberculosis and
their mycobacterial isolates [108, 117]. Polymorphisms in either TLR2 and SLC11A1 (NRAMP1) were associated with higher change of being infected with strains belonging to the evolutionary successful *M. tuberculosis* Beijing genotype. Globally, *M. tuberculosis* shows strong geographical differences [118, 119], and this might be triggered by evolutionary pressure from the innate immune system (“coevolution”). Besides *M. tuberculosis*, also, host immune gene polymorphisms show strong geographical differences. The studies of Caws et al. and Van Crevel et al. [108, 117] provide support for the hypothesis that evolutionary adaptation of particular *M. tuberculosis* lineages to certain human populations. For instance, in the case of TLR2 in the study of Caws et al., a certain *M. tuberculosis* genotype family might have a higher or lower affinity for TLR2 expressed in individuals with a particular TLR2 genotype, leading to differences in downstream signalling and subsequent events after recognition of *M. tuberculosis*. Clearly, this concept needs to be investigated in terms of innate immune recognition by examining a number of PRR genes in TB patients in relation to their infective *M. tuberculosis* genotypes. Comparing host-mycobacterial genotype relationships of more successful *M. tuberculosis* genotypes like the Beijing family and less successful genotypes will help increase the understanding of the concept of “coevolution”, virulence and innate host defense to *M. tuberculosis*.

Other important new areas of research related to innate immunity have been initiated recently, and their relationship with tuberculosis remains to be answered. One of the important cellular responses associated with anticytocidal defense has been suggested to be the process of autophagy. Autophagy has been also shown to modulate the inflammation [120], especially through its interaction with the peptidoglycan receptor NOD2 [121, 122]. One important question to be answered is whether there is a role for autophagy in the induction of an inflammatory response by MTB. What is the explanation for the apparent redundancy in the pattern recognition, and which PRR is most important in which stage of the disease? Answers to these questions are needed in order to develop rational immunotherapeutic interventions like addition of TLR-agonists to candidate vaccines.

More can also be learned from studies in human patients. For instance, patients with advanced HIV-infection have virtually no T-cell immunity. However, even in settings which are hyperendemic for TB, some HIV-infected patients will never develop TB. Certainly, these individuals must have a very effective innate host response against MTB. A pivotal approach will be to combine genetic with functional studies; what does a SNP associated with susceptibility to TB mean in terms of the function of the immune response?

Another suggestion is to study an increased number of SNPs in more PRRs in the same population and to assess the cumulative effects of various combinations of SNPs to obtain a stronger association with disease. A striking observation is that only loss-of-function mutations are investigated. Could it be that gain-of-function mutations of PRRs might influence the immune response to MTB as well?

Finally, one of the most important challenges for the coming years is to translate the knowledge gained in the basic science of immune responses to mycobacteria into improved or novel immune-based treatment strategies, ranging from a better vaccine to immunotherapy.

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