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Choroid plexus transcriptome and ultrastructure analysis reveals a TLR2-specific chemotaxis signature and cytoskeleton remodeling in leukocyte trafficking

Amin Mottahedin^a, C. Joakim Ek^a, Katarina Truvé^b, Henrik Hagberg^{c,d}, and Carina Mallard^{a,*}

^aCentre of Perinatal Medicine and Health, Department of Physiology, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

^bBioinformatics Core Facility, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

^cCentre for the Developing Brain, Division of Imaging Sciences and Biomedical Engineering, King's College London, King's Health Partners, St. Thomas' Hospital, London, UK

^dCentre of Perinatal Medicine and Health, Department of Obstetrics and Gynecology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Abstract

Perinatal infection and inflammation are major risk factors for injury in the developing brain, however, underlying mechanisms are not fully understood. Leukocyte migration to the cerebrospinal fluid (CSF) and brain is a hallmark of many pathologies of the central nervous system including those in neonates. We previously reported that systemic activation of Toll-like receptor (TLR) 2, a major receptor for gram-positive bacteria, by agonist Pam3CSK4 (P3C) resulted in dramatic neutrophil and monocyte infiltration to the CSF and periventricular brain of neonatal mice, an effect that was absent by the TLR4 agonist, LPS. Here we first report that choroid plexus is a route of TLR2-mediated leukocyte infiltration to the CSF by performing flow cytometry and transmission electron microscopy (TEM) of the choroid plexus. Next, we exploited the striking discrepancy between P3C and LPS effects on cell migration to determine the pathways regulating leukocyte trafficking through the choroid plexus. We performed RNA sequencing on the choroid plexus after administration of P3C and LPS to postnatal day 8 mice. A cluster gene analysis revealed a TLR2-specific signature of chemotaxis represented by 80-fold increased expression of the gene *Ccl3* and 1000-fold increased expression of the gene *Cxcl2*. Ingenuity pathway analysis (IPA) revealed TLR2-specific molecular signaling related to cytoskeleton organization (e.g. actin signaling) as well as inositol phospholipids biosynthesis and degradation. This included upregulation of genes such as *Rac2* and *Micall2*. In support of IPA results, ultrastructural analysis by TEM revealed clefting and perforations in the basement membrane of

*Corresponding author. carina.mallard@neuro.gu.se (C. Mallard).

Authors' contribution

AM, JE, HH and CM designed the experiments and analyzed the data. AM and JE performed the experiments. AM and KT analyzed the sequencing data. AM drafted the manuscript. All authors contributed to the writing of final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbi.2019.02.004>.

the choroid plexus epithelial cells in P3C-treated mice. In summary, we show that the choroid plexus is a route of TLR2-mediated transmigration of neutrophils and monocytes to the developing brain, and reveal previously unrecognized mechanisms that includes a specific chemotaxis profile as well as pathways regulating cytoskeleton and basement membrane remodeling.

Keywords

Choroid plexus; Inflammation; Infection; Leukocyte migration; Cytoskeleton; Basement membrane; Remodeling; Toll-like receptor; Inositol; Micall2

1 Introduction

Leukocyte trafficking to the brain and/or CSF is the hallmark of many pathological conditions of the CNS including neonatal and adult meningitis, encephalitis, multiple sclerosis and neonatal hypoxic-ischemic (HI) brain injury (Kim, 2010; Liesz et al., 2011; Ransohoff and Brown, 2012; Shechter et al., 2013). Although the leukocyte recruitment to the brain is considered a physiological response to a local infection or injury, pathological consequences of leukocyte presence in the brain are documented in studies in several models of neurological diseases (Ajami et al., 2011; Liesz et al., 2011; Liu et al., 2010; Zattoni et al., 2011). In a mouse model of neonatal HI brain injury, for example, it was shown that leukocytes infiltrate the brain from hours to several weeks after the insult (Albertsson et al., 2014; Winerdal et al., 2012). In an LPS-sensitized model of neonatal HI brain injury, blocking lymphocyte infiltration to the brain ameliorated the injury outcome (Yang et al., 2014) suggesting a detrimental effect of leukocyte presence in the brain following HI. Our recent study revealed that peripheral administration of a TLR2 agonist, Pam3CSK4 (P3C), 14 h before HI insult suppresses brain mitochondrial respiration and increases the injury in Perinatal infection and inflammation are major risk factors for injury in the developing brain, however, underlying mechanisms are not fully understood. Leukocyte migration to the cerebrospinal fluid (CSF) and brain is a hallmark of many pathologies of the central nervous system including those in neonates. We previously reported that systemic activation of Toll-like receptor (TLR) 2, a major receptor for gram-positive bacteria, by agonist Pam3CSK4 (P3C) resulted in dramatic neutrophil and monocyte infiltration to the CSF and periventricular brain of neonatal mice, an effect that was absent by the TLR4 agonist, LPS. Here we first report that choroid plexus is a route of TLR2-mediated leukocyte infiltration to the CSF by performing flow cytometry and transmission electron microscopy (TEM) of the choroid plexus. Next, we exploited the striking discrepancy between P3C and LPS effects on cell migration to determine the pathways regulating leukocyte trafficking through the choroid plexus. We performed RNA sequencing on the choroid plexus after administration of P3C and LPS to postnatal day 8 mice. A cluster gene analysis revealed a TLR2-specific signature of chemotaxis represented by 80-fold increased expression of the gene *Ccl3* and 1000-fold increased expression of the gene *Cxcl2*. Ingenuity pathway analysis (IPA) revealed TLR2-specific molecular signaling related to cytoskeleton organization (e.g. actin signaling) as well as inositol phospholipids biosynthesis and degradation. This included upregulation of genes such as *Rac2* and *Micall2*. In support of IPA results, ultrastructural analysis by TEM revealed clefting and perforations in the basement membrane of the

choroid plexus epithelial cells in P3C-treated mice. In summary, we show that the choroid plexus is a route of TLR2-mediated transmigration of neutrophils and monocytes to the developing brain, and reveal previously unrecognized mechanisms that includes a specific chemotaxis profile as well as pathways regulating cytoskeleton and basement membrane remodeling. neonatal mice (Mottahedin et al., 2017b). In neonatal and adult bacterial meningitis, infiltration of inflammatory cells to CSF is considered a diagnostic hallmark (Hoffman and Weber, 2009) but the mechanisms of leukocyte invasion and the effects on neurological outcome is yet to be fully understood. Epidemiological studies also suggest that neonatal sepsis and meningitis are risk factors for cerebral palsy, a neurological syndrome that can result from brain injury during the perinatal period (Drougia et al., 2007; Kulak et al., 2010).

Toll-like receptors (TLRs) are the main components of the innate immune system that recognize and react to infections or damage-induced molecules by signaling to downstream inflammatory pathways (Akira et al., 2006; Piccinini and Midwood, 2010). TLRs mediate recruitment of immune cells to the site of inflammation by their downstream products such as chemokines and cytokines. We previously showed that peripheral administration of the TLR2 ligand, P3C, induces marked infiltration of neutrophils and monocytes to the CSF and brain of neonatal mice. In contrast, administration of the TLR4 agonist LPS did not cause significant infiltration of cells, despite eliciting a similar level of peripheral inflammation as P3C (Mottahedin et al., 2017b). We also observed an increase in number of leukocytes at CNS immune cell trafficking sites, most notably the choroid plexus. Here we first confirm that the choroid plexus is a route of TLR2-induced neutrophil and monocyte infiltration to the CSF by flow cytometry and transmission electron microscopy. Next, we exploited the remarkable difference between P3C- and LPS-induced leukocyte migration to the CNS as a research strategy to hypothesize that genes regulated specifically by P3C in the choroid plexus are likely involved in leukocyte trafficking. RNA sequencing of the choroid plexus of neonatal mice injected with P3C or LPS revealed significant differences in expression level of hundreds of genes. Gene cluster analysis and ingenuity pathway analysis identified a TLR2-specific chemotaxis signature as well as genes related to cytoskeleton rearrangement. In agreement with these results, ultrastructural analysis showed a previously unrecognized phenomenon in the choroid plexus epithelium, basement membrane cleaving and perforation. Altogether, the results suggest novel mechanisms of leukocyte migration to the brain and potential therapeutic targets to ameliorate neuroinflammation induced by meningitis or other CNS pathologies.

2. Materials and methods

2.1. Animals

C57B1/6J mice were purchased from Charles River Laboratory (Germany). All animals were housed and bred at Laboratory for Experimental Biomedicine, University of Gothenburg, with ad libitum access to food and water and on a 12-hour light-dark cycle. All experiments were performed in accordance with the Swedish Board of Agriculture, and the experimental procedures were approved by the Gothenburg animal ethics committee.

2.2. Flow cytometry of the choroid plexus

Neonatal mice at postnatal day (P) 8 were treated intraperitoneally (i.p.) with 5 mg/kg P3C (Pam3CSK4, Invivogen) or saline and at 6 h and 14 h after treatment mice were given an overdose of thiopental, and perfused via the heart with PBS. Choroid plexuses were dissected out from lateral and forth ventricles under a microscope and placed in a petri dish with ice-cold PBS. Choroid plexus from two mice were pooled and enzymatically digested in collagenase type IV (Life Technologies) (450 unit per ml PBS, 45 min, 37 °C), and then triturated several times using a pipette (Baruch et al., 2015). Cells were passed through a 70 µm filter and washed with PBS. Unspecific binding sites were blocked by rat anti-mouse CD16/CD32 (BD Biosciences) for 30 min at 4 °C, followed by incubation with antibodies CD45 APC-Cy7 (Clone 30-F11; BD Pharmingen), CD11b FITC (Clone MI/70; eBiosciences), Ly6C PE-Cy7 (Clone AL-21; BD Pharmingen), Ly6G PE (Clone 1A8; BD Pharmingen) for 1 h at 4 °C. BD FACSCanto (BD Bioscience) was used for flow cytometry. Cells were gated based on forward and side scatter properties (Fig. 1A) followed by gating for live and single cells (Fig. 1B and C) and myeloid cells (Fig. 1D). Neutrophils were identified as CD45 + CD11b + Ly6C^{int}Ly6G + and inflammatory monocytes were identified as CD45 + CD11b + Ly6C^{hi}Ly6G- (Fig. 1E). Staining for neutrophils and monocytes was performed because these cells were most prevalent in the CSF after P3C administration (Mottahedin et al., 2017a).

2.3. Choroid plexus dissection for RNA sequencing

Eight-day old mice were injected i.p. with P3C (5 mg/kg body weight), LPS (0.3 mg/kg body weight) or saline. These dosages of P3C and LPS were chosen as they induce comparable systemic inflammation (Mottahedin et al., 2017a). Pups were sacrificed by an overdose of thiopental sodium followed by an incision in heart. Lateral ventricle choroid plexuses were dissected at 6 h and 14 h after P3C or LPS injections in RNase-free PBS and immediately transferred to RNeasy lysis buffer (Qiagen) and kept at 4 °C overnight. The time points were selected based on leukocyte migration kinetics to the CSF (Mottahedin et al., 2017a). The RNeasy lysis buffer was then removed and samples were frozen on dry ice and kept in -80 °C freezer until further process. Choroid plexuses from lateral ventricles of 5 pups were pooled for each sample.

2.4. RNA extraction and preparation

Total RNA was extracted using Trizol (Qiazol; Qiagen) and miRNAeasy Kit (Qiagen) according to the manufacturer's protocols. The quantity was assessed on a Nanodrop 2000 (Thermoscientific). RNA quality was checked using Experion RNA StdSense Chips (Bio-Rad) and by an Experion Automated Electrophoresis System (Bio-Rad). Five hundred ng RNA was used for library preparation using TruSeq Stranded mRNA and Total RNA Sample Preparation Kit (Illumina).

2.5. RNA sequencing data analysis

Libraries were sequenced on an Illumina NextSeq 500 with read length of 2 × 100 nucleotides. Approximately 30 million reads were obtained from each sample.

The quality of sequences was checked running the tool FastQC (v. 0.11.2). Mouse assembly mm10 was downloaded from UCSC and used as reference genome. The mapping was performed with the tool Star (v. 2.3.10) (Dobin et al., 2013). Flagstat files with statistics were produced with the tool Samtools (v. 0.1.19) (Li et al., 2009). Counts of reads mapped to each gene was performed with the tool htseq (v. 0.6.1) (Anders et al., 2015). Differential expression was assessed using DESeq2 (v. 1.2.10) (Love et al., 2014). The p-values were corrected for multiple comparisons applying the Benjamini-Hochberg procedure. For cluster analysis, DESeq2 package was used to normalize the count data and variance-stabilizing transformation of the count data was applied.

Dendrograms were created using Pearson correlation and complete-linkage clustering on genes with cut-off p-value of < 0.05 . The GO (gene ontology) terms were obtained for the gene clusters using DAVID (Huang da et al., 2009). Venny software (v 2.0) was used to compare the list of genes significantly regulated by different treatments. To understand the molecular pathways regulated specifically by P3C, differentially regulated genes were analyzed by Ingenuity Pathway Analysis (IPA, version 31813283, Qiagen) with a cut-off p-value of < 0.05 and no other filtering. The results were sorted based on the pathway activation z-score. The heatmap in Fig. 4 was obtained using ClustVis (Metsalu and Vilo, 2015).

2.6. Transmission electron microscopy

Eight-day old mice were injected i.p. with P3C (5 mg/kg), LPS (0.3 mg/kg) or saline. Mice were sacrificed at 14 h as described above. The choroid plexus from lateral ventricles were dissected out immediately in a Petri dish filled with modified Karnovsky fixation solution (2.5% glutaraldehyde, 2% paraformaldehyde, 0.05 M Na-cacodylate and 0.02% Na-azide). The tissues were post-fixed in the same solution for 2h room temperature, washed with 0.15 M Na-cacodylate in PBS and then post-fixed in 1% OsO₄ in water +1% K₄Fe in 0.15 M Na-cacodylate for 2 h at 4 °C. The tissues were then put in 0.5% Unranyl acetate in water for 15 min in the dark, dehydrated in graded ethanol series and embedded in agar 100 resin. Polymerization was performed by incubating the samples in 40 °C for 15 h and 60 °C for 48 h. Ultrathin sections were cut at 70 nm thickness using a Leica EM UC7 ultramicrotome, mounted on specimen grids and post-stained in 1% unranyl acetate for 5 min followed by 3 min incubation in Reynolds solution (1:7 lead acetate in 0.01 M NaOH). Images were acquired using a Talos L120C electron microscope equipped with 4 × 4k CMOS Ceta camera.

2.7. Statistics

The statistics for RNA sequencing data are described above. Flow cytometry data was analyzed by One-way ANOVA followed by Dunnett's post-hoc test to correct for multiple comparisons. Data are presented as Mean $\pm$ SEM and $p < 0.05$ considered as significant. Graphpad Prism v 6.01 was used for performing statistics.

3. Results

3.1. Choroid plexus is a route of TLR2-mediated leukocyte migration to the CSF

We previously showed that peripheral administration of P3C, but not LPS, results in neutrophil and monocyte infiltration to the CSF, which was associated with their presence in the periventricular compartments including the choroid plexus. (Mottahedin et al., 2017a). To confirm the latter, we performed flow cytometry (Fig. 1A–E) on dissected choroid plexuses at 6h and 14 h after P3C injection. There was an increase in number of neutrophils and inflammatory monocytes (Fig. 1F) at both time points. TEM ultrastructural analysis did not show infiltrating cells in the epithelium of the choroid plexus of mice treated with saline (Fig. 1G) or LPS (Fig. 1H). However, infiltrating leukocytes were observed in the epithelium of choroid plexus in P3C-treated mice (Fig. 1I).

3.2. RNA sequencing of choroid plexus after TLR ligand administration

To explore the mechanism of leukocyte trafficking through the choroid plexus induced by P3C, RNA sequencing on dissected choroid plexus was performed. To specify the pathways and genes underlying the leukocyte migration we compared the P3C-induced transcriptome to LPS-induced transcriptome. LPS at the stated dosage promotes a peripheral inflammatory response comparable to P3C but does not cause leukocyte infiltration to the CNS (Mottahedin et al., 2017a). First, to understand inter-group and intra-group variability we performed principal component (PC) analysis. The results showed a 49% variance in PC1 and 17% variance in PC2 (Fig. 2A). As expected the control samples (saline 6h and 14 h) were grouped together on PC1 axis. The TLR treatment groups were segregated although P3C 6 h and LPS 14 h samples showed slightly more variability. Administration of P3C and LPS led to significant regulation of thousands of genes at both 6 h and 14 h time points (Fig. 2B–E). In general, there were more genes regulated at 6 h (Fig. 2B and D for P3C and LPS respectively) compared to 14 h time point (Fig. 2C and E for P3C and LPS respectively), and more genes were upregulated than downregulated. The number of regulated genes was higher in the LPS groups at both time points.

3.3. Differential gene expression by P3C and LPS

The top 30 most significantly regulated genes (based on p-value) by P3C and LPS in comparison to control group are shown in Table 1. At the 6 h time point, NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) and TNF (tumor necrosis factor) related genes were the most upregulated by P3C (Table 1A). The most significantly upregulated cytokines and chemokines were *IL1b*, *Ccl3*, *Csfl* and *Ccl9* (Table 1A). Cell adhesion protein *Icam1* and tight junction/cytoskeleton remodeling protein *Micall2* (also called *Jrab*) were among top upregulated genes by P3C as well (Table 1A). Among top genes downregulated by P3C at 6 h were genes involved in cellular junction such as *Gjb2* and *Pcdhl8* (Table 1A). At 14h time point, genes related to leukocyte recruitment such as *Madcam1* top the list of upregulated genes by P3C compared to control (Table 1B). Among LPS-induced (LPS vs control) top upregulated genes were those related to interferon response (e.g. *Ifit2* and *Jr7*) at both time points (Table 1C and D). Similar to P3C, LPS also downregulated the expression of genes involved in cellular junctions, *Gjb2* and *Pcdhl8* (Table 1C and D).

Il1b, *Ccl3* and *MicaU2* were among top 10 genes differentially regulated by P3C compared to LPS at 6 h time point (Table 2A). At 14 h time point, *Marco* and *Madcam1* were top differentially regulated genes (Table 2B).

3.4. Cluster analysis and GO term ontology reveals TLR2-specific chemotaxis signature

To identify P3C- and LPS-specific GO terms, cluster analysis was performed at both time points on all genes significantly regulated by P3C and LPS when compared to saline (7249 genes, $p < 0.05$). The largest cluster of genes upregulated by LPS at 6 h (Fig. 3 and Table 3I) was related to immune response and defense to viruses. LPS also downregulated a vast cluster of genes related to phosphorylation and mitochondria (Fig. 3 and Table 3E). Interestingly, P3C specifically induced upregulation of a gene cluster corresponding to chemotaxis (Fig. 3 and Table 3G) mainly at the 6 h time point. P3C also partly induced upregulation of gene clusters related to cytoskeleton organization (Fig. 3 and Table 3C) and cilium morphogenesis (Fig. 3 and Table 3F) at 6h and 14 h time points respectively. At 14 h time point, P3C upregulated a cluster of genes related to receptor internalization and cell migration (Fig. 3 and Table 3H).

The P3C-specific chemotaxis signature (Fig. 3 and Table 3G) included 22 genes of which 21 genes were significantly upregulated by P3C at 6 h time point (Fig. 4). LPS also significantly regulated 19 genes within this GO term. However, P3C induced several folds more increase in expression of these genes. Strikingly, expression of *Ccl3* and *Cxcl2* were ~30 and ~50 times higher, respectively, in P3C group compared to LPS (Fig. 4).

3.5. Ingenuity pathway analysis (IPA) reveals TLR2-specific cytoskeleton remodeling pathways

To further identify biological pathways underlying leukocyte migration, P3C-specific genes were explored using Ingenuity Pathway Analysis (IPA). At 6h and 14 h time points 1064 and 687 genes were specifically regulated by P3C respectively compared to LPS (Fig. 5A and C). In agreement with gene cluster analysis results, IPA predicted activation of pathways involved in cytoskeleton organization including actin signaling (z-score = 3.05 at 6 h, z-score = 1.8 at 14 h; Fig. 5B and D and Fig. S1), phospholipase C signaling (z-score = 3; Fig. 5B and Fig. S2) and superpathway of inositol phosphate compounds (z-score = 2.85; Fig. 5B and Fig. S3) at 6 h time point as well as leukocyte extravasation signaling (z-score = 2.1; Fig. 5D and Fig. S4) at 14 h time point.

3.6. TLR2-specific basement membrane cleaving and perforation

To validate IPA results suggesting P3C effects on cytoskeleton-related pathways TEM was performed to assess ultrastructure of choroid plexus. P3C did not induce changes in tight junction structure (Fig. 6A), neither any marked changes in the structure of cilia (Fig. 6B). However, in P3C-treated mice cleaving, vacuolization and extensive perforations were observed in the basement membrane of the choroid plexus epithelium (Fig. 6C–E, Supplementary video 1).

4. Discussion

Leukocyte migration to the CNS is a hallmark of many neurological diseases including neonatal encephalopathy (Smith et al., 2018). Leukocyte accumulation in CSF and subarachnoid spaces is also observed in some CNS pathologies, most markedly in meningitis (Wilson et al., 2010). The mechanisms by which leukocytes pass through the barriers of the brain and their role in progression of neurological diseases remain yet to be elucidated. The choroid plexus and CSF both harbor immune cells under physiological conditions and therefore the choroid plexus is suggested as a “gate” to the CNS where immune cells can cross from the blood to the brain to maintain the immune homeostasis of the CNS (Schwartz and Baruch, 2014; Shechter et al., 2013). Peripheral inflammation may change CNS immune homeostasis. For example, TNF α release during hepatic inflammation in adult mice activate microglial cells to secrete chemokines such as MCP-1 to recruit inflammatory monocytes into the brain (D’Mello et al., 2009). Moreover, CSF pleocytosis has been reported in infants with urinary tract infections (Syrogiannopoulos et al., 2001). We previously reported that peripheral administration of a TLR2 ligand leads to recruitment of neutrophils and monocytes to CSF and brain but surprisingly administration of LPS, a TLR4 ligand, did not result in infiltration of peripheral immune cells, although the level of systemic inflammation was similar (Mottahedin et al., 2017a). Here, by employing flow cytometry and TEM, we demonstrate that the choroid plexus is a route of TLR2-mediated leukocyte entry to the CNS. However, our study does not rule out other potential entry routes such as the leptomeninges (Shechter et al., 2013).

To understand the mechanisms of leukocyte recruitment and transmigration through the choroid plexus we performed RNA sequencing and whole transcriptome analysis. The difference between TLR2 and TLR4 ligands in inducing cell infiltration to CSF was exploited to avoid common inflammatory pathways and specifically identify genes and pathways regulating leukocyte migration. The analysis revealed several potential factors that may underlie the leukocyte migration:

- TLRs signals through various adapter molecules. While TLR2 has MyD88 as the adapter molecule, TLR4 signals via both Myd88 and TRIF. As expected, P3C activated MyD88-dependant downstream signaling including NF- κ B, P38, MAPK, while LPS induced both MyD88-dependant as well as MyD88-independent TRIF-mediated signaling leading to interferon response, which is similar to LPS effects previously described on the choroid plexus in adult mice (Marques et al., 2009). NF- κ B is a family of transcription factors having a pivotal role in regulating inflammation. Interestingly, P3C, but not LPS, highly upregulated *RelB* and *Njkb2* (p100/p52) of NF- κ B which constitutes the non-canonical NF- κ B signaling. Studies on the role of RelB/p52 NF- κ B in regulation of cell migration are contradictory. While RelB $^{-/-}$ fibroblasts persistently release chemokines such as MCP-1 suggesting a role for RelB in inhibiting chemotactic factors (Xia et al., 1997), another study suggests a role for p52 in CXCL12-mediated migration of macrophages and fibroblasts (Kew et al., 2012).
- As expected, both P3C and LPS induced expression of many cytokines and chemokines, however, cluster analysis revealed a P3C-specific chemotaxis

signature (Fig. 4). Expression of key cytokines and chemokines such as *Tnf*, *Il1b*, *Ccl3*, *Cxcl2*, *Ccl2*, and *Ccl4* was significantly higher in the choroid plexus of P3C-treated animals. Based on p-value ranking, *Ccl3* was the most differentially regulated chemokine by P3C. Its induction level by P3C was also several folds higher than that by LPS. Differential induction of *Ccl3* by P3C and LPS in primary human monocytes has been previously reported (Parker et al., 2004) suggesting *Ccl3* as a chemotactic factor that may play a key role in leukocyte recruitment and migration.

- IPA and cluster analysis predicted P3C-specific activation of pathways regulating cytoskeleton organization such as actin signaling which may be required for both transcellular and paracellular leukocyte transmigration. In addition, among top genes regulated by P3C were genes related to tight junction remodeling and cytoskeleton rearrangement such as *MicaU2* (also called *Jrab*) and *Rnd1*. It has been shown that *MicaU2* is involved in endocytosis of occludin in epithelial barrier of intestine (Terai et al., 2006) and *Rnd1* in actin filament disassembly (Nobes et al., 1998). However, the expression of most genes encoding for tight junction proteins either were not altered or only slightly changed, either by P3C or LPS. Ultra-structural analysis also did not show any marked changes by P3C in the tight junctions. Nevertheless, acute remodeling of the tight junction barrier without downregulation of their gene expression may be sufficient for paracellular transmigration of cells through epithelial barriers (Turner et al., 2014). In addition, there is evidence for transcellular migration of cells through the choroid plexus *in vitro* (Steinmann et al., 2013; Wewer et al., 2011). Our TEM analysis supports this possibility. A requirement for leukocyte transmigration through the epithelium is traversing the basement membrane. We showed a marked change in the basement membrane structure as manifested by clefting, massive perforations and vacuolization. While these structural changes in the basement membrane have been reported in epithelial tissues during morphogenesis (Wang et al., 2017), to our knowledge this is the first report of such observation in epithelial tissues induced by immune activation and leukocyte migration. Thus, their potential role in cell migration warrants further functional investigation. Altogether, RNA sequencing data analysis and ultrastructural investigation suggest possibility of cytoskeleton remodeling in the choroid plexus of P3C-treated animals that might allow the transmigration of leukocytes.

The results of this work raise the question why systemic activation of TLR2 should lead to leukocyte invasion of CNS through the choroid plexus? It is plausible that this is a mechanism evolved by the immune system to fight bacterial infection of CNS. TLR2 deficient mice are highly susceptible to bacterial meningitis (Echchannaoui et al., 2002), but whether this is due to lack of TLR2 in the CNS or periphery is not known. Moreover, whether P3C can pass the barriers of the brain to induce direct effects on CNS is unknown. It therefore remains to be understood if TLR2 activation in the brain parenchyma is required for leukocyte infiltration to the CNS. Strikingly, bacteria seem to be able to hijack TLR2 signaling to alter the epithelial tight junctions in order to access and invade the epithelium of

the intestine (Clarke et al., 2011). Therefore, there might have been an evolutionary competition between bacteria and the immune system to control the barriers' function of the CNS. If this hypothesis is correct, some bacteria evolved to modulate brain barrier through TLR2 in order to access the CNS while immune system and the brain barriers evolved to respond by recruiting leukocytes to the CNS in case of TLR2 activation.

In summary, our study reveals a key role for choroid plexus in mediating TLR2-mediated leukocyte recruitment and migration. We propose a model (Supplementary video 2) of TLR2-induced leukocyte transmigration through choroid plexus in which a TLR2-specific chemotaxis profile recruits leukocytes to the choroid plexus followed by a remodeling of basement membrane and cytoskeleton of epithelial cells which facilitate leukocyte migration. This study highlights several previously underappreciated genes and signaling pathways that might be involved in the process that may open new avenues of research targeting neuroinflammation in different CNS diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Choroid plexus is the route of TLR2-induced leukocyte infiltration to the central nervous system. (A-E) Gating strategy for the flow cytometry on the choroid plexus of neonatal mice. CD45 + CD11b + Ly6C^{hi}Ly6G⁻ cells identified as inflammatory monocytes and CD45 + CD11b + Ly6C^{int}Ly6G⁺ cells identified as neutrophils. (B) Flow cytometry data showing infiltration of inflammatory monocytes and neutrophils into the choroid plexus after systemic administration of TLR2 ligand, P3C, to neonatal mice (n = 8–9). Data represent mean ± SEM. One-way ANOVA followed by Dunnett's post-hoc test was applied to determine the statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001 (G–I) Transmission electron microscopy of choroid plexus of neonatal mice (n = 4–5) injected i.p. with saline (G), LPS (H) or P3C (I). Infiltrating leukocytes (I; red arrows in the left image and magnified in the right image) were observed in the choroid plexus after P3C administration.

Fig. 2.

Systemic activation of TLR2 and TLR4 induce a marked change in gene expression in the choroid plexus. (A) Principal component analysis of the choroid plexus RNA sequencing data shows separation of treatment (P3C or LPS) and control groups ($n = 5$ per group) at PC1 plane. (B-E) Upregulation or downregulation of genes in the choroid plexus of neonatal mice 6 h (B) and 14 h (C) after systemic administration of P3C, and 6 h (D) and 14 h (E) after systemic administration of LPS. Red dots indicates genes that are significantly

regulated. Benjamini-Hochberg procedure was performed to determine statistical significance.

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Fig. 3.

Cluster analysis of RNA sequencing data identifies chemotaxis- and cytoskeleton organization signature induced by P3C in the choroid plexus. Complete-linkage hierarchical clustering and heatmap of transformed count data followed by Gene Ontology identified a P3C-specific cytoskeleton organization gene cluster as well as a chemotaxis gene signature (In red). Each row represents a gene and each column represents a sample ($n = 5$). The dendrograms of gene clustering were removed for a simpler visualization. The cut-off p-value (Comparison of each treatment group to its corresponding control) for inclusion of

genes was $p < 0.05$. The letters (a-i) on the right correspond to 9 identified gene clusters and the letters in Table 3, GO ontology.

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Fig. 4.

Genes in chemotaxis signature identified by hierarchical clustering are several folds more upregulated by P3C than by LPS. The heat map corresponds to differential gene expression in comparison to the control. The numbers indicates the fold change in gene expression. The cut-off p-value is $p < 0.05$ (P3C vs saline). ns = not significant.

Fig. 5. Ingenuity pathway analysis (IPA) identifies P3C-specific cytoskeleton remodeling pathways. Genes specifically regulated by P3C at 6 h (A) and 14 h (C) were analysed by IPA. Top 11 pathways (Ranked by z-score, red line) identified by IPA are shown in B (6h) and D (14 h). Among top pathways were pathways regulating cytoskeleton (e.g. actin signaling at both 6 h and 14 h and 3-phosphoinositide signaling at 6 h). The green line indicates the p-value for each pathway. The cut-off p-value was set at $p < 0.05$.

Fig. 6.

Transmission electron microscopy reveals P3C-induced structural changes in the choroid plexus. (A) No marked differences were observed in the structure of tight junctions between the neonatal mice treated with saline (left) and mice treated with P3C (right). (B) No marked differences were observed in the structure of cilia between the mice treated with saline (left) and mice treated with P3C (right). (C) Extensive clefting and perforations (red arrows) observed in the basement membrane of the choroid plexus in mice treated with P3C (right) in comparison with those treated with saline (left). (D) Magnified images seen in C. (E) Perforations in the basement membrane of the choroid plexus of mice treated with P3C. Choroid plexuses from the lateral ventricle; n=5 and 8 sections per animal were analyzed.

Table 1

Top 30 genes (ranked by p-values) significantly regulated by P3C (6 h and 14 h, A and B) and LPS (6h and 14 h, C and D) in comparison to control group. Upregulated (red), downregulated (blue). FC = fold change.

A					
Gene	p-value	FC	Gene	p-value	FC
Nfkbia	3.E-221	9.2	9930014A18	5.E-55	-2.2
Tnfaip3	4.E-178	20.7	Gjb2	4.E-52	-5.4
Junb	4.E-145	17.3	Fam84b	8.E-37	-2.0
Nfkbiz	7.E-133	7.6	Flrt3	2.E-31	-2.1
Birc3	4.E-121	14.8	Gpr34	1.E-27	-5.7
Relb	1.E-114	26.2	Pcdh18	7.E-27	-1.8
Nfkb2	8.E-112	21.2	Slc23a2	3.E-26	-1.8
Icam1	5.E-102	60.4	Plk2	3.E-25	-1.6
Gm15987	4.E-87	43.6	Npr3	2.E-24	-1.9
Tnfr1	3.E-82	4.5	Msx1	3.E-23	-1.7
Cyp11b1	2.E-81	4.0	P2ry12	3.E-21	-3.8
Icosl	3.E-81	30.2	Slc5a3	3.E-21	-1.6
Map3k8	3.E-77	4.0	Slc40a1	1.E-20	-2.2
Sema4c	7.E-75	4.4	Nr3c1	2.E-20	-1.3
Pik3r5	2.E-72	20.3	Cdr2	3.E-20	-1.8
Il1b	1.E-71	36.0	Sostdc1	7.E-20	-1.8
Ccl3	1.E-71	83.8	Spon2	2.E-19	-2.5
Fkbp5	9.E-69	7.1	Dlgap1	2.E-19	-1.6
2810474O19Rik	1.E-68	3.0	Nav3	2.E-18	-2.1
Slc13a3	2.E-68	36.2	Galnt10	6.E-17	-1.7
Micall2	2.E-68	7.2	Fam46d	6.E-17	-2.6
Csf1	1.E-66	7.9	Rhpn2	1.E-16	-1.6
Nfkbie	1.E-65	48.5	Ahr	1.E-16	-1.8
Osmr	5.E-64	4.1	Brwd3	1.E-16	-1.7
Ccl9	5.E-62	16.3	Gabra4	3.E-16	-1.7
Adh1	6.E-61	5.4	Mrc1	3.E-16	-3.2
Tlr1	1.E-59	8.8	Mrap2	4.E-16	-2.0
Stat3	1.E-59	2.6	Pcdh7	5.E-16	-1.4
Maff	2.E-59	8.3	Ighm	6.E-16	-3.7
Cybb	2.E-57	7.6	Epha4	7.E-16	-1.7
B					
Gene	p-value	FC	Gene	p-value	FC
Slc13a3	9.E-106	79.2	Gpr34	3.E-43	-12.2
Madcam1	2.E-87	27.6	Mrc1	8.E-42	-6.7
Pik3ap1	5.E-84	4.7	P2ry12	6.E-38	-5.8
Adgre1	2.E-77	6.0	Nr1d2	2.E-35	-2.3

A					
Gene	p-value	FC	Gene	p-value	FC
Saa3	2.E-70	500.2	Csf1r	1.E-34	-2.3
Pik3r5	2.E-70	19.4	Trem2	3.E-32	-11.3
Arntl	3.E-70	2.4	ErbB4	3.E-32	-1.5
C3	3.E-66	394.9	Per3	6.E-31	-2.2
Tlr1	2.E-63	11.2	Plk5	5.E-26	-2.9
Rac2	2.E-59	9.2	Cpz	2.E-25	-2.0
Itgal	2.E-59	14.2	Slc40a1	7.E-23	-2.3
Vav1	3.E-59	5.6	Col26a1	1.E-22	-1.9
Nfkbiz	1.E-53	3.8	P2ry13	2.E-22	-3.5
Lcn2	2.E-52	507.1	Tlr7	1.E-21	-4.3
Cd74	5.E-51	14.7	Cd33	4.E-20	-7.6
Itgam	6.E-51	5.7	Pf4	9.E-20	-5.0
Hck	2.E-50	7.8	Cd86	2.E-18	-5.8
C1s1	2.E-49	6.7	Cpxm1	2.E-18	-1.6
Ms4a6d	1.E-48	8.8	Itga8	4.E-18	-1.8
Chil1	5.E-46	11.0	Clec7a	8.E-18	-5.7
Fgl2	1.E-45	5.6	Siglech	5.E-17	-8.3
Ifitm3	1.E-45	4.6	Slc35f1	3.E-16	-1.8
Slc15a3	2.E-44	7.8	Dbp	2.E-15	-2.1
Clec4a2	2.E-44	12.4	Tef	2.E-15	-1.5
Gpx3	2.E-43	5.1	Tll1	3.E-15	-1.6
Fyb	1.E-41	4.4	Wisp1	9.E-15	-1.9
H2-K1	3.E-41	3.8	Itgax	1.E-14	-3.3
C1ra	4.E-41	6.4	Optc	5.E-14	-3.8
Aldh3a1	8.E-41	33.1	Mmp2	6.E-14	-1.3
Cxcl16	7.E-40	4.9	Tgfbi	3.E-13	-1.6
C					
Gene	p-value	FC	Gene	p-value	FC
Osmr	3.E-179	10.5	Pcdh18	4.E-92	-3.2
Cyp1b1	7.E-178	7.5	9930014A18Rik	2.E-88	-2.8
Lgals9	3.E-161	7.2	Abca9	2.E-79	-2.9
St3gal1	1.E-113	8.1	Gjb2	4.E-79	-8.3
Fkbp5	8.E-112	12.1	Tet1	6.E-68	-2.0
Stat3	3.E-110	3.6	Spon2	3.E-67	-8.5
Rsad2	3.E-108	16.3	Dlgap1	1.E-61	-2.4
Il4ra	5.E-108	5.8	Kdr	5.E-55	-2.9
Ifit2	4.E-106	20.7	Cemip	2.E-54	-4.0
Socs3	1.E-104	7.8	Itga8	3.E-54	-2.8
Irf7	8.E-102	16.3	Kcne2	2.E-52	-2.7
Zfyve28	2.E-98	11.4	Flrt3	2.E-43	-2.4

A					
Gene	p-value	FC	Gene	p-value	FC
Gbp9	4.E-98	15.2	Jakmip2	1.E-42	-2.5
Rnf213	1.E-97	5.8	Galnt10	1.E-42	-2.3
Gpx3	5.E-97	11.4	Dusp3	2.E-42	-1.8
Ifi204	1.E-94	29.5	Plau	2.E-41	-6.4
Ly6a	2.E-88	11.2	Pcdh12	1.E-38	-4.3
Brca2	2.E-88	4.0	Sdpr	1.E-36	-1.9
Plac8	4.E-87	24.5	Fam84b	2.E-36	-2.0
Tha1	3.E-86	6.4	Igf1	3.E-36	-2.0
Gbp2b	7.E-86	67.1	Vstm4	8.E-36	-2.2
Map3k6	8.E-85	9.1	Cped1	2.E-35	-2.2
Tgm2	4.E-84	7.9	Gabra4	4.E-34	-2.1
Ampd3	1.E-83	3.5	Kank4	1.E-33	-3.0
Gbp2	4.E-83	80.1	Dlst	5.E-33	-1.6
Isg15	8.E-83	22.1	Thbs2	6.E-33	-2.0
Pyhin1	4.E-81	28.9	Adam12	1.E-32	-2.1
Tnfrsf1a	6.E-81	2.6	Eml1	2.E-32	-1.5
Ifitm3	2.E-80	7.4	Cnn2	2.E-32	-3.1
Tmem229b	8.E-80	3.8	E030013119Rik	8.E-32	-6.4
D					
Gene	p-value	FC	Gene	p-value	FC
Gpx3	3.E-96	11.2	Col26a1	6.E-34	-2.1
Ifitm3	2.E-73	7.0	Cpxm1	4.E-31	-1.9
Saa3	2.E-71	524.1	Per3	8.E-31	-2.2
Irf7	2.E-71	10.8	Nr1d2	2.E-28	-2.1
Arntl	3.E-71	2.5	Slc40a1	8.E-25	-2.4
Lpl	1.E-65	4.5	Myl9	3.E-22	-2.5
H2-T23	4.E-64	6.5	Tll1	2.E-21	-1.7
Ly6a	8.E-59	7.2	Cpz	4.E-20	-1.9
A2m	3.E-54	3.2	Tet1	2.E-19	-1.5
Gbp4	4.E-51	31.3	Plk5	6.E-19	-2.5
Iigp1	1.E-48	87.9	Tef	2.E-18	-1.6
Nwd1	2.E-47	1.9	Dbp	4.E-18	-2.3
Nlrc5	4.E-47	20.2	SrpX2	4.E-18	-3.4
Rnf213	3.E-46	3.4	Fbln2	5.E-17	-2.2
Gbp9	1.E-45	6.6	Itga8	9.E-16	-1.8
Stat1	2.E-45	8.7	Hmcn1	4.E-15	-1.9
Isg15	2.E-45	12.6	Slc35f1	9.E-15	-1.7
Plac8	2.E-43	10.5	Abca8a	2.E-14	-2.0
Tgtp2	3.E-43	33.2	Ccdc80	4.E-14	-1.6
Pik3ap1	6.E-42	3.1	Erbb4	4.E-13	-1.3

A					
Gene	p-value	FC	Gene	p-value	FC
Xdh	1.E-41	19.6	Esm1	5.E-13	-3.1
Irf9	1.E-41	3.6	Optc	8.E-13	-3.8
Gbp2	1.E-41	22.7	Socs2	9.E-13	-1.7
Rtp4	2.E-41	7.5	Slc24a3	1.E-12	-1.6
Serping1	4.E-41	2.6	Slc23a2	7.E-12	-1.5
Lcn2	3.E-40	236.0	Ces2e	7.E-12	-2.8
Oasl2	4.E-39	13.5	Fhl2	8.E-12	-1.7
Zbp1	7.E-39	32.7	Mfap4	9.E-12	-2.1
Itgam	8.E-39	4.6	Vwa5b1	1.E-11	-1.7
H2-K1	1.E-38	3.6	Col5a2	1.E-11	-1.5

Table 2

Top 30 (ranked by p-values) genes differentially regulated between P3C and LPS at 6 h (A) and 14 h (B).

A		
Gene	p-value	FC
Tnfaip3	1.E-83	6.7
Klhl25	1.E-69	3.0
Cd83	5.E-57	9.9
Slc25a25	2.E-56	2.1
Ccl3	1.E-53	30.1
Nfkbia	9.E-52	2.8
Il1b	1.E-49	15.0
Micall2	2.E-46	4.9
Csfl	1.E-42	5.2
Jakmip2	1.E-41	2.5
Gm15987	2.E-41	4.1
Sema4c	3.E-38	2.8
Igsf9	3.E-37	7.0
Icosl	5.E-36	8.4
Tnf	6.E-35	13.7
Gdpd5	7.E-35	2.9
Foxj1	7.E-33	2.2
Junb	8.E-33	3.0
Samd4	1.E-31	1.9
Birc3	4.E-30	3.6
Elf3	4.E-30	11.6
Kdr	4.E-30	2.2
Itga8	2.E-29	2.2
Adgre1	2.E-29	3.0
Dusp3	1.E-28	1.6
Cxcl2	3.E-28	50.2
Marco	4.E-28	18.5
Pde4dip	4.E-27	1.5
Pxn	1.E-26	1.7
Dzip11	2.E-26	1.7
B		
Gene	p-value	FC
Marco	1.E-38	17.1
Madcam1	2.E-34	7.1
Pik3r5	3.E-34	4.3
Slc13a3	4.E-28	8.2
Kif9	9.E-27	1.5

A		
Gene	p-value	FC
4430402I18Rik	9.E-27	2.2
Gm15987	1.E-25	5.0
Ccl3	2.E-24	11.4
Chil1	4.E-24	5.7
Vav1	3.E-23	2.6
Sla	3.E-21	3.1
Col22a1	3.E-21	5.5
Lrmp	3.E-21	4.8
Slamf9	3.E-21	4.5
Adgre1	3.E-21	2.5
Pglyrp2	5.E-21	98.4
Pigz	5.E-20	4.7
Nfkbiz	5.E-20	2.2
Aldh3a1	7.E-19	9.1
Olfm4	8.E-18	12.1
C1qtnf2	9.E-17	4.1
Rgs9	1.E-16	2.6
Itgal	2.E-16	3.0
2410004P03Rik	1.E-15	1.5
Nphs1	1.E-15	16.2
Clec4a2	1.E-15	2.8
Spef2	3.E-15	1.4
Pik3cg	3.E-15	2.3
Cfap126	3.E-14	1.7
Lyn	1.E-13	1.6

Table 3

(A–D) top 5 (ranked by p-values) GO terms corresponding to gene clusters a–i shown in Fig. 3.

A				
Term	Count	%	p-value	Fold Enrichment
GO:0007049~cell cycle	69	11.77	5.17E-22	3.89
GO:0006334~nucleosome assembly	28	4.78	1.01E-18	9.33
GO:0051301~cell division	49	8.36	2.26E-18	4.54
GO:0007067~mitotic nuclear division	41	7.00	2.74E-17	5.13
GO:0032776~DNA methylation on cytosine	11	1.88	1.32E-08	11.91
B				
Term	Count	%	p-value	Fold Enrichment
GO:0007049~cell cycle	22	10.78	4.44E-07	3.72
GO:0007067~mitotic nuclear division	14	6.86	2.74E-06	5.25
GO:0051301~cell division	16	7.84	3.23E-06	4.45
GO:0007099~centriole replication	4	1.96	4.40E-04	25.98
GO:0051984~positive regulation of chromosome segregation	3	1.47	8.93E-04	62.35
C				
Term	Count	%	p-value	Fold Enrichment
GO:0000122~negative regulation of transcription fr. RNApol.II	36	9.16	3.14E-07	2.64
GO:0030036~actincytoskeletonorganization	14	3.56	2.61E-06	5.27
GO:0001525~angiogenesis	18	4.58	2.66E-06	4.03
GO:0016477~cellmigration	16	4.07	3.09E-06	4.48
GO:0007155~celladhesion	26	6.62	4.81E-06	2.87
D				
Term	Count	%	p-value	Fold Enrichment
GO:0006810~transport	61	13.5	3.48E-04	1.58
GO:0002376~immune system process	20	4.44	5.43E-04	2.47
GO:0002504~ag presentation of peptide or polysacch.via MHCII	4	0.89	7.15E-04	20.98

A				
Term	Count	%	p-value	Fold Enrichment
GO:0050729~positive regulation of inflammatory response	7	1.56	2.11E-03	5.25
GO:0048002~ag processing and presentation of peptide antigen	3	0.67	2.60E-03	35.41
E				
Term	Count	%	p-value	Fold Enrichment
GO:0016310~phosphorylation	84	5.28	1.34E-07	1.81
GO:0007005~mitochondrion organization	19	1.19	2.76E-05	3.10
GO:0030206~chondroitin sulfate biosynthetic process	7	0.44	2.01E-04	7.11
GO:0006468~proteinphosphorylation	68	4.27	2.80E-04	1.56
GO:0043069~negative regulation of programmed cell death	7	0.44	3.29E-04	6.60
F				
Term	Count	%	p-value	Fold Enrichment
GO:0016126~sterol biosynthetic process	8	2.44	9.04E-08	20.22
GO:0060271~cilium morphogenesis	15	4.57	2.08E-07	6.02
GO:0030030~cell projection organization	14	4.27	3.41E-07	6.33
GO:0042384~cilium assembly	12	3.66	3.04E-06	6.35
GO:0006694~steroid biosynthetic process	8	2.44	3.24E-05	8.80
G				
Term	Count	%	p-value	Fold Enrichment
GO:0006954~inflammatory response	46	5.71	1.43E-13	3.62
GO:0006935~chemotaxis	22	2.73	1.95E-09	5.05
GO:0006955~immune response	33	4.10	7.27E-09	3.28
GO:0007249~I-kappaB kinase/NF-kappaB signaling	12	1.49	1.01E-08	10.15
GO:0030593~neutrophil chemotaxis	16	1.99	2.43E-08	6.28
H				
Term	Count	%	p-value	Fold Enrichment
GO:0031623~receptor internalization	10	3.52	2.43E-09	18.45

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A				
Term	Count	%	p-value	Fold Enrichment
GO:0002376~immune system process	18	6.34	1.88E-05	3.47
GO:0030335~positive regulation of cell migration	12	4.23	1.02E-04	4.36
GO:0007155~cell adhesion	19	6.69	1.10E-04	2.89
GO:0055114~oxidation-reduction process	23	8.10	1.31E-04	2.51
I				
Term	Count	%	p-value	Fold Enrichment
GO:0002376~immune system process	120	5.59	2.45E-30	3.08
GO:0051607~defense response to virus	68	3.17	1.33E-24	4.01
GO:0009615~response to virus	44	2.05	2.81E-21	5.15
GO:0035458~cellular response to interferon-beta	30	1.40	2.88E-20	7.20
GO:0045087~innate immune response	96	4.47	1.38E-15	2.36