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DIFFERENT TLR AGONISTS TEND TO ELICIT AN IMMUNE-REGULATORY CYTOKINE AND CHEMOKINE PHENOTYPE IN HUMAN MICROGLIA

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ABSTRACT

Microglia, the macrophage-like cells in the CNS, express different TLRs that recognize conserved microbial structures and endogenous danger signals. Preliminary studies have indicated that human microglia cells express a wide range of different TLR family members as opposed to astrocytes and oligodendrocytes which primarily express TLR2, TLR3 and TLR4. Here, we show that cultured human microglia consistently express all TLR family members at readily detectable levels, with relatively high levels of TLR1, TLR2, TLR3 and TLR4. Levels of individual TLRs are differentially modulated by cytokines and TLR agonists themselves.

In order to shed more light on the impact of TLR activation, we examined the response of human microglia to agonists for TLR3, 4, 7/8 or 9 by evaluating the induction of 40 different cytokines and chemokines. Cultured microglia in the absence of TLR agonists are already activated to some extent, and secrete marked amounts of various chemokines. In the presence of different TLR agonists, a strikingly similar cytokine and chemokine response profile was observed. In most cases, enhanced production was observed of the cytokines IL-13, IL-10, IL-11, IL-12p40, TGF-β, TNF-α and IL-1β and of the chemokines, CCL1, CCL5, CXCL10 and CCL15. Very limited, if any, induction of IL-12 was observed. Interestingly, this response profile is exemplary for an immune-regulatory macrophage phenotype rather that being representative for classically or alternatively activated macrophages. Our findings therefore suggest that human microglia tend to mount an immune-regulatory response to different TLR agonists.

INTRODUCTION

Microglia, the macrophage-like cells resident within the CNS, play an essential role in inflammatory, ischemic and degenerative diseases of the CNS[1,2]. They detect invading pathogens via various receptors, including Toll like receptors (TLRs)[3]. TLRs are evolutionarily conserved homologues of the *Drosophila* Toll gene, which recognize conserved structural motifs[4,5]. TLRs play an important role in inflammation, development and repair in the CNS [6,7-10]. Stimulation of TLRs with different agonists leads to the initiation of signaling cascades that involves different adaptor molecules such as MyD88, TIRAP/Mal, TRIF and TRAM. These signaling cascades lead to activation of down-stream effector molecules such as NF-κB or IRF3, initiating the production of inflammatory mediators as well as IFN-inducible genes [11,12]. In certain cases, TLR-mediated signaling has been shown to trigger unique response pathways. As an example, we have previously shown that TLR3 activation on astrocytes triggers production of a variety of mediators that counteract gliosis and promote neuronal survival, angiogenesis and remyelination. In organotypic human brain slice cultures the collective activity of mediators which are released by astrocytes upon TLR3 activation leads to significantly enhanced survival of neurons. These findings suggest that at least when expressed on astrocytes TLR3 activates neuroprotective and tissue repair responses rather than pro-inflammatory host defense responses[13]. No such response was observed by TLR4 stimulation, indicating that at least in the case of astrocytes, fundamental differences exist in the functional response to different TLR agonists. Whether or not this is also the case for human microglia has so far remained unclear.

Under normal conditions, TLRs are expressed at low levels in the brain parenchyma, but they are induced in inflammatory neurological disorders such as multiple sclerosis (MS)[14] and during meningitis[15]. The expression of different TLRs (1-10) has been detected in cultured primary human as well in murine and primate microglia, predominantly inside intracellular vesicles [14,16,17] . Murine microglia express detectable levels of mRNA encoding TLR1-9 and preliminary data for human microglia have suggested that they equally express several different TLRs[14]. Activation of TLR3 on human microglia has been reported to induce IL-12, TNF-α, IL-6, CXCL-10 and IL-10, and has been suggested to promote Th1 polarization of $CD4^+$ T cells[18,19]. TLR2-mediated responses in human microglia have been reported to induce IL-6 and IL-10[18]. Group B streptococcus activates murine microglia, but also induces apoptosis in these cells via TLR2[20,21]. Also TLR9 is functional in microglia, as evidenced by microglial activation by CpG DNA both *in vitro* and *in vivo*. In response to CpG DNA, accumulation of TNF-α, IL-12p40, IL-12p70, NO and co-stimulatory molecules has been documented[22,23]. In the above studies, however, only a limited number of cytokines and chemokines have been evaluated so far, leaving open the question to what extent microglial response to different TLR agonist really differ.

To more closely compare the functional response mediated by different TLRs in human microglia, we examined by protein profiling the release of 40 cytokines and chemokines by cultured human adult microglia. Microglia isolated from fresh post-mortem brain samples were examined under normal culturing conditions in the presence of GM-CSF, and after treatment with different TLR agonists including poly I:C, LPS, R848 and bacterial DNA, which serve as selective agonists for TLR3, TLR4, TLR7/8 and TLR9, respectively. We show that untreated cultured microglia express all TLR family members, albeit at different levels, and that they already produce high levels of IL-6, CXCL8, CCL2, CCL3, CCL4 and CCL8 when cultured in the absence of any TLR agonist. Treatment of microglia with any of the above four TLR agonists induced a cytokine and chemokine response profile that was remarkably similar in all cases. Interestingly, the response tended to include production of the cytokines IL-13, IL-10, TGF-β and TNF-α, and the chemokine CCL1 in most cases. Induction of IL-12 was generally not observed. This response profile of mixed pro-and anti-inflammatory mediators is generally regarded as typical for an immune-regulatory macrophage phenotype. Thus, different TLR agonists appear to promote such a regulatory phenotype in human microglia rather than a classically activated phenotype.

MATERIAL EN METHODS

Isolation and culturing of human adult microglia cells

Microglia were obtained from non-affected post mortem subcortical white matter after rapid autopsy provided by The Netherlands Brain Bank. Characteristics of the donors are stated in Table 1.

Table 1

Donor	Sex	Age	PM delay	Clinical history
01-002	f	83	03:30	Alzheimer's disease with vascular encephalopathy
01-012	m	76	06:15	Pick's disease;
01-027	f	50	14:35	Olivopontocerebellar trophy
01-036	f	63	10:55	Parkinson's disease
01-042	f	71	06:45	Alzheimer's disease
02-024	f	75	05:30	Control
03-052	m	53	05:30	Multiple sclerosis
03-057	f	68	03:50	Alzheimer's disease

TLR agonist treatment of microglia

Poly I:C (50 μg/mL, Pharmacia Biotech, Amersham Pharmacia Biotech, Buckinghamshire, UK), ultra pure LPS (200 ng/mL, Invivogen, San Diego, CA), the small synthetic antiviral imidazoquinoline compound R-848 (1 μg/mL, Invivogen, San Diego, CA) and synthetic oligonucleotides that contain unmethylated CpG motifs; ODN2216 (5 μM, Invivogen, San Diego, CA) were used as ligands for TLR3, TLR4, TLR7/8 and TLR9, respectively to stimulate cells. Microglia isolated from three different donors were treated with the different agonists for 24 h, cell supernatants were harvested, and cells were lysed in RNAzolB for RNA extraction.

Real-time PCR

Total cellular RNA was isolated using RNA-Bee™ as previously described (Bsibsi et al, 2002). Subsequently, RNA was reverse transcribed into cDNA and levels of TLR1-10 and GAPDH as a reference were determined by quantitative real-time PCR. The following fluorogenic molecular beacons and primers (Biolegio, Nijmegen, the Netherlands) were used:
GAPDH: sense primers: 5' CAATGCCTCCTGCACCACCAAC3';
anti-sense primer: 5' AGGGGCCATCCACAGTCTTCT 3';
Beacon:cgctcgCACCCCTGGCCAAGGTCATCCAcgagcg;
TLR1: sense primer 5' GAAGAAAGTGAATTTTTAGTTGATAGGTCA 3';
anti-sense primer: 5' ACAGTGATAAGATGTCAGAAGTCCAAAG 3';
beacon: cgtgccATCCACGTTCCTAAAGACCTATCCCAGAggcacg;
TLR2: sense primer 5'GAAATGTGAAAATCACCGATGAAAG 3';
anti-sense primer: 5'TCCACTTTACCTGGATCTATAACTCTGTC 3';
beacon cgtgccTTTGATGACTGTACCCTTAATGGAGTTggcacg;
TLR3: sense primer 5 'CAGTACATCGAGTTCTTGGTTTCAAA 3';
anti-sense primer:5'GAGAAATGTTCCCAGACCCAATC 3';
beacon: cgtgccCAGACAGACAGAACAGTTTGAATATGCAGCggcacg;
TLR4: sense primer 5 'TAAAGAATTTAGAAGAAGGGGTGCC 3';
anti-sense primer: 5'CAACAATCACCTTTCGGCTTTTA 3';
beacon:cgtgccGAGACTTTATTCCCGGTGTGGCCAggcacg.
TLR5: sense primer 5'CCAATGTCACTATAGCTGGGCC 3'
anti-sense primer: 5'TCCTCTTCATCACAACCTTCCG3';
beacon: cgtgccATATTGTGTGTACCCTGACTCGTTCTCTGGggcacg
TLR6: sense primer 5'ATTGAAAGCATTCGTGAAGAAGATTT3';
anti-sense primer: 5'CATCTCAGAAAACACGGTGTACAAA3';
beacon: cgtgccGACTTGACAATAGAACATATCACGAACCAggcacg
TLR7: sense primer 5'GTCAGGAAATCTCATTAGCCAAACTC3';
anti-sense primer: 5'CTTATATCCAGAACTTCCAGTTTGTGAA3';
beacon: cgtgccTGAAATTCCAACCTTTAGCAGAGCTGAGggcacg
TLR8: sense primer 5'GTCTGATAATTTCATCACACACATAACG3';
anti-sense primer: 5'CCATTTGATTGTATACCGGGATTT3';

beacon: cgtgccTCACTAAAATAAATCTAAACCACAACCCCAggcacg
TLR9: sense primer 5'CTGGGAAACCTCCGAGTGCT';
anti-sense primer: 5'AGACAGGTGGGCAAAGGACAC3';
beacon: cgtgccTTCCAGGGCCTAACACAGCTggcacg
TLR10: sense primer 5'GGTTAAAAGACGTTCATCTCCACG3';
anti-sense primer: 5'CTGAGATACCAGGGCAGATCAAAG3';
beacon: cgtgccTGTGGTTATTATGCTAGTTCTGGGGTTGGggcacg
GAPDH and TLR beacons contained 5' fluorescein FAM, and the 3'quencher Dabcyl. Thermal cycling consisted of 95 ºC for 5 min and 40 cycles of 95 ºC for 30 s, 56 ºC for 20 s, 56 ºC for 20 s and 72 ºC for 30 s. PCR was performed using an ABI PRISM® 7700 sequence detection system (Applied Biosystems, Foster City, CA). Data were analyzed using sequence detector version 1.7 software.

Cytokine and chemokine profiling

Cytokine and chemokine profiling of culture supernatants was performed using the human inflammatory antibody array (RayBiotech, Inc. Norcross, GA) according to the manufacturer instructions.

RESULTS

The expression and regulation of TLRs in adult human microglia

To determine the expression pattern of TLRs, adult human microglia were isolated from six different donors and levels of TLR-encoding mRNA levels were assessed using quantitative real time PCR. As shown in Fig. 1 untreated cultured microglia constitutively express all TLRs 1-10 at detectable levels. Whereas TLRs, 1, 2, 3, 4 and 10 were expressed at relatively high levels, the expression of TLR5, 6, 7, 8, and 9 was low. This expression profile of TLRs was found to be very similar between all microglia cultures.

Next, we investigated the effect of the pro-inflammatory cytokines IL-1β, IFN-γ and TNF-α on the mRNA levels of TLRs in microglia from three different donors. Increases of greater that two fold were considered to be significant. As illustrated in Fig. 2, IL-1β induced expression of TLR1 (4 fold), TLR4 (3 fold), TLR8 (4 fold) and TLR9 (5 fold). IFN-γ selectively induced TLR8 (8 fold) while the expression of the other TLRs remained unchanged.

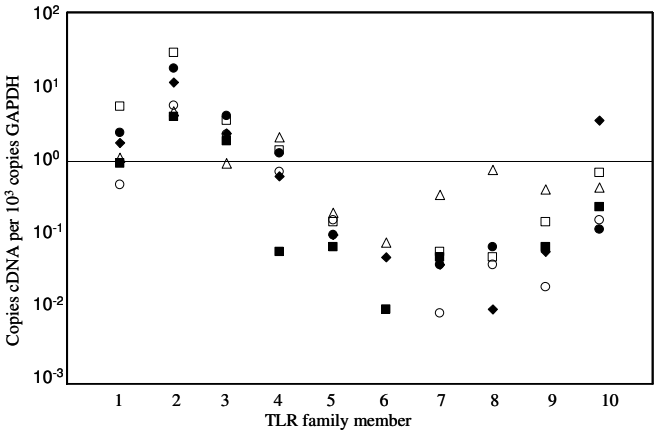

Figure 1: TLR expression profile in cultured but otherwise untreated microglia. Transcript levels in six different microglia cultures were evaluated by quantitative RT PCR. Each symbol indicates one donor.

Stimulation of microglia with TNF-α had surprisingly little effect on TLR transcript levels. The lack of responses at this level does not reflect total lack of microglia responsiveness to TNF-α, since expression levels of various cytokines and chemokines did change in response to the same dose of TNF-α.

To examine whether perhaps TLR agonists themselves alter expression levels of TLRs, microglia from three different donors were stimulated with 100 μg/mL zymosan, 50 μg/mL poly I:C, 200 ng/mL ultra pure LPS, 1 μg/mL R849 and 5 μM ODN2216, which are TLR2, 3, 4, 8 and 9 agonists, respectively. As shown in Fig. 3, TLR expression is modulated by these TLR agonists. Treatment of microglia with zymosan, poly I:C, LPS and ODN2206 led to a marked 3- to 17-fold increase of TLR3 mRNA levels, where, poly I:C had the strongest effect on TLR3 induction (17 fold). TLR7-10 encoding transcripts were marginally induced, where, TLR7 was only induced by poly I:C, TLR8 was induced by his own agonist R848 and TLR9 and TLR10 were induced by poly I:C, LPS and ODN2116.

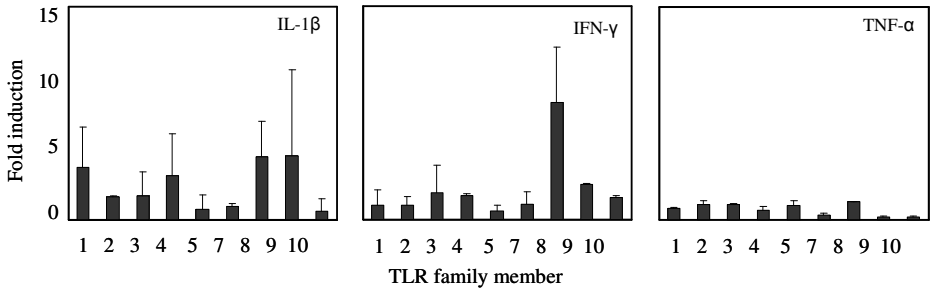

Figure 2: The effects of cytokines on TLR expression in microglia. Human microglia isolated from four different donors were stimulated with IL-1β (500 U/ml), IFN-γ (500 U/ml) or TNF-α (500 U/mL) for 24 h and TLR transcript levels were evaluated using quantitative RT PCR. The data shown represent the average of the four different experiments, plus or minus standard deviation

These data confirm and extend our previous report [14] on the broad TLR profile of adult human microglia and reveal marked expression especially of TLR1, 2, 3, 4 and 10 on cultured cells. The most striking feature of the results is the dominant and the strong induction of TLR3 in particular by poly I:C, while changes in expression levels of the other TLRs were more modest.

Cytokine and chemokine profiling of untreated human microglia

The main goal in our study was to compare the profiles of cytokine and chemokine responses to agonists for different TLR family members. In the present study, we focused on the TLR family members 3, 4, 7, 8 and 9 since these were expressed at relatively high levels in untreated microglia and/or subject to induction by cytokines or TLR agonists. Since cultured microglia are already activated to some extent, and morphologically different from the resting ramified cells found in normal brain tissue, we first examined the cytokine and chemokine production profile of cultured microglia in the absence of any TLR agonist. Media from untreated microglial cultures were incubated with membranes containing an array of 40 human anti-cytokine/chemokine antibodies in duplicate, and the density of each recognition signal at the corresponding position was determined. The relative intensities for each mediator were normalized to control spots on the same membrane. Fig. 4 illustrates the average cytokine and chemokine profiles of untreated microglia, which were very similar in all cultures examined.

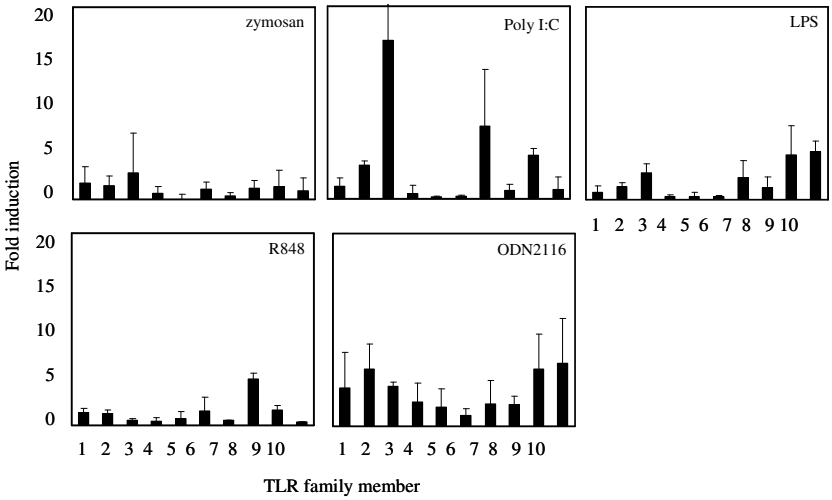

Figure 3: The effects of TLR agonists on TLR expression in microglia. Human microglia isolated from four different donors were stimulated for 24 h with zymosan (100 μg/ml), poly I:C (50 μg/ml), LPS (200 ng/ml), R-848 (1 μg/ml) or ODN2116; Cpg oligonucleotide type B (5 μM) for 24 h and qantitative real time PCR was performed to asses levels of TLR mRNAs. The data shown represent the average of the four different experiments, plus or minus standard deviation.

Apart from the presence of large amounts of IL-6, a striking abundance of chemokines was found, including CXCL8, CCL2, CCL3, CCL4 and CCL8. As expected, also levels of GM-CSF were high in the supernatant, since this growth factor was used as a culture medium supplement to promote survival and proliferation of microglia in culture. Fig.4 shows that the secretion levels of the different chemokines and IL-6 are comparable to or even higher than those of GM-CSF, suggesting that these mediators are indeed secreted at biologically relevant levels. Several of the above mediators are present at such high levels in the culture medium of untreated microglia that any further induction by subsequent treatment with a TLR agonist will probably be very limited, if at all detectable, for this reason alone. This is particularly likely to apply to IL-6, CXCL-8 (IL-8) and CCL2.

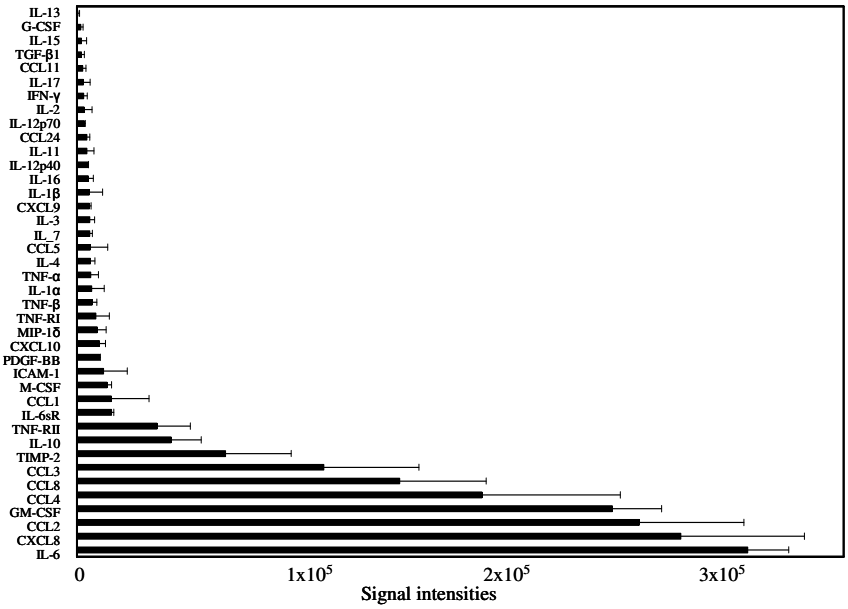

Figure 4: Secretion levels of cytokines and chemokines in cultured but otherwise untreated human microglia. Cultured media from three different microglia cultures were incubated with membranes containing an array of 40 human protein anti-cytokine/chemokine antibodies. Using a biotin-labeled mix of secondary antibodies, cytokine and chemokine binding was monitored in duplicate for each sample.

TLR-mediated production of cytokines and chemokines

Finally, we examined the impact of different TLR agonists on the production of the various cytokines and chemokines by microglia. To this end, we supplied microglial cultures with either poly I:C, LPS, R848 or ODN2216, and culture media were collected 24 h after addition of these TLR agonists. Table 2 summarizes the averaged data from three separated experiments each time performed using cells derived from a different donor. Changes in levels of cytokines or chemokines greater than 1.5-fold were considered as meaningful. The most striking aspect of the results obtained is the observation that cytokine and chemokine responses to each of the different TLR agonists were remarkably similar.

The most strongly induced cytokines after TLR agonist treatments were IL-13 and, to a lesser extent, TNF-α. IL-13 showed the most pronounced change in response to the

different TLR agonists, being consistently induced about 15 fold. Also TNF-α was markedly induced up to 13-fold by the four agonists. In addition, all TLR agonist enhanced the production of TGF-β, IL-15, IL-1β, IL-6sR and CCL5. IL-10, IL-11 and IL-12p40 were induced by three of the agonists, but not by ODN2216. The chemokine CCL1 was also induced by three agonists, except for poly I:C. Of note is also the absence of any marked induction of IL-12p70 induction. Only stimulation with LPS and R846 led to a detectable but rather minor elevation of IL-12p70.

Fig. 5 visualizes the major changes in cytokine and chemokine production upon treatment with different TLR agonists, and illustrates that overall, the different TLR agonists all induced very similar cytokine and chemokine response in microglia. ODN2216 tended to induce a somewhat more narrow response profile. Several mediators which were clearly induced by the other TLR agonists such as CXCL10, IL-10 and IL-12p40 failed to be induced by ODN2216. On the other hand, ODN2216 did induce the major responsive mediators such as CCL5, IL-13, TGF-β and IL-6sR at similar levels as compared to other agonists. We therefore do not attribute the more limited effects of ODN2216 to dosing issues.

Table 2: **Cytokine and chemokine induction in human microglia by TLR agonists.** For each mediator, the response is expressed as average protein expression relative to that in an untreated control culture of the same isolate. Data represent the average of three different experiments, plus or minus standard deviation.

Mediator	Poly I:C	LPS	R846	ODN2216
CCL1	0.53±0.07	**1.84±0.72**	**1.81±0.44**	**1.55±0.34**
CCL2	0.74±0.09	0.53±0.15	0.84±0.17	1.24±0.94
CCL3	1.18±0.15	1.14±0.27	1.14±0.17	1.06±0.13
CCL4	0.79±0.08	0.90±0.11	1.00±0.14	1.03±0.06
CCL5	**7.65±2.46**	**7.31±1.66**	**5.07±1.70**	**5.03±2.54**
CCL8	0.98±0.10	0.82±0.18	1.01±0.06	0.94±0.11
CCL11	0.99±0.06	1.42±0.30	**1.53±0.34**	1.18±0.10
CCL24	0.89±0.07	**1.65±0.56**	**1.58±0.64**	1.25±0.22
CXCL8	0.85±0.10	0.72±0.095	0.93±0.19	1.16±0.28
CXCL9	1.24±0.29	**1.70±0.49**	1.44±0.38	0.97±0.11
CXL10	**3.78±1.18**	**3.52±1.25**	1.30±0.05	1.02±0.17
CCL15	**1.89±0.76**	**2.80±0.84**	**2.14±0.56**	1.27±0.35
IL-1α	0.55±0.22	**2.18±0.93**	**1.91±0.73**	1.09±0.25
IL-1β	**1.75±0.65**	**4.14±2.22**	**4.59±2.84**	**2.26±1.40**
IL-2	0.91±0.04	**2.09±0.96**	**2.26±1.21**	**1.58±0.63**
IL-3	0.84±0.08	1.15±0.19	1.20±0.24	0.87±0.08
IL-4	1.29±0.01	**1.57±0.25**	**1.56±0.19**	1.22±0.01
IL-6	0.82±0.02	0.64±0.07	0.89±0.23	1.10±0.33
IL-7	0.97±0.19	1.43±0.53	**1.86±0.54**	1.19±0.12
IL-10	**2.00±0.31**	**1.85±0.46**	**2.44±0.65**	0.75±0.23
IL-11	**1.73±0.06**	**2.13±0.59**	**2.37±0.65**	1.21±0.16
IL-12p40	**4.69±1.31**	**2.67±0.45**	**4.41±0.50**	1.40±0.11
IL-12p70	0.89±0.0	**1.52±0.28**	1.35±0.26	1.06±0.20
IL-13	**13.85±7.50**	**17.74±9.98**	**18.85±10.96**	**15.40±9.48**

Table 2: Continued

MEDIATOR	POLY I:C	LPS	R846	ODN2006
IL-15	**3.34±2.29**	**1.97±1.00**	**2.26±0.95**	**1.64±0.52**
IL-16	1.05±0.23	1.38±0.55	**1.61±0.50**	1.25±0.22
IL-17	1.08±0.23	1.33±0.43	1.46±0.29	1.20±0.19
TNF-α	**13.74±3.99**	**6.56±1.18**	**6.55±0.33**	**2.26±0.94**
TNF-β	1.20±0.16	1.36±0.38	1.46±0.33	1.06±0.08
IFN-γ	0.78±0.20	**1.69±0.52**	**1.52±0.52**	1.08±0.23
TGF-β1	**2.48±0.20**	**4.71±2.24**	**3.68±2.08**	**2.74±1.37**
G-CSF	0.90±0.12	**1.68±0.41**	**1.83±0.42**	1.34±0.14
GM-CSF	0.90±0.01	0.79±0.08	0.86±0.11	1.12±0.20
M-CSF	**1.50±0.37**	**1.72±0.45**	**1.71±0.62**	1.20±0.30
ICAM-1	0.93±0.15	**1.62±0.68**	**1.55±0.65**	1.04±0.29
IL-6sR	**2.96±1.22**	**3.56±1.43**	**3.97±2.01**	**3.27±1.25**
TNF-RI	1.16±0.26	1.23±0.33	1.20±0.26	1.07±0.13
TNF-RII	0.94±0.22	1.04±0.22	1.35±0.18	1.08±0.27
PDGF-BB	1.04±0.12	1.49±0.27	1.42±0.30	0.98±0.08
TIMP-2	0.73±0.13	0.87±0.03	0.74±0.01	0.78±0.16

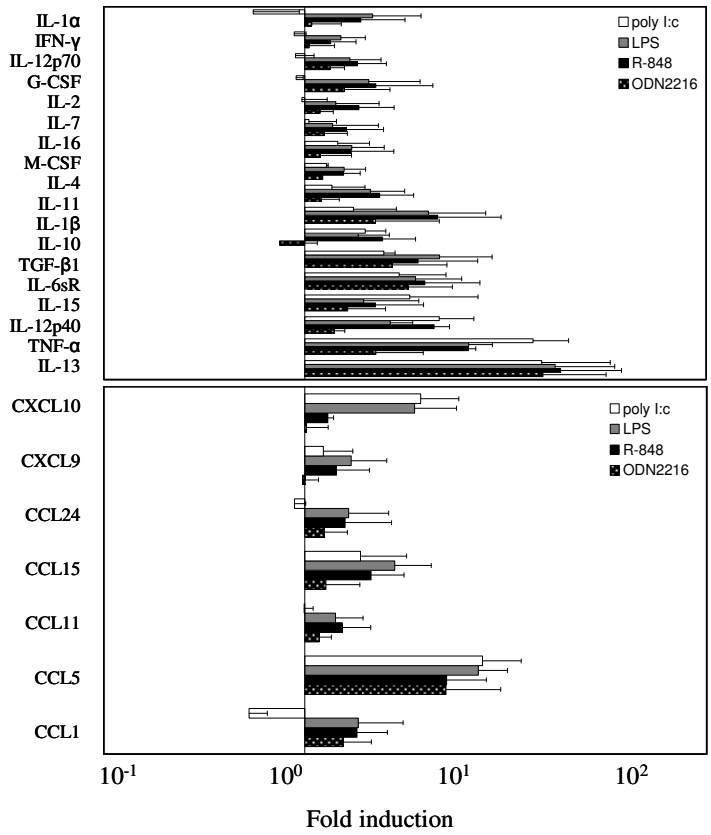

Figure 5: The impact of TLR agonists on cytokine and chemokine production in human microglia. Cultured human microglia cells were treated with poly I:C (50 μg/ml), LPS (200 ng/ml), R-848 (1 μg/ml) or ODN2116 (5 μM) for 24 h and supernatants were monitored for the presence 40 different cytokines and chemokines using antibody arrays. The production levels were compared to untreated microglia from the same donor. The data represent the average results of three separate experiments, plus or minus standard deviation

DISCUSSION

The present study was aimed at comparing the functional response by human microglia to activation via either TLR3, TLR4, TLR7/8 or TLR9. These five TLRs were selected since cultured microglia without any additional treatment express relatively high levels of TLR3 and TLR4 and they display marked induction of TLR7, TLR8 and TLR9 upon activation with certain cytokines or TLR agonists. The major finding of this study is that different TLR agonists elicit rather similar cytokine and chemokine responses, involving a range of mediators that are typical for an immune-regulatory phenotype of macrophages. Especially the dramatic 14-to 19-fold induction of the cytokine IL-13 was found to consistently dominate the response.

Under our standard culturing condition, microglia already display a distinct state of activation as compared to resting microglia *in vivo*. Cells are bi-polar and secrete particularly high levels of different chemokines including CXCL8, CCL2, CCL4, CCL8 and CCL3. This probably results from mechanical and metabolic stress during isolation and confrontation with serum components and growth factors in culture medium. Whereas the chemokines secreted by untreated microglia are well known to be relevant to leukocyte recruitment, it should be pointed out that they are also relevant to glial cells themselves. Human astrocytes, microglia and neurons express receptors for all these chemokines[24] and activated microglia demonstrate increased migratory response to different chemokines and to injury[25]. Thus, production of various chemokines as listed above may well represent the first level of microglial responses to injury or insult, mediating also glial and neuronal migration within the CNS itself.

Previous data on human or primate microglia have indicated expression of a range of different TLRs[14,17,18]. In the present study, we more closely evaluated relative expression levels of each of these, and we add TLR10 to the human microglial repertoire. In agreement with previous studies, the present results indicate that TLR expression on microglia is differentially modulated by cytokines and TLRs agonists. Interestingly, microglia display strong and selective induction of TLR3 (17 fold) upon activation with poly I:C. Intriguingly, TLR3 was also strongly and selectively induced in human astrocytes by cytokines or TLR agonists (chapter 5). TLR3 on astrocytes mediates a comprehensive neuroprotective response rather that a polarized pro-inflammatory reaction (chapter 5) . Since neuronal survival is also promoted by adding poly I:C to organotypic human brain slice cultures, and therefore in the presence of microglia, also microglial TLR3 may contribute to the previously documented neuroprotective TLR3 response in the CNS.

The main finding of our study, however, is that different TLR agonists tend to trigger a very similar cytokine and chemokine response profile in microglia. The microglial response is observable in cell culture by morphological changes including shortening of cellular processes and enlargement of their soma, in line with the report of Ebert et al[26]. At the level of cytokine and chemokine production, the response is dominated by secretion of strongly elevated levels of IL-13 and TNF-α. In addition, TGF-β, IL-1β, IL-15, IL-10, IL-6sR and IL-12p40 tend to become secreted at markedly elevated levels, while IL-12p70 is not. IL-13 is a well known anti-inflammatory cytokine which inhibits production f IL-1β, TNF-α and nitric oxide from activated microglia and macrophages, thus suppressing the cytotoxic and inflammatory functions of macrophages. Also, IL-13 induces death of overactivated microglia cells[27-33], underpinning its immune-regulatory functions. TGF-β1, a pleiotrophic cytokine with diverse actions such as chemoattraction, angiogenesis, neuroprotection and suppression of inflammation, was also consistently induced upon activation with different TLRs agonists. The immune-regulatory functions of TGF-β1 are equally well know, also in the context of neuroinflammation[34]. In addition to cytokines and chemokines, TLR agonists led to the release of colony stimulating factors (CSFs), which regulate survival, proliferation and differentiation of hematopoietic cells, including microglia themselves. M-CSF has neuroprotective functions and promotes phagocytosis by murine microglia. In addition, M-CSF can also act as a chemotactic signal to induce proliferation and migration of microglia cells to the damaged area [35-37]. G-CSF exerts a neuroprotective effect through the direct activation of anti-apoptotic pathway. Administration of G-CSF for example leads to reduction of infarction volume and mortality and improvement of neurological behavior after cerebral ischemia [38,39]. Among the main chemokines induced are CCL1, CCL5, CCL15, CCL11 and CXCL10, all of which can act as chemotactic signals for microglial themselves. CCL5 for instance readily mediates microglial migration via CCR3 and CCR5[40]. CCL1 is a particularly interesting chemokine, induced by all agonists except poly I:C. Production of CCL1 is selectively found in macrophages that display a regulatory phenotype, typically induced by concomitant activation of macrophages with a TLR agonist and immune complexes[41]. Apparently, release of CCL1 from microglia does not require a second signal in addition to a TLR agonist. CCL1 is an important chemokine since it selectively recruits CCR8-expressing regulatory T cells and Th2 cells[42]. Thus, it is a key signal to promote control of inflammatory pathways. That the different TLR agonists indeed all tend to induce a regulatory phenotype in microglia, as suggested by the production of CCL1, is further emphasized by the coordinate secretion of TNF-α as well as TGF-β (and IL-10 in most cases), which is found in neither classically activated or alternatively activated macrophages.

In conclusion, our results show that TLRs are broadly expressed in human microglia, and differentially modulated by TLR agonists and cytokines. While minor differences are found in the cytokine and chemokine response profiles to different TLR agonists, the

overwhelming quality of each response appear to be an immune-regulatory one. Thus, microglia may be distinct from other macrophage-like cells in containing a default TLR response pathway that consistently promotes an immune-regulatory phenotype, thus contributing to a functional immune-privileged state of the CNS.

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