Human TLR10 is an anti-inflammatory pattern-recognition receptor

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Toll-like receptor (TLR)10 is the only pattern-recognition receptor without known ligand specificity and biological function. We demonstrate that TLR10 is a modulatory receptor with mainly inhibitory effects. Blocking TLR10 by antagonistic antibodies enhanced proinflammatory cytokine production, including IL-1β, specifically after exposure to TLR2 ligands. Blocking TLR10 after stimulation of peripheral blood mononuclear cells with pam3Csk4 (Pam3Cys) led to production of 2,065 ± 106 pg/mL IL-1β (mean ± SEM) in comparison with 1,043 ± 51 pg/mL IL-1β after addition of nonspecific IgG antibodies. Several mechanisms mediate the modulatory effects of TLR10: on the one hand, cotransfection in human cell lines showed that TLR10 acts as an inhibitory receptor when forming heterdimers with TLR2; on the other hand, cross-linking experiments showed specific induction of the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra, 16 ± 1.7 ng/ml, mean ± SEM). After cross-linking anti-TLR10 antibody, no production of IL-1β and other proinflammatory cytokines could be found. Furthermore, individuals bearing TLR10 polymorphisms displayed an increased capacity to produce IL-1β, TNF-α, and IL-6 upon ligation of TLR2, in a gene-dose-dependent manner. The modulatory effects of TLR10 are complex, involving at least several mechanisms: there is competition for ligands or for the formation of heterodimer receptors with TLR2, as well as PI3K/Akt-mediated induction of the anti-inflammatory cytokine IL-1Ra. Finally, transgenic mice expressing human TLR10 produced fewer cytokines when challenged with a TLR2 agonist. In conclusion, our knowledge we demonstrate for the first time that TLR10 is a modulatory pattern-recognition receptor with mainly inhibitory properties.

TLR10 | immunology | cytokines | SNPs

Highly conserved molecular structures of invading microorganisms are recognized by immune cells through pattern-recognition receptors, of which Toll-like receptors (TLRs) are the most documented family. In humans, 10 members of the TLR family have been described (1). In general, specific ligation of TLRs leads to induction of proinflammatory mediators, such as cytokines and chemokines. One member of the TLR family, however, TLR10, is considered an orphan receptor because of its still-unknown ligands and function. Human TLR10 is encoded on chromosome 4 within the TLR2 gene cluster, together with TLR1, TLR2, and TLR6, and shares all structural characteristics of the TLR family (2, 3). However, TLR10 differs from other TLRs by its lack of a classic downstream signaling pathway (4), despite its interaction with the myeloid differentiation primary response gene 88 adaptor protein (3). TLR10 is predominantly expressed in tissues rich in immune cells, such as spleen, lymph node, thymus, tonsil, and lung (2). Expression of TLR10 can be induced in B cells, dendritic cells, eosinophils, and neutrophils (3, 5, 6), as well as on nonimmune cells, such as trophoblasts (7). TLR1 and TLR6 are known to form heterodimers with TLR2, and this was shown for TLR10 as well (3, 8). It is therefore rational to hypothesize that if TLR10 has a biological function, which it exerts through the formation of heterodimers with TLR2. In the present study we report the surprising regulatory function of human TLR10 on TLR2-driven cytokine production exerted through competition for ligands, on the one hand, and specific induction of the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra), on the other hand. In addition, we demonstrated that hTLR10 transgenic mice produced fewer cytokines in vivo when exposed to a potent TLR2 ligand.

Results

Blocking TLR10 Increases Cytokine Production by Primary Human Cells. To investigate the biological function of TLR10, freshly isolated human peripheral blood mononuclear cells (PBMCs) were exposed for 24 h to the TLR2 ligand pam3Csk4 (Pam3Cys) in the presence or absence of a specific antibody directed against TLR10. Production of IL-1β and IL-6 was increased 2-fold and 1.5-fold, respectively, in the presence of the anti-TLR10 antibody (P < 0.001) (Fig. 1 A and B). This effect of the TLR10-antibody was also observed for IL-8 production (P < 0.05), and to a somewhat lesser extent for TNF-α (Fig. 1 C and D). Similar data were obtained when the cells were exposed to viable Borrelia burgdorferi, the causative organism of Lyme disease that is mainly recognized by TLR1/TLR2 heterodimers (9, 10). No difference in IL-10 production was observed after blocking TLR10 with the specific antibody (Fig. S1 A). Additionally, we detected no differences in mRNA expression levels of suppressor of cytokine signaling (SOCS)-1 and of SOCS-3 after blocking TLR10 molecules (Fig. S1 B and C, respectively). Blocking of TLR10 did not change cytokine induction after PBMC stimulation with ligands specific for TLR4

Significance

We demonstrate the biological role of TLR10, the only member of the Toll-like receptor (TLR)-family so far without a known function. We show that TLR10 acts as an inhibitory receptor, with suppressive effects. Blocking TLR10 by specific antibodies significantly upregulated TLR2-mediated cytokine production. Additionally, we show that individuals carrying loss-of-function SNPs in TLR10 display upregulation of TLR2-mediated cytokine production. After challenging human TLR10 transgenic mice with TLR2 ligand pam3Csk4 (Pam3Cys), less inflammation could be observed when compared with wild-type mice. Taking these data together, we show that TLR10 is the only pattern-recognition receptor within the TLR family that is able to dampen TLR2 responses, thereby suppressing immune responses through production of IL-1Ra.

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Functional Polymorphisms of the Human TLR10 Gene. A strong argument in favor of TLR10 being a functional receptor is that the TLR10 gene is under selection pressure in European populations; nonsynonymous polymorphisms within the human TLR10 gene are considered to influence TLR10 function (11). Two of these SNPs, N241H (rs11096957) and M326T (rs11466653), have been found to be associated with reduced risk of prostate cancer and were described to be possibly damaging to the TLR10 gene (11, 12). We investigated whether the presence of these SNPs in TLR10 affects the TLR2-induced cytokine response by PBMCs obtained from healthy individuals with different TLR10 genotypes. Within the group of the 112 volunteers screened, 40 were identified as bearing the wild-type allele, 60 were heterozygous, and 12 were homozygous for the N241H SNP. PBMCs isolated from individuals bearing the three different genotypes were incubated with RPMI Pam3Cys, live B. burgdorferi, or lipopolysaccharide (LPS) for 24 h. Thereafter, cytokine concentrations were measured in the supernatants. Cells of

**TLR10 Overexpression Inhibits TLR2-Mediated Signaling.** To explore the mechanisms through which TLR10 exerts the suppressive function, HEK293 cells were transfected with either TLR2 alone, or TLR2 together with TLR1, TLR6, or TLR10. HEK cells were cultured in the presence of either the TLR2 ligand Pam3Cys or B. burgdorferi for 24 h. IL-8 production was observed when HEK293 cells transfected with TLR2 alone were exposed to TLR2 ligands. In contrast, HEK293 cells cotransfected with both TLR2 and TLR10 showed a strong reduction of IL-8 production (Fig. 2 A and B). The inhibitory effect of TLR10 transfection was also observed when cotransfected TLR2/10 HEK cells were exposed to the TLR2/6 ligand Pam2CGDLPKPKSF (FSL-1) (Fig. 2B). To determine the degree of specificity for TLR2, we cotransfected HEK cells with TLR5 and TLR10. TLR10 cotransfection had no effects on the IL-8 production induced by flagellin (Fig. 2C). Thus, TLR10 acts primarily as an inhibitory receptor for TLR2 ligands.

**RNA Silencing of TLR10 Results in Enhanced Cytokine Production.** To corroborate these findings, we performed a series of experiments using siRNA to silence the gene for TLR10 in monocyte-derived macrophages. To assess the efficiency of the siRNA approach, we determined the TLR10 mRNA levels in the silenced macrophages (Fig. 2D). As commonly seen with siRNA in primary cells, we achieved a 50% inhibition of the messenger mRNA encoding TLR10. After incubation of the cells with the specific TLR10 siRNA, cells were exposed to B. burgdorferi for 24 h. Up-regulation of IL-6 production by the TLR10-silenced macrophages was observed, which was not seen with scramble-silencing RNA (Fig. 2E). No differences in TNF-α production could be observed (Fig. 2F).

Fig. 1. Blocking TLR10 results in higher cytokine production. The $5 \times 10^5$ PBMCs from 11 to 17 individuals were preincubated for 1 h at 37 °C with either 10 μg/mL aTLR10 antibody (aTLR10, clone 3C10C5) or 10 μg/mL IgG isotype control (IgG). After preincubation, cells were stimulated for 24 h with TLR2 ligand Pam3Cys (10 μg/mL), or live B. burgdorferi (B.b.). After stimulation, cell supernatants were collected and proinflammatory cytokines were measured using ELISA (A–D). Bars represent the mean and the SEM. Paired t test; *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 2. TLR10 transfected HEK cells inhibit cytokine production. HEK293 cells were transiently transfected with TLR2, TLR12, TLR6, or TLR2/10. (A) Twenty-four hours after transfection, cells were stimulated for 24 h with different concentrations of live B. burgdorferi (B.b.). (B) HEK293 cells transiently transfected using different ratios of plasmid concentrations of TLR10 plasmid. Cells were stimulated 24 h after transfection for 22 h with specific TLR ligands Pam3Cys or FSL-1. IL-8 production (in pg/mL) was measured in the cell-free supernatant using ELISA. (C) HEK293 cells transiently transfected with TLR5 or TLR5/10 plasmids. After 24 h of recovery, cells were stimulated for 22 h with a dose–response of recombinant flagellin. IL-8 production was measured by ELISA. Bars represent the mean of six separate experiments ± SEM. Mann-Whitney U test, two-sided. **p < 0.01. (D) Monocytederived macrophages of eight individuals were transfected with scrambled siRNA as a negative control (NC siRNA) or siRNA directed against TLR10 (TLR10 siRNA). (E) After transfection, monocytederived macrophages were stimulated with 1.10^6 live B. burgdorferi per mL. (F) Twenty-four hours after stimulation, IL-6 or TNF-α protein levels were measured in micrograms per milliliter (pg/mL) in the supernatants using specific sandwich-ELISAs.
individuals heterozygous or homozygous for the N241H polymorphism displayed strongly increased production of IL-1β, IL-6, IL-8, and TNF-α when stimulated with TLR2 ligands compared with cells with wild-type TLR10 (Fig. 3 A–D). In contrast, cytokine production induced by stimulation of other TLR ligands (TLR3, TLR4, TLR5, TLR9) were not affected by the TLR10 polymorphisms, again underscoring the selective interaction of TLR10 with TLR2 (Fig. 3 E–H). TLR4 stimulation using LPS in a larger cohort also did not lead to altered cytokine production in individuals bearing TLR10 polymorphisms (Fig. S2).

For the M326T SNP, 105 volunteers were identified as bearing the wild-type allele and 7 volunteers as being heterozygous for the SNP. Despite the low prevalence of the M326T polymorphism, increased cytokine production was also observed with cells bearing this SNP. Two other polymorphisms, rs11096955 and rs4129009, were recently found to cause structural effects in the TLR10 molecule (13, 14). Additionally, these polymorphisms led to increased cytokine production (Figs. S3 and S4). Taken together, these gene dosage effects on cytokine production provide an additional proof that TLR10 inhibits TLR2-induced cytokines, with no effect on the TLR4 signaling pathway.

Our findings in these volunteers concur with the recent report showing that variation in the TLR10/TLR1/TLR6 locus is the major genetic determinant of interindividual differences in TLR2-mediated cytokine responses (14). This study showed that SNPs in the TLR10 gene affect the cytokine responses elicited by specific TLR2 ligands, determined by whole-blood assays.

**Signal Transduction.** To explore whether passive competition with other stimulatory TLR2 coreceptors, such as TLR1 or TLR6, is solely responsible for the TLR10 effects or whether active signaling mechanisms are also involved, we assessed which signaling pathways may be induced by TLR10. Anti-TLR10 antibodies were coated onto culture plates and freshly isolated PBMCs were added for 24 h, a standard method for cross-linking and stimulation of receptors with unknown specific ligands. Afterwards, RNA sequencing of stimulated cells was performed to obtain a comprehensive picture of TLR10-induced effects. RNA sequencing data analysis and comparison with RNA sequencing data of IgG–cross-linked PBMCs has identified several genes that were up-regulated; pathway analysis performed on the genes transcribed after TLR10 ligation points to modulatory pathways, such as wound healing, chemokines, and growth factors (Fig. 4 A and Table S1). Among these genes, the gene encoding for IL-1 receptor antagonist (IL-1RN) stood out as being very strongly up-regulated (55.5-fold up-regulation) (Fig. 4 A). IL-1α is the natural inhibitor of excessive IL-1 signaling and it blocks both IL-1α and IL-1β (15, 16). As a consequence, IL-1α down-modulates cytokine responses in several immune cells exposed to specific ligands of pathogens.

The remarkable up-regulation of IL-1Ra mRNA expression by the PBMCs after cross-linking TLR10 was confirmed by PCR (Fig. 4 B). In addition, enhanced protein levels of IL-1Ra were found when PBMCs were incubated for 24 h with plate-bound anti-TLR10 antibody, compared with IgG isotype control (Fig. 4 C). Individuals bearing TLR10-dysfunctional polymorphisms were found to produce less IL-1Ra after stimulation (Fig. 4 D and E and Fig. S5 A and B). In these cross-linking experiments, no IL-8, IL-6, TNF-α, or IL-β production could be detected (Fig. 4 F). After blockade with the specific anti-TLR10 antibody on PBMCs, IL-1Ra levels were also up-regulated compared with isotype control (Fig. 5 C).

To prove the inhibitory role of TLR10 through IL-1Ra production, lung epithelial cells were incubated with supernatants from either IgG or anti-TLR10 antibody cross-linked PBMCs. Stimulation of A549 cells with active IL-1β led to significant induction of IL-8 (Fig. 4 G, light gray bar). When the supernatants of aTLR10–cross-linked PBMCs were preincubated with a specific neutralizing antibody against IL-1Ra, this effect could no longer be observed (Fig. 4 G, dark gray bars).

Because it has been reported that the PI3K/Akt pathway is needed for the induction of IL-Ra production (17, 18), we assessed whether TLR10 effects may be mediated by PI3K/Akt pathway. PI3K/Akt inhibition resulted in complete inhibition of IL-1Ra production (17, 18), we assessed whether TLR10 effects may be mediated by PI3K/Akt pathway. PI3K/Akt inhibition resulted in complete inhibition of IL-1Ra production (17, 18).

**Human TLR10 Inhibits Inflammation in Vivo.** Wild-type mice do not express functional TLR10 because of sequence gaps and retroviral insertions in the TLR10 gene (4). To explore the biological effect of TLR10 in vivo, we generated human (h) TLR10-trans-
TLR10 induces IL-1Ra through MAPK-dependent pathways. Ten micrograms per milliliter anti-TLR10 antibody or 10 μg/mL IgG isotype control were coated to 24-well flat-bottom plates and incubated for 2 h at 37 °C. After washing and blocking, 5 × 10^5 PBMCs from two donors were added and incubated overnight at 37 °C. (A) Heatmap of the genes found to be up- and down-regulated after adding anti-TLR10 antibody. A magnification of the upper part of the heatmap is shown in the table (Right). Numbers represent fragments per kilobase per million mapped reads values. (B) mRNA expression levels and (C) protein secretion of IL-1Ra were determined. Ten micrograms per milliliter anti-TLR10 antibody or 10 μg/mL IgG isotype control were coated to 96-well flat-bottom plates. After blocking, 5 × 10^4 PBMCs were added to the coated plates and incubated for 2 h at 37 °C. Bars represent mean ± SEM for at least four donors. (D) The 5 × 10^5 PBMCs from individuals without polymorphisms (wt; wild-type; white bars), individuals with the I690L SNP (D) or I775V SNP (E) in one of both alleles (he; heterozygous; gray bars), and individuals with the SNP in both alleles (ho; homozygous; black bars), were stimulated for 24 h at 37 °C with RPMI, or B. burgdorferi. IL-1Ra was measured in the supernatant using ELISA. Results are shown in picograms per milliliter (pg/mL). Bars represent the means ± SEM. (F) Cytokine secretion by PBMC after cross-linking anti-TLR10 antibody. Detection limit IL-1α, IL-6, TNF-α, and IL-8 are, respectively, 39 pg/mL, 156 pg/mL, 78 pg/mL, and 1,560 pg/mL. (G) 5 × 10^5 A549 cells in RPMI (10% FCS) were preincubated for 30 min at 37 °C with supernatant from cross-linking experiments. In these cross-linking experiments, either IgG or specific anti-TLR10 antibodies (10 μg/mL) were cross-linked to PBMCs for 24 h. Before addition to the A549 cells, this supernatant was preincubated for 1 h at 37 °C with either specific neutralizing antibodies against IL-1Ra (aIL-1Ra), or isotype control antibodies (IgG Goat), both 10 μg/mL. Thereafter, the A549 cells were either stimulated for 24 h with medium or 3 pg/mL active IL-1β. Bars represent means plus SEM, paired t testing. Experiment represents six separate measurements. **P < 0.01. (H) IL-1Ra mRNA expression levels or protein in picograms per milliliter (pg/mL) (I) in PBMCs (in the absence/presence of anti-TLR10 coated antibodies) with or without PI3k inhibitors 3MA and Wortmannin (Wort). (J) Model of TLR10 function. TLR10 inhibits MAPK signaling, and also induces PI3k which finally results in IL-1Ra production.

**Discussion**

In the present article we describe the surprising modulatory effects exerted by TLR10 upon the inflammatory reaction exerted through other members of the TLR2 receptor family. TLR10 was the only member of the TLR family without a well-defined biological function; our data show that it is, to our knowledge, also the first TLR receptor with inhibitory properties. TLR10's modulatory effects are complex, involving at least several mechanisms: competition for ligands, or for the formation of heterodimer receptors with TLR2, as well as PI3K/Akt-mediated induction of the anti-inflammatory cytokine IL-1Ra.

These data lead to the conclusion that TLR10 is the only TLR family member that acts as an inhibitory receptor, whereas ligand of all other TLRs leads mainly to proinflammatory cytokine production. Additional reports also hint to a modulatory function of TLR10. Mullia et al. provide evidence that TLR10, which is expressed on trophoblast during gestation, induces apoptosis through activation of caspase-3 (7). Mullia et al. also found the trigger for this response to be a TLR2 ligand (i.e., peptidoglycan); no effect on IL-6 production was found in these studies (7). Very recently, a study described that TLR10 acts as a proinflammatory TLR, which is the opposite of the suppressive function of TLR10 we report here (19). Because Regan et al. (19) use only siRNA approaches to demonstrate the biological function of TLR10, it is difficult to compare the results of the two studies.

**Fig. 4.** TLR10 induces IL-1Ra through MAPK-dependent pathways. Ten micrograms per milliliter anti-TLR10 antibody or 10 μg/mL IgG isotype control were coated to 24-well flat-bottom plates and incubated for 2 h at 37 °C. After washing and blocking, 5 × 10^5 PBMCs from two donors were added and incubated overnight at 37 °C. (A) Heatmap of the genes found to be up- and down-regulated after adding anti-TLR10 antibody. A magnification of the upper part of the heatmap is shown in the table (Right). Numbers represent fragments per kilobase per million mapped reads values. (B) mRNA expression levels and (C) protein secretion of IL-1Ra were determined. Ten micrograms per milliliter anti-TLR10 antibody or 10 μg/mL IgG isotype control were coated to 96-well flat-bottom plates. After blocking, 5 × 10^4 PBMCs were added to the coated plates and incubated for 2 h at 37 °C. Bars represent mean ± SEM for at least four donors. (D) The 5 × 10^5 PBMCs from individuals without polymorphisms (wt; wild-type; white bars), individuals with the I690L SNP (D) or I775V SNP (E) in one of both alleles (he; heterozygous; gray bars), and individuals with the SNP in both alleles (ho; homozygous; black bars), were stimulated for 24 h at 37 °C with RPMI, or B. burgdorferi. IL-1Ra was measured in the supernatant using ELISA. Results are shown in picograms per milliliter (pg/mL). Bars represent the means ± SEM. (F) Cytokine secretion by PBMC after cross-linking anti-TLR10 antibody. Detection limit IL-1α, IL-6, TNF-α, and IL-8 are, respectively, 39 pg/mL, 156 pg/mL, 78 pg/mL, and 1,560 pg/mL. (G) 5 × 10^5 A549 cells in RPMI (10% FCS) were preincubated for 30 min at 37 °C with supernatant from cross-linking experiments. In these cross-linking experiments, either IgG or specific anti-TLR10 antibodies (10 μg/mL) were cross-linked to PBMCs for 24 h. Before addition to the A549 cells, this supernatant was preincubated for 1 h at 37 °C with either specific neutralizing antibodies against IL-1Ra (aIL-1Ra), or isotype control antibodies (IgG Goat), both 10 μg/mL. Thereafter, the A549 cells were either stimulated for 24 h with medium or 3 pg/mL active IL-1β. Bars represent means plus SEM, paired t testing. Experiment represents six separate measurements. **P < 0.01. (H) IL-1Ra mRNA expression levels or protein in picograms per milliliter (pg/mL) (I) in PBMCs (in the absence/presence of anti-TLR10 coated antibodies) with or without PI3k inhibitors 3MA and Wortmannin (Wort). (J) Model of TLR10 function. TLR10 inhibits MAPK signaling, and also induces PI3k which finally results in IL-1Ra production.
Using a set of complementary studies, including hTLR10tg mice, we showed that TLR10 acts as an inhibitory TLR that controls mainly TLR2-driven signals. However, we have to stress that the effects of TLR10 observed here are not exclusively inhibitory: the transcriptomic analysis clearly shows an important set of genes that are strongly up-regulated upon engagement of TLR10. The biological relevance of this particular set of genes remains to be explored more extensively in future studies.

Within the TLR family, TLR10 is most closely related to TLR1 and TLR6, the two TLRs that form heterodimers with TLR2 and hence function as coreceptors (2). Given the results of our cotransfection experiments, it is highly likely that the inhibitory effects of TLR10 are mediated through an interaction of TLR10 with TLR2 and perhaps also with TLR1 and TLR6 (Fig. 4).

Regarding the molecular mechanisms through which TLR10 exerts its inhibitory effect, broadly three different mechanisms could be envisaged: (i) competition for ligands with the stimulatory TLRs, (ii) competition for dimerization with the other members of the TLR2 family (TLR1, TLR6), and (iii) TLR10-specific direct inhibitory signaling. Our cotransfection experiments plead for either of the first two mechanisms, whereas the experiments showing TLR10-dependent induction of IL-1Ra suggests that the latter pathway is also operational. The in vivo experiments with the hTLR10tg mice support all three of these possible mechanisms. The link between TLR10 and IL-1Ra is corroborated by a recent publication reporting that allelic variants of the TLR10 gene may influence the susceptibility and time-course of hearing loss of Meniere disease (13). Based on favorable clinical experience with recombinant IL-1Ra (anakinra) treatment in Meniere disease, a controlled clinical trial this drug is underway (15). It may be speculated that loss-of-function of the TLR10 is involved in a lack of sufficient IL-1Ra production in Meniere disease.

Currently, it is unknown whether there are specific ligands for TLR10 other than those are also binding TLR2. Because TLR10 expressed on the transfected cells responded to Pam3Cys, FSL-1, and B. burgdorferi, it is likely that these TLR2 ligands also directly interact with TLR10. Interestingly, the effect of TLR10 occurs with both ligation of the TLR2/TLR1 complex (by Pam3Cys) and of the TLR2/TLR6 complex (by FSL-1), indicating that TLR2 ligation is crucial. Because transfection may have led to overexpression of the receptors, we cannot rule out that some spontaneous heterodimerization of TLR10 with TLR2 has occurred.
The finding of an inhibitory TLR among the agonistic TLRs is not entirely surprising. TLRs belong to the Toll/IL-1R family, and within that family other inhibitory molecules are known. Examples are IL-1R type II and single-lg IL-1-related receptor (SIGIRR). Such receptors may exert their suppressive effect through competition with the stimulatory receptors (e.g., the decoy receptor IL-1RII), whereas others act through inhibitory intracellular signals (e.g., SIGIRR). Members of other classes of pattern-recognition receptor also display suppressive activities, such as the C-type lectin receptors DC-SIGN and mannose receptor (20, 21). However, up-regulation of inhibitory cytokines (e.g., IL-36Ra, IL-38) and antagonists belonging to the IL-1 superfamily was not seen by mRNA sequencing after cross-linking anti-TLR10 antibodies.

The finding of a suppressive function of TLR10 has important consequences. First of all, this study elucidates a biological function for the last human TLR without a known role, and contributes to our understanding of TLR biology. Also, understanding the function of TLR10 raises opportunities for better insight into pathology. Genetic variability in TLR10 may change the balance between pro- and anti-inflammatory responses, and hence modulate the susceptibility to infection and to autoimmune disease. Such insights may be relevant for the development of novel treatment strategies based on modulation of the function of TLR10.

Experimental Procedures

Blood Samples. Human PBMCs were isolated by differential centrifugation using Ficoll-Paque (GE Healthcare) from buffy-coats of healthy blood donors (Sanquin Bloodbank). For the genetic studies, DNA of a cohort of healthy volunteers was used as described previously (22).

Reagents. RPMI 1640 (Dutch modification) was used as PBMC culture medium. Before use, RPMI was supplemented with 50 mg/mL gentamycin, 2 mM L-glutamine, and 1 mM pyruvate. LPS (Escherichia coli serotype 055:B5; Sigma) was repurified as previously described (23). Synthetic Pam3Cys was purchased from EMC Microcollections. Borrelia spirochetes and Candida albicans were cultured and prepared as described previously (22, 24). Anti-TLR10 antibody was purchased from Abcam (Clone 3C10CS, and Clone 158C1114, endotoxin-free) and mouse IgG1 monoclonal antibody was used as isotype control. SB202190 (p38 MAPK inhibitor, 0.3 μM); SP600125 (JNK1/2/3 inhibitor, 5 μM), and U0126 (MEK1/2 inhibitor, 3 μM) were purchased from Superarray Bioscience Corporation, 3MA was purchased from Sigma, and Wortmannin was purchased by Biologend. In experiments using pharmacological inhibitors, control cells were treated with an equivalent concentration of vehicle (DMSO).

PBMC Stimulations and TLR10 Blockade. Samples of venous blood were drawn after informed consent was obtained. Experiments were conducted according to the principles expressed in the Declaration of Helsinki. Venous blood was drawn from the cubital vein into 10-mL EDTA tubes (Monoject). PBMCs were isolated according to standard protocols, with minor modifications. See SI Experimental Procedures for more information.

A549 Culture and Stimulations. See SI Experimental Procedures for more information regarding A549 culture and stimulations.

Cytokine Measurements. IL-8 production by HEK cells was determined using PeliKine Compact ELISA kits (Sanquin). Cytokines were measured using specific sandwich ELISA kits for IL-1α, TNF-α, and IL-1Ra. IL-6 and IL-10 were measured using PeliKine Compact ELISA kits (Sanquin). The detection limit of the different ELISA kits was 20 pg/mL.

Plasmid DNA isolation. See SI Experimental Procedures for more information regarding plasmid DNA isolation.

Transfection of HEK293 Cells with TLR10. HEK293 were cultured in DMEM F12 medium (Gibco, Life Technologies), 100 μM Penicillin-100 μg streptomycin (Invitrogen), and 7.5% (vol/vol) nonheat inactivated FBS (HyClone, Thermo Scientific). See SI Experimental Procedures for more information.

TLR10 Silencing by siRNA. Adherent monocytes were cultured into macrophages for 6 d in the presence of 10% (vol/vol) human pool serum and 50 ng/mL macrophage-colony stimulating factor (ProSpec). See SI Experimental Procedures for more information.

mRNA Extraction, DNA Isolation, and PCR Analysis. See SI Experimental Procedures for more information.

RNA Sequencing. See SI Experimental Procedures for more information regarding RNA sequencing.

Generation of the hTLR10tg Mice. A constitutive human TLR10 knock-in mouse was generated by Taconic Artemis using targeted transgenesis. Using recombination-mediated cassette exchange (RMCE), a CAG promoter cassette, the human TLR10 ORF, and the hGH polyadenylation signal and an additional polyadenylation signal was inserted into the ROSA26 locus. This obtained RMCE vector was transfected into TaconicArtemis-CS7BL/6 ES cell line. Using positive Neomycin selection, recombinant clones were selected. Positively selected blastocysts were transferred into embryos and chimeric mice were produced. Using a Caliper LabChip GX device, sample analysis has been performed. Animals were fully back-crossed to C57Black6 background before they were used for experiments. Genotyping of the mice was performed using PCR and 1.5% agarosegel electrophoresis using primers wild-type-forward: 5′CCTCTCCCTCTGATACCTGAACCTC- wild-type-reverse: 5′ CGATGCTTAACTACCTGATAGG; TLR10 cond-forward: 5′GACACGAGGAGGTAGGC- TC; TLR10 cond-reverse: 5′CTTCTCAAGATAGCATGGG; positive control-forward: 5′ GAGACTCTGGTACACTCC; positive control-reverse: 5′ CCTTCAGAAGAGCTGGGAC. PCR conditions were 95 °C 5 min; 35 cycles of 95 °C 30 s, 60 °C 30 s, 72 °C 60 s, followed by 72 °C 10 min. Male wild-type and hTLR10tg mice between 12 and 16 wk of age were used. The mice were fed sterilized laboratory chow (Hope Farms) and water ad libitum. The experiments were approved by the Ethics Committee on Animal Experiments of the Radboud University Medical Centre, Nijmegen, The Netherlands. To examine the inflammatory response, both wild-type and hTLR10tg mice were injected intraperitoneally with 50 μg Pam3Cys in 100 μL PBS. After 4 h, mice were killed. Cytokine concentrations in EDTA plasma or in supernatants of ex vivo stimulated splenocytes were measured using ELISA, as described previously (10).

Statistics. Data were analyzed using nonnormally distributed two-tailed Mann–Whitney U test or paired t-testing (GraphPad Prism, GraphPad Software). Values of P < 0.05 were considered statistically significant: * P < 0.05; ** P < 0.01; *** P < 0.001. The means ± SEM of three or more independent experiments are reported.

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