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TLR Cross-Talk Specifically Regulates Cytokine Production by B Cells from Chronic Inflammatory Disease Patients

Madhumita Jagannathan,* Hatice Hasturk,† YanMei Liang,‡ Hyunjin Shin,§ Jeremy T. Hetzel,¶ Alpdogan Kantarci,† Daniel Rubin,¶ Marie E. McDonnell,¶ Thomas E. Van Dyke,† Lisa M. Ganley-Leal,2§§ and Barbara S. Nikolajczyk2,3§

Chronic systemic inflammation links periodontal disease and diabetes to increased incidence of serious comorbidities. Activation of TLRs, particularly TLR2 and TLR4, promotes chronic systemic inflammation. Human B cells have been generally thought to lack these TLRs. However, recent work showed that an increased percentage of circulating B cells from inflammatory disease patients express TLR2 and TLR4, and that TLR engagement on B cells resulted in unexpected changes in gene expression. New data show that B cells from inflammatory disease patients secrete multiple cytokines in response to different classes of TLR ligands. Furthermore, the B cell response to combinations of TLR ligands is cytokine- and ligand-specific. Some cytokines (IL-1β and IL-10) are predominantly regulated by TLR4, but others (IL-8 and TNF-α) are predominantly regulated by TLR2, due in part to TLR-dictated changes in transcription factor/promoter association. TLR2 and TLR9 also regulate B cell TLR4 expression, demonstrating that TLR cross-talk controls B cell responses at multiple levels. Parallel examination of B cells from periodontal disease and diabetes patients suggested that outcomes of TLR cross-talk are influenced by disease pathology. We conclude that disease-associated alteration of B cell TLR responses specifically regulates cytokine production and may influence chronic inflammation. The Journal of Immunology, 2009, 183: 7461–7470.

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2 L.M.G.-L. and B.S.N. contributed equally to this work.

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The Journal of Immunology
of proinflammatory cytokines under some conditions (22, 23). Engagement of other TLR family members, such as TLR9, can alternatively alter cellular responses to either TLR2 or TLR4 ligands in myeloid cells (23, 24). Appropriate TLR cross-talk therefore plays an important role in mounting an effective immune response to the complex combinations of TLR ligands presented by pathogens, commensal bacteria, and endogenous ligands.

Studies aimed at understanding the role of innate immune cells and TLR function in systemic inflammatory disease, with the exception of studies on TLR9, have largely focused on myeloid cells. However, B cells also function as a critical arm of the innate immune system, in part due to their ability to respond to TLR ligands and secrete cytokines (25). The role of B cell TLR engagement and subsequent cytokine production in chronic inflammatory diseases, including periodontal disease and diabetes, is poorly characterized. Our studies suggest that activated human B cells can circulate throughout the body (26); therefore, B cells may play an ongoing role in systemic manifestations of inflammatory diseases. Periodontal disease patients vs healthy donors have an elevated percentage of TLR2- and TLR4-positive B cells. New data show that these B cells constitutively and inducibly secrete elevated levels of cytokines, the latter in response to TLR ligands. These results also uncovered a high degree of specificity in B cell cytokine production in response to combinations of TLR ligands. Finally, B cells from periodontal disease and diabetes patients responded differently to combinations of TLR ligands. Based on this analysis of biologically important outcomes of TLR pathway cross-talk in human inflammatory disease patients, we conclude that clinical treatments and vaccines aimed at regulating immune responses through TLRs must test the complex response of B cells to combinations of TLR ligands.

Materials and Methods

Cells

Human samples were obtained following informed consent under a Boston University Institutional Review Board-approved protocol. Peripheral blood was collected into heparinized tubes by venous puncture. B cells were purified from whole blood using Histopaque 1077 to isolate the peripheral blood mononuclear layer, then negatively selected with magnetic beads to purify the CD19+ B cells according to the manufacturer’s protocol (Miltenyi Biotec). Only B cell preparations that were >95% pure were used in cytokine analyses. Most contaminating cells in all preparations were CD3+ T cells; monocyte contamination was <1%. B cells were stimulated for 24 h before analysis of secreted cytokines. The initial B cell concentration for all cultures was 10^6/ml, and all cultures were incubated in U-bottom wells. Nondiabetic periodontal disease (PD) patients had a diagnosis of localized aggressive periodontitis (27) but no other known disease. The PD patients that provided B cells for cytokine analyses are summarized in supplemental Table S1.4 In brief, the PD patients were characterized by periodontal infection with multiple organisms including P. gingivalis and Actinobacillus actinomycetemcomitans. Clinical and radiographic criteria of PD were: age of onset around the circumpuberal period (<13 years old), and alveolar bone loss localized around the first permanent molars and incisors (27). Additionally, a subject’s periodontal diagnosis was further confirmed by neutrophil functional analysis (28). Systemically healthy donors who (27). Additionally, a subject’s periodontal diagnosis was further confirmed by neutrophil functional analysis (28). Systemically healthy donors who

Flow cytometry

B cells were isolated by negatively selecting magnetic beads and were >95% pure as assayed by flow cytometry with anti-CD11b, anti-CD3, and anti-CD19 (Fig. 1). Rested B cells (1 h at 37°C) were stimulated with ligands at 1 μg/μl (approximately equimolar amounts) for 6–24 h for chromatin immunoprecipitation (ChIP) or cytokine analyses, respectively. ChIPs were completed as published (18). Alternatively, DNA was amplified with IL-8 primers: 5’-TGG GCCATGTTGCAA-3’; 5’-ACTTAT GCACCTC ATCTTCTATT-3’. Cytokines were quantified on a Luminex 200 using a 10-plex detection kit that measured IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, TNF-α, and GM-CSF (Invitrogen). TLR4 ligands have been described (31, 32).

Statistical analyses

The Mann-Whitney U test was used for nonparametric comparisons of groups for B cells from peripheral blood. Wilcoxon matched-pairs tests were used for cytokine analyses. Linear associations of continuous variables were assessed with Spearman’s rank correlation coefficient (Prism). ChIPs were analyzed by Student’s t test. All statistical tests were two-tailed, and p values of <0.05 defined statistical significance.

Results

B cells from PD patients express functional TLR2

Our work on B cells from inflamed tonsil and inflammatory bowel disease patients demonstrated that B cells from inflammatory environments are surface TLR2-positive and respond to TLR2 ligand by secreting IL-8 (26, 30). To test whether B cells from another chronic inflammatory disease, aggressive periodontitis, are similarly altered, we measured the percentage of TLR2-positive B cells in whole blood and inflamed gingiva from PD patients (Fig. 1A–C). PD patients had a modest but statistically significant increased percentage of TLR2-positive B cells compared with healthy donors (Fig. 1B). A high percentage of B cells in inflamed gingiva also express TLR2 (Fig. 1C), but the absence of healthy B cells from noninflamed gingiva prevented a comparison to gingival B cells from PD patients (18). TLR2-positive B cells were equivalently represented in the CD19+/CD38+, CD19+/CD38+, and CD19+/CD27+ populations (not shown). This finding is consistent with data showing similar expression levels of TLRs 1, 2, 7, 9, and 10 among naive, germinal center, and memory B cells (33).

To determine whether the modest elevation of circulating TLR2-positive B cells in PD patients correlated with increased TLR2 responsiveness, we stimulated highly purified CD19+ B cells from PD patients (Fig. 1D) with the prototypic TLR2 ligand Pam3CSK4 (Pam3). B cells from PD patients secrete IL-8 in response to TLR2 engagement (Fig. 1, E and F). This contrasts with the lack of IL-8 production by B cells from healthy donors (Fig. 1F). Differences in IL-8 secretion between B cells from healthy vs PD donors were statistically significant (Fig. 1F; compare open and filled bars; p < 0.05 under all conditions), including media incubation. These data suggest that B cells from PD patients are constitutively activated, perhaps by in vivo ligands from common periodontal pathogens, such as P. gingivalis (34). We conclude that modest TLR2 elevation on B cells from PD patients confers significant increases in TLR2 ligand response.
A theoretical source of IL-8 in our cultures is the small number of contaminating cells, especially monocytes, that secrete high levels of cytokines in response to TLR2 (and TLR4) ligand. The lack of IL-8 in samples from healthy donors (Fig. 1F) discounts this possibility. However, to further test the role of contaminating non-B cells in constitutive and inducible IL-8 production, we determined the relationship between B cell purity (% CD19<sup>+</sup>) and cytokine production (ng/ml) by Spearman’s rank correlation. No correlation emerged (\( p > 0.1 \); not shown), confirming that contaminating cells are unlikely sources of IL-8 or other cytokines in these cultures. Furthermore, preliminary examination of monocyte-contaminated B cell preparations indicated that <1% contaminating monocytes would insignificantly contribute to overall cytokine levels measured in our highly purified B cell populations (<10% of total cytokine production, data not shown). We conclude that contaminating monocytes are unlikely sources of TLR2 (and TLR4)-induced IL-8 in our purified B cell cultures. These findings support our conclusion that the relatively modest increase in TLR2-positive B cells in PD patients confers robust responsiveness to TLR2 ligand as measured by IL-8 secretion.

To identify mechanisms driving IL-8 production by B cells from PD patients, we examined IL-8 promoter association with a transcription factor that activates IL-8 in myeloid cells, c-Jun (35). c-Jun constitutively associated with the IL-8 promoter in freshly isolated B cells from PD patients (Fig. 1G), and association was maintained after 24 h in culture (Fig. 1I, leftmost open bars). c-Jun/IL-8 association is further increased in B cells activated through TLR2 (Pam3; Fig. 1I). These data suggest that TLR2 activates...
c-Jun, resulting in constitutive and inducible IL-8 production in B cells from PD patients.

To confirm that B cells from PD patients produce IL-8 in vivo, and to further test similarities between circulating and gingival B cells (Fig. 1, A and C), we measured intracellular IL-8 expression in fresh ex vivo peripheral blood and gingival B cells from PD patients. Blood B cells from PD patients, but not healthy donors, were intracellular IL-8-positive (Fig. 1J). Likewise, B cells isolated from inflamed gingiva produced IL-8 (Fig. 1K). These data confirm that B cells, rather than contaminating cells, are the source of IL-8 in our ex vivo experiments, and they support the likelihood that B cells produce IL-8 in vivo. We conclude that activation of the IL-8 promoter by TLR2 engagement and c-Jun/IL-8 association drives IL-8 production by B cells in PD patients.

**B cells from PD patients constitutively secrete IL-1β**

In addition to IL-8, multiple proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, have been implicated in PD and in a second inflammatory disease that is a strong risk factor for PD, diabetes (36–39). To more comprehensively identify the role B cells play in chronic inflammatory disease, we first measured secretion of additional cytokines by fresh ex vivo B cells from healthy or PD patients. On average, B cells from PD patients vs healthy donors constitutively secreted elevated levels of IL-1β (Fig. 2). All B cells constitutively secreted low levels of GM-CSF, IFN-γ, IL-10, and TNF-α (Fig. 2) and undetectable levels of IL-2, IL-4, and IL-5 (not shown). IL-6 production was highly variable in all assays hence uninterpretable (not shown). We conclude that B cells from PD patients may contribute to chronic systemic inflammation through constitutive IL-8 and IL-1β secretion.

**B cell TLR2 engagement activates selected cytokines and other signatures of inflammatory cells**

To more broadly evaluate the role of increased B cell TLR expression (Fig. 1 and Ref. 18) in chronic inflammatory disease, we measured secretion of additional cytokines by B cells responding to TLR ligands. The first series of analyses focused on cytokine secretion by B cells responding to single TLR ligands. B cells from PD patients responded to the TLR2 ligand Pam3 by...
significantly increasing production of all cytokines (supplemental Fig. S1), although some cytokine levels were indistinguishable, on average, from levels produced by TLR2-stimulated B cells from healthy donors (Fig. 3A; GM-CSF and IFN-γ). A weak TLR2 response in B cells from healthy donors is consistent with published data (30, 33). However, a subset of cytokines, specifically IL-1β and TNF-α, are secreted at significantly elevated levels (p < 0.05) by TLR2-stimulated B cells from PD vs healthy donors (Fig. 3A). IL-10 secretion was also preferentially induced in B cells from PD patients, but this increase was statistically insignificant (p > 0.05), due to greater variability in inducible IL-10 secretion. IL-2, IL-4, and IL-5 were undetectably secreted by all B cells responding to all stimuli tested (not shown). These data further support the conclusion that elevated surface TLR2 expression on B cells from PD patients may have proinflammatory outcomes, and they suggest that constitutively elevated IL-1β production by B cells from PD patients (Fig. 2) could result from TLR2 engagement by in vivo ligands.

TLR2 and TLR4 regulate each other in mouse B cells (40); therefore, we asked how elevated TLR2 activity might affect TLR4 in B cells from PD patients. To test whether TLR2 engagement affected TLR4 gene activation in B cells from PD patients, we treated B cells from PD patients with TLR2 ligand (Pam3) and measured inducible association of the TLR4-activating transcription factor PU.1 (41) with the TLR4 promoter (Fig. 3B). PU.1 association with the TLR4 promoter increased ~5-fold in TLR2-stimulated B cells, and it increased somewhat less (3-fold) in cells stimulated with TLR2 ligand (Pam3) plus TLR4 ligand (rLPS). As expected, TLR2 ligand also up-regulated surface TLR4 expression as assayed by flow cytometry (Fig. 3C). We conclude that TLR2 ligand activates TLR4 expression in B cells from PD patients. Interestingly, these findings identify a mechanism by which in vivo TLR2 ligands may regulate TLR4-mediated B cell activation in PD (18).

**B cell TLR4 engagement activates a subset of cytokines activated by TLR2**

To further explore potential physiological outcomes of TLR2-mediated TLR4 activation in B cells from PD patients, we measured B cell responses to TLR4 ligands. We measured cytokine responses to rLPS, the TLR4 ligand that alters gene expression in B cells from PD patients (18). Although, on average, rLPS increased production of most cytokines in PD B cells (GM-CSF, IFN-γ, IL-1β, and IL-10; supplemental Fig. S2), only IL-8 and TNF-α production was significantly higher in B cells from PD vs healthy donors (Figs. 1F and 3D). Interestingly, increased IL-8 secretion in response to rLPS did not correlate with inducible c-Jun/IL-8 promoter association (Fig. 1A), indicating that, at least in some cases, IL-8 can be activated in the absence of c-Jun binding. Note that rLPS failed to activate IL-8 production by monocytes (Fig. 1G), indicating that TLR4 may function differently on B cells compared to monocytes. We conclude that TLR4 significantly activates only a subset of TLR2-activatable cytokines in B cells from PD vs healthy donors.

**TLR9 engagement activates cytokine and TLR4 expression by B cells from healthy and PD donors**

To more comprehensively identify outcomes of TLR activation in B cells from inflammatory disease patients compared with healthy donors, we stimulated B cells with the TLR9 ligand CpG and measured cytokine production. CpG-mediated TLR9 activation up-regulated multiple cytokines in B cells from both PD and healthy donors, on average, to an equivalent level (Fig. 3E and supplemental Fig. S3). Differences in cytokine production between PD and healthy donors were not significant (p > 0.1). These data suggest that the MyD88-dependent TLR pathway, presumably activated by all three TLR ligands tested, is not fundamentally hyperactive in B cells from PD patients. Furthermore, as with the TLR2 ligand, the TLR9 ligand activated TLR4 expression, as measured by an increased mean fluorescence intensity of TLR4 staining on CpG-treated B cells (Fig. 3F). We conclude that activation of TLRs up-regulated on B cells from PD patients (TLR2 and TLR4) selectively increases cytokine production, but that activation of TLRs expressed on B cells from healthy donors (i.e., TLR9) generally increases cytokine production in all B cells. Furthermore, our demonstration that TLR2 and TLR9 activate TLR4 expression predicts that the outcome of B cell TLR engagement may be influenced by cross-talk between members of the TLR family.

**Cytokine-specific outcomes of TLR costimulation identify B cell cytokines regulated predominantly by TLR2 vs TLR4**

TLR family cross-talk results in alteration of cytokine production, compared with cytokine production in the presence of single TLR ligands (42). The paucity of TLR2 and TLR4 expression on B cells from healthy donors (43) had previously made TLR2/4/9 cross-talk studies irrelevant in B cells. However, the demonstrations that (1) TLR2 and TLR4 are up-regulated on B cells from inflammatory disease patients (18, 26) (Fig. 1) and (2) TLR2 ligand activates TLR4 (Fig. 3, B and C) make investigation of TLR2/4 cross-talk critical for identifying roles for B cells in disease pathogenesis. Understanding up-regulated TLR cross-talk is also essential for safe vaccine design for the large number of individuals affected by inflammatory diseases. To begin testing how TLR cross-talk affects B cell cytokine production, we stimulated these cells in vitro with TLR2 ligand (Pam3) in combination with the TLR4 ligand rLPS. Levels of some cytokines, specifically IFN-γ, TNF-α, and IL-8, were indistinguishable in B cells stimulated with Pam3 with or without rLPS (Figs. 1F and 4, A and B). The lack of an effect of rLPS on Pam3-induced IL-8 production (Fig. 1F) is consistent with the indistinguishable change in c-Jun/IL-8 promoter association in Pam3 with or without rLPS-stimulated B cells (Fig. 1I). In contrast, Fig. 4C shows that B cells from PD patients stimulated with Pam3 plus rLPS secreted significantly lower levels of IL-10, on average, compared with B cells stimulated with TLR2 ligand (Pam3) alone (p = 0.037). IL-1β was modestly, but statistically significantly lower in B cells stimulated with TLR2 plus TLR4 ligand (Pam3 plus rLPS) vs TLR2 ligand (Pam3) alone (Fig. 4D; p = 0.027; 10 of 12 samples had lower IL-1β levels in Pam3 plus rLPS vs Pam3-stimulated cultures). The biological significance of this modest IL-1β decrease remains to be determined. However, decreased IL-1β protein secretion corresponds to significantly decreased association between the IL-1β promoter and an IL-1β activating transcription factor, c-Jun (44), in cells treated with Pam3 plus rLPS vs Pam3 alone (Fig. 4E). The ability of rLPS to decrease Pam3-mediated IL-10 and IL-1β production in B cells contrasts with the inability of rLPS to affect Pam3-induced IL-1β mRNA production by monocytes (Fig. 4F). This finding further supports the conclusion that TLR4 has different functional consequences in B cells vs monocytes. As expected, B cells from healthy donors produced low amounts of cytokines in response to all stimuli (Fig. 4, A–D, open bars). We conclude that rLPS predominantly regulates both IL-1β and IL-10 genes in the presence of TLR2 ligand (Pam3). This result contrasts with similar levels of TNF-α production by B cells stimulated with TLR2 alone or in combination with TLR4 ligand, and suggests that TLR2-
mRNA in stimulated vs unstimulated monocytes. The latter value was set to equal 1. Shown are averages and SD of three independent experiments.

cient numbers and variability of measurements for IL-8 (number of cells for additional analysis. Furthermore, insuffi-
tations) because not all samples in Fig. 3 yielded a sufficient
comparison with values from Fig. 3 (single TLR ligand stimula-
ized with the TLR2 ligand Pam3 in the presence or absence of the TLR4 ligand rLPS. Shown is average and SD of (A) IFN-γ, (B) TNF-α, (C) IL-10, and (D) IL-1β production. Analysis was described in Fig. 3. Values of \( p > 0.1 \) for all comparisons are emphasized by NS; \( * \), \( p < 0.05 \). The number of Pam3-stimulated samples is as in Fig. 3. For rLPS, \( n=11 \) for IL-1β, and \( n=10 \) for IFN-γ, TNF-α, and IL-10. For Pam3 plus rLPS, \( n=12 \) for IL-1β, and \( n=11 \) for IFN-γ, TNF-α, and IL-10. E, ChIP analysis of transcription factor association with the IL-1β promoter in B cells from healthy (left) or PD (right) donors stimulated as indicated. \( * \), \( p=0.04 \) (by paired Student’s t test) in c-Jun/IL-1β promoter association in B cells from PD patients stimulated with Pam3 alone or in combination with rLPS. Shown are averages and SEM from two to three healthy and three PD donors. F, IL-1β mRNA production by monocytes stimulated with the indicated ligand(s) for 20 h. mRNA was normalized to GAPDH and quantified by the ΔΔC\(_T\) method. The y-axis shows mRNA in stimulated vs unstimulated monocytes. The latter value was set to equal 1. Shown are averages and SD of three independent experiments. G–I, B cells from healthy or PD donors were stimulated with TLR2 (Pam3) or TLR4 (LPS 1690) ligand alone and in combination as indicated. NS emphasizes no significant difference between Pam3 and Pam3 plus 1690-stimulated B cells from PD patients. Results for (G) TNF-α, (H) IL-10, or (I) IL-1β are shown. Bars show average of \( n=4 \) for 1690-stimulated cells, and \( n=6 \) for Pam3 plus 1690-stimulated cells.

TLR4-activated pathways are redundant for only a subset of cytokines.

To determine how TLR4 ligand from a periodontal pathogen (P. gingivalis LPS 1690) influences B cell activation, we stimulated purified B cells from PD or healthy donors with LPS 1690 in the presence or absence of TLR2 ligand (Pam3). LPS 1690 alone had little effect on cytokine production (Fig. 4, G–I and supplemental Fig. 5A). Furthermore, LPS 1690 insignificantly changed Pam3-induced cytokine production (Figs. 4, G–I). Overall, data in Fig. 4 indicate that engagement of TLR2 and TLR4 on B cells from PD patients has cytokine-specific (IL-10 and IL-1β vs TNF-α) and ligand-specific (LPS 1690 vs rLPS) outcomes.

**TLR2 and TLR4 insignificantly affect TLR9-mediated cytokine production by B cells from healthy and PD donors**

To further test the possibility that TLR cross-talk regulates cytokine production by B cells, we stimulated cells with CpG alone or in combination with Pam3 or rLPS to activate TLR9, TLR2, or TLR4, respectively, and measured cytokine production. B cells from PD and healthy donors produced similar levels of cytokines in response to all stimulation conditions (Fig. 5). Note that cytokine values in Fig. 5 cannot be quantitatively compared with values from Fig. 3 (single TLR ligand stimulations) because not all samples in Fig. 3 yielded a sufficient number of cells for additional analysis. Furthermore, insufficient numbers and variability of measurements for IL-8 (\( n = 3–4 \)) made any conclusions on IL-8 production in response to TLR9 ligand unreliable. Regardless of these caveats, multiple conclusions are supported by these data. First, the TLR-triggered MyD88-dependent pathway that lies downstream of TLR2, TLR4, and TLR9 (45, 46) is probably fundamentally similar in B cells from PD and healthy donors. This finding strengthens our conclusion that cytokine production is regulated through ligand-specific (unknown) mechanisms. Second, these data show that TLR2 or TLR4 up-regulation does not alter response to a TLR normally expressed in human B cells, TLR9 (43, 47, 48). The precise response of B cells is therefore determined by the availability of specific TLR ligands. Finally, the similar response of B cells from healthy and PD donors indicates that B cell TLR9-mediated cytokine production plays a critical role in these patients.

**IL-1β production uncovers differences in the TLR responses of B cells from PD vs DM patients**

IL-1β is a key proinflammatory cytokine in multiple chronic inflammatory diseases with systemic manifestations, including PD and DM (49, 50). Because IL-1β secretion is constitutive by B cells from PD patients (Fig. 2), but is further regulated by TLR2 and TLR4 ligands (Figs. 3A and 4D), we asked whether IL-1β is similarly regulated in B cells from DM patients, who often have confounding oral inflammation. In contrast to B cells from PD patients, B cells from DM patients do not constitutively secrete IL-1β at levels that are significantly greater, on
average, than levels secreted by B cells from healthy donors (Fig. 6A). We next considered the possibility that TLR ligands regulate inducible IL-1β production by B cells from DM patients. Ligands to TLR2, TLR4, and TLR9 modestly (albeit statistically significantly) increased IL-1β secretion (Fig. 6B). This result contrasts with the significant increase in IL-1β production by B cells from PD patients responding to TLR2 ligand (Pam3; Fig. 3A). We conclude that TLR-mediated IL-1β regulation differs in B cells from DM vs PD patients.

To test whether B cells from DM patients, as with other innate immune cell types (but in contrast to B cells from PD patients), synergistically activate cytokine production in response to combinations of TLR ligands, we measured responses of cells stimulated with multiple TLR ligands. Fig. 6C shows that the oral pathogen TLR4 ligand LPS 1690 insignificantly altered IL-1β production by B cells from either DM or PD patients ($p > 0.1$ in Pam3 vs Pam3 plus LPS 1690 samples, NS). Although the TLR4 ligand rLPS modestly decreased IL-1β TLR2 (Pam3)-activated IL-1β production in B cells from PD patients (Fig. 6D), TLR4 ligand did not affect IL-1β production by TLR2-stimulated B cells from DM patients (Fig. 6D, NS). Similarly, Pam3 further activated TLR4-mediated IL-1β production by B cells from DM patients, but not PD patients (Fig. 6E). Under all stimulation conditions, IL-1β production by B cells from DM patients was low. However, the data support the idea of fundamental differences in TLR pathway “wiring” in B cells from DM vs PD patients. Preliminary analysis of additional cytokines produced by B cells from DM patients is consistent with this interpretation (not shown). Taken together, these new findings on B cell cytokine production in PD and DM raise the possibility that alteration of TLR pathways, and therefore outcomes of TLR activation, significantly influence systemic inflammatory responses by altering B cell cytokine production, most potently in PD patients.

Discussion

Our data identify disease-, stimulus-, and cytokine-specific B cell responses to combinations of TLR ligands. Findings herein also suggest mechanistic differences to explain the elegant specificity of human B cell responses to disease-associated combinations of TLR ligands. This response occurs despite modest elevation of surface TLRs, indicating surface expression may underestimate ligand responsiveness. Many pathogens, including the periodontal pathogen *P. gingivalis*, produce ligands that activate TLR2 and TLR4 (13, 51–53), two TLRs elevated on the surface of B cells from PD patients. Similarly, multiple TLRs would be engaged in B cells responding to TLR-activating vaccine adjuvant or confounding PD in the multitude of diabetic individuals with naturally elevated levels of TLR4 ligands, including free fatty acids and/or systemic endotoxin (7, 54, 55). Understanding the response of B cells to combinations of TLR ligands will aid in rational vaccine strategies that use TLR ligands as adjuvants and are targeted to a broad cross-section of individuals, inevitably including PD and DM patients.

Both TLR expression and TLR function differ between B cells from inflammatory disease patients and human myeloid cells. One interpretation of these findings is that B cell TLRs are “hard wired” differently from myeloid cell TLRs. A second possibility is that mechanisms driving cytokine activation are fundamentally different in B cells vs myeloid cells. The results from ChIP assays and B cells from either DM or PD patients ($p > 0.1$ in Pam3 vs Pam3 plus LPS 1690 samples, NS). Although the TLR4 ligand rLPS modestly decreased IL-1β TLR2 (Pam3)-activated IL-1β production in B cells from PD patients (Fig. 6D), TLR4 ligand did not affect IL-1β production by TLR2-stimulated B cells from DM patients (Fig. 6D, NS). Similarly, Pam3 further activated TLR4-mediated IL-1β production by B cells from DM patients, but not PD patients (Fig. 6E). Under all stimulation conditions, IL-1β production by B cells from DM patients was low. However, the data support the idea of fundamental differences in TLR pathway “wiring” in B cells from DM vs PD patients. Preliminary analysis of additional cytokines produced by B cells from DM patients is consistent with this interpretation (not shown). Taken together, these new findings on B cell cytokine production in PD and DM raise the possibility that alteration of TLR pathways, and therefore outcomes of TLR activation, significantly influence systemic inflammatory responses by altering B cell cytokine production, most potently in PD patients.

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Both TLR expression and TLR function differ between B cells from inflammatory disease patients and human myeloid cells. One interpretation of these findings is that B cell TLRs are “hard wired” differently from myeloid cell TLRs. A second possibility is that mechanisms driving cytokine activation are fundamentally different in B cells vs myeloid cells. The results from ChIP assays and B cells from either DM or PD patients ($p > 0.1$ in Pam3 vs Pam3 plus LPS 1690 samples, NS). Although the TLR4 ligand rLPS modestly decreased IL-1β TLR2 (Pam3)-activated IL-1β production in B cells from PD patients (Fig. 6D), TLR4 ligand did not affect IL-1β production by TLR2-stimulated B cells from DM patients (Fig. 6D, NS). Similarly, Pam3 further activated TLR4-mediated IL-1β production by B cells from DM patients, but not PD patients (Fig. 6E). Under all stimulation conditions, IL-1β production by B cells from DM patients was low. However, the data support the idea of fundamental differences in TLR pathway “wiring” in B cells from DM vs PD patients. Preliminary analysis of additional cytokines produced by B cells from DM patients is consistent with this interpretation (not shown). Taken together, these new findings on B cell cytokine production in PD and DM raise the possibility that alteration of TLR pathways, and therefore outcomes of TLR activation, significantly influence systemic inflammatory responses by altering B cell cytokine production, most potently in PD patients.

Discussion

Our data identify disease-, stimulus-, and cytokine-specific B cell responses to combinations of TLR ligands. Findings herein also suggest mechanistic differences to explain the elegant specificity of human B cell responses to disease-associated combinations of TLR ligands. This response occurs despite modest elevation of surface TLRs, indicating surface expression may underestimate ligand responsiveness. Many pathogens, including the periodontal pathogen *P. gingivalis*, produce ligands that activate TLR2 and TLR4 (13, 51–53), two TLRs elevated on the surface of B cells from PD patients. Similarly, multiple TLRs would be engaged in B cells responding to TLR-activating vaccine adjuvant or confounding PD in the multitude of diabetic individuals with naturally elevated levels of TLR4 ligands, including free fatty acids and/or systemic endotoxin (7, 54, 55). Understanding the response of B cells to combinations of TLR ligands will aid in rational vaccine strategies that use TLR ligands as adjuvants and are targeted to a broad cross-section of individuals, inevitably including PD and DM patients.

Both TLR expression and TLR function differ between B cells from inflammatory disease patients and human myeloid cells. One interpretation of these findings is that B cell TLRs are “hard wired” differently from myeloid cell TLRs. A second possibility is that mechanisms driving cytokine activation are fundamentally different in B cells vs myeloid cells. The results from ChIP assays and
the relatively “normal” activation of the MyD88-dependent pathway in B cells preliminarily discount this explanation. A third possibility is that TLR function differs, regardless of cell type, in patients with inflammatory disease compared with healthy individuals. This scenario emphasizes the importance of testing responses to TLR ligands in primary cells from patients before clinical trials to identify disease-specific outcomes that cannot be predicted by analysis of cells from healthy donors. Additionally, the demonstration that B cells from individual donors with PD tend to exhibit similar cytokine responses (supplemental Fig. S5) suggests that efforts to further understand and then harness TLR cross-talk to regulate cytokine production by B cells are likely to have widespread efficacy.

The change in cytokine production by B cells was low for some cytokines, regardless of statistically significance. A modest change in average cytokine production may hold higher significance in the subset of patients with more responsive B cells, and it therefore may identify subsets of patients within our cross-sectional cohort. Regardless, cytokines often function through feedback loops that can amplify the short-term signals measured in our 24-h cultures. Low levels of IL-1β production in our 24-h cultures. Low levels of IL-1β are particularly effective in activating a positive feedback loop to achieve robust IL-1β production in an autocrine/paracrine mechanism (56). Changes in the absolute value of cytokine production may therefore inaccurately estimate the physiological relevance of such changes in the complex in vivo environment. Future studies would benefit from a model organism that, unlike mice, generally houses a low percentage of TLR2/4 B cells. This model remains to be identified.

Periodontal disease is a common confounder of diabetes, and therefore the response of B cells in diabetic patients could result solely from oral inflammation. However, the distinct differences in IL-1β response in B cells from PD vs DM patients demonstrates that the B cells are functionally different, regardless of the co-occurrence of PD in diabetics. One possible explanation is that PD may be different from the chronic periodontitis more common in DM patients. A direct comparison of the role of PD vs diabetes in B cell function will require analysis of additional DM patients with healthy gingiva, who represent only ~30% of our clinical population. Alternatively, analysis of chronic periodontitis patients without confounding DM would determine the role that this type of PD might play in B cells responses in DM patients with confounding oral inflammation. These comparisons will be important to determine whether B cells in PD patients are likely to respond uniquely to treatments developed to counter TLR activation in diabetes.

The paradigm for TLR cross-talk summarized from various studies on myeloid lineage cells is shown in Fig. 7A. Based on our data, we propose a revised model to describe the effect of TLR cross-talk through surface molecules up-regulated in B cells from inflammatory disease patients (Fig. 7, B and C). Our data support the conclusion that selected cytokines (in gray) are predominantly regulated by TLR4 (leftmost arrow) and IL-10, gray) even in the presence of ligands for both. → Indicates that dominant regulation of cytokine production by TLR2 or TLR4 may involve direct interference of TLR-activated pathways, although this possibility remains to be tested. Decreased cytokine production was demonstrated for only one of two TLR4 ligands tested. Our data also show that TLR2 and TLR4 ligands do not detectably change TLR9-mediated B cell cytokine production (thick black arrow). The response of inflammatory disease patient B cells to other TLR2, TLR4, or TLR9 ligands remains to be determined. Interestingly, studies in wild-derived mouse strains support our conclusion that TLR responses differ between B cells and myeloid cells in outbred populations (59).

FIGURE 7. Models explaining biological outcomes of TLR cross-talk in hematopoietic cells. A, TLR cross-talk in myeloid cells is complex. Numerous studies show myeloid cells, predominantly dendritic cells and macrophages, respond to TLR2/4 coengagement by synergistically increasing IL-6 and TNF-α production (22, 23), although some studies indicated cytokine production decreases (20) or does not change in human monocytes responding to a combination of TLR2 and TLR4 vs a single TLR ligand alone (21). TLR2/4 cross-talk is represented by a gray double-headed arrow. TLR2 and TLR9 (leftmost double-headed black arrow) also cooperate to increase cytokine responses, although synergy in TNF-α production (in gray) is minimal (23). Coengangement of TLR4 and TLR9 (rightmost black double-headed arrow) resulted in additive/synergistic activation of IL-12 and IL-6 (in black) but not other cytokines (24). At least some of the apparent inconsistencies in these results are likely due to different outcomes triggered by different ligands that bind the same TLR. B and C, Model for TLR cross-talk in B cells from inflammatory disease patients. Our studies show that B cells from these patients express TLR2 and TLR4 and regulate select cytokines using mechanisms than likely vary from those used by myeloid cells. TLR2/4 cross-talk did not result in additive cytokine activation in B cells. We instead identified cytokines that are predominantly regulated by (B) TLR2 (IL-8, TNF-α, gray) or (C) TLR4 (IL-1β and IL-10, gray) even in the presence of ligands for both. → Indicates that dominant regulation of cytokine production by TLR2 or TLR4 may involve direct interference of TLR-activated pathways, although this possibility remains to be tested. Decreased cytokine production was demonstrated for only one of two TLR4 ligands tested. Our data also show that TLR2 and TLR4 ligands do not detectably change TLR9-mediated B cell cytokine production (thick black arrow). The paradigm for TLR cross-talk summarized from various studies on myeloid lineage cells is shown in Fig. 7A. Based on our data, we propose a revised model to describe the effect of TLR cross-talk through surface molecules up-regulated in B cells from inflammatory disease patients (Fig. 7, B and C). Our data support the conclusion that selected cytokines (in gray) are predominantly regulated by TLR2 (B) or TLR4 (C). TLR responses are influenced by idiosyncrasies of the inflammatory disease (PD vs DM, not shown in model). TLR2 and TLR4 insignificantly influence TLR9-mediated cytokine production (thick black arrow), in contrast to the generally additive effects of multiple TLR ligands on cytokine production in dendritic cells (24, 57, 58). This model makes testable predictions for understanding mechanisms responsible for the high specificity of TLR-triggered cellular responses likely to play important roles in disease pathogenesis.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.
References


Figure S1: Jagannathan et al.

PD

MvsP GM-CSF

Healthy

MvsP GM-CSF

IlvsP IFN-γ

MvsP FN-γ

IlvsP IL-1β

MvsP IL-1β

IlvsP IL-10

MvsP IL-10

IlvsP TNF-α

MvsP TNF-α
Supplemental Fig. 1:

Two point graphs showing cytokine production (designated at top of each graph) by B cells from PD (left column) and healthy (right column) donors. “MvsP” indicates a comparison between cells incubated in media (left dot of graph) and cells from the same donor incubated in Pam3 (right dot of graph). P values from paired nonparametric analysis are shown in upper left of each graph. N’s for each analysis are as in main text. Note that even minimal absolute differences in cytokine production can achieve statistical significance if variability is low (for example IFN-γ and IL-1β in B cells from healthy donors).
Figure S2: Jagannathan et al.

**PD**

- MvsR GM-CSF
- MvsR IFN-γ
- MvsR IL-1β
- MvsR IL-1κ
- MvsR TNFα

**Healthy**

- MvsR GM-CSF
- MvsR IFN-γ
- MvsR IL-1β
- MvsR IL-1κ
- MvsR TNFα
Supplemental Fig. 2:

Two point graphs showing cytokine production (designated at top of each graph) by B cells from PD (left column) and healthy (right column) donors. “MvsR” indicates a comparison between cells incubated in media (left dot of graph) and cells from the same donor incubated in rLPS (right dot of graph). P values from paired nonparametric analysis are shown in upper left of each graph. N’s for each analysis are as in main text.
Figure S3: Jagannathan et al.

PD

1 MvsCpG GM-CSF

0.0066

Healthy

MvsCpG GM-CSF

0.0625

1 MvsCpG IFN-g

0.0370

MvsCpG IFN-g

0.5708

1 MvsCpG IL-1b

0.0064

MvsCpGIL-1b

0.0513

1 MvsCpG IL-10

0.3277

MvsCpGIL-10

0.0313

1 MvsCpG TII-F-a

0.0099

MvsCpG TII-F-a

0.0025
Supplemental Fig. 3:

Two point graphs showing cytokine production (designated at top of each graph) by B cells from PD (left column) and healthy (right column) donors. “MvsCpG” indicates a comparison between cells incubated in media (left dot of graph) and cells from the same donor incubated in CpG (right dot of graph). P values from paired nonparametric analysis are shown in upper left of each graph. N’s for each analysis are as in main text.
Figure S4: Jagannathan et al.

PD

Mvs1690 GM-CSF

Healthy

Mvs1690 GM-CSF

Mvs1690 IFNγ

Mvs1690 IFN-γ

Mvs1690 IL-1β

Mvs1690 IL-1β

Mvs1690 IL-10

Mvs1690 IL-10

Mvs1690 TNF-α

Mvs1690 TNF-α
Supplemental Fig. 4:

Two point graphs showing cytokine production (designated at top of each graph) by B cells from PD (left column) and healthy (right column) donors. “Mvs1690” indicates a comparison between cells incubated in media (left dot of graph) and cells from the same donor incubated in *P. gingivalis* LPS 1690 (right dot of graph). P values from paired nonparametric analysis are shown in upper left of each graph. N’s for each analysis are as in main text.
Figure S5: Jagannathan et al.

Cytokine production by B cells from individual donors
Figure S5: Cytokine production by B cells from 9 individual PD patients emphasizes similarities among individual results. Concentration of each cytokine shown on the X-axis secreted by B cells from donors 1-9. Results from each treatments are shown as a single dot for each cytokine to indicate the overall pattern of cytokine production by B cells from that donor. Log scale on Y axis minimizes differences apparent in other figures. Media controls have lowest levels of cytokine production shown in each cluster of dots (not indicated).
<table>
<thead>
<tr>
<th></th>
<th>PD Median</th>
<th>Healthy Median</th>
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<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>32 (20-38)</td>
<td>31 (27-52)</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td><strong>Males (%)</strong></td>
<td>7 (50%)</td>
<td>8 (50%)</td>
</tr>
<tr>
<td><strong>Race n(%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Caucasian</td>
<td>2 (14.3)</td>
<td>6 (37.5)</td>
</tr>
<tr>
<td>-African American</td>
<td>11 (78.6)</td>
<td>8 (50)</td>
</tr>
<tr>
<td>-Asian</td>
<td>1 (7.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>-Hispanic</td>
<td>1 (7.1)</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>-Unknown</td>
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Table S2. Descriptive statistics of DM subjects (N=11 total).

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<thead>
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<th>Metric</th>
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<tbody>
<tr>
<td>Age (years)</td>
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<tr>
<td>A1c (%)</td>
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<td>6.0 to 14.9</td>
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<tr>
<td>Duration DM (years)</td>
<td>10</td>
<td>5 to 32</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>32.4</td>
<td>17.0 to 43.1</td>
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<tr>
<td>CRP* (mg/dL)</td>
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<td>0.1 to 16.4</td>
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<table>
<thead>
<tr>
<th>Category</th>
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<tbody>
<tr>
<td>Type 2 diabetes</td>
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</tr>
<tr>
<td>Males</td>
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<td>54.5</td>
</tr>
<tr>
<td>Race</td>
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<td></td>
</tr>
<tr>
<td>-Caucasian</td>
<td>6</td>
<td>54.5</td>
</tr>
<tr>
<td>-African American</td>
<td>5</td>
<td>45.5</td>
</tr>
</tbody>
</table>

DM=Diabetes mellitus; A1c=Hemoglobin A1c; BMI=Body mass index; CRP=C-reactive protein DM= diabetes mellitus  
* CRP values available for 9 of 11 subjects