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

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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.

C hronic inflammation underlies many diseases, including periodontal disease and diabetes mellitus (1, 2). These diseases are generally categorized by pathological changes, but many factors mediating morbidity likely stem from the systemic inflammation shared among these diseases (3, 4). In general, pathological inflammation results from inappropriate immune responses to seemingly innocuous stimuli, such as commensal microbes or host-derived ligands. Some chronic inflammatory diseases, such as periodontal disease, are thought to initiate as a failure in homeostasis with commensal bacteria. The resulting local inflammation is mirrored by increased systemic inflammation, which likely explains the link between periodontal disease and cardiovascular disease (5). Similarly, the systemic inflammatory environment in diabetes patients probably stems from elevated levels of endogenous ligands, such as free fatty acids or advanced glycation end products, that can stimulate innate immune cells to produce proinflammatory cytokines (6–8). Elevated circulating endotoxin levels in diabetes patients (9), perhaps originating from compromised mucosal surfaces, may further exacerbate the systemic inflammatory response implicated in the most serious complications of this disease.

TLRs are involved in mounting pathogenic inflammatory responses to commensal organisms and host-derived ligands in chronic inflammatory diseases, for instance periodontal disease and diabetes (10–12). The likely role of TLRs in human systemic inflammatory disease is supported by multiple reports. First, both TLR2 and TLR4 are receptors for products of major periodontal pathogens, including Porphyromonas gingivalis (13). Second, TLR4 polymorphisms that alter TLR4 function associate with occurrence of periodontal disease and diabetes, at least in some cohorts (14, 15). Third, TLR2 and TLR4 are strongly implicated in diabetes by demonstrations that either inhibiting or genetically deleting each receptor in mice protects against a key characteristic of type 2 diabetes, insulin resistance (6, 16, 17). These observations suggest that altering expression, and thus function, of TLRs can promote inflammation in chronic diseases. Interestingly, multiple studies, including analyses of TLR4+ B cells from periodontal disease patients, show that cellular responses to TLR4 ligands can be a mixture of prototypic pro- and anti-inflammatory responses (18, 19).

The concept that cross-talk among TLR family members defines the innate immune response has emerged from studies on immune system sentinel cells, especially dendritic cells and macrophages. TLR2 and TLR4 activate these cells through a MyD88-dependent pathway. TLR4 also activates the TRIF/TRAM pathway in response to selected ligands. Pathway cross talk explains why in at least some cases, TLR4 engagement has the same biological outcome as engagement of TLR2 and TLR4 (20, 21). However, TLR2 and TLR4 ligands can also synergize to activate production

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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 isolate CD19<sup>+</sup> 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<sup>+</sup>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<sup>5</sup>/ml, and all cultures were incubated in U-bottom wells. Nondiabetic periodontal disease (PD)<sup>4</sup> 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.<sup>5</sup> In brief, the PD patients were characterized by periodontal infection with multiple organisms including <i>P. gingivalis</i> and <i>A. actinomycetemcomitans</i>. 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 had no sign of periodontal disease other than mild gingivitis were matched to PD patients based on age, sex, and race when possible. Patients and healthy donors were recruited from the Clinical Research Center and the Boston University School of Medicine community and were nonsmokers. Gingival tissue was collected from patients with PD as part of their routine periodontal surgical treatment. Human Mono Mac-6 monocytes were cultured and simulated as published (29). Patients with diabetes (DM) were recruited from the Endocrinology, Diabetes and Nutrition Clinic at Boston University School of Medicine community and were nonsmokers. Healthy donors were recruited from the Clinical Research Center and the University Institutional Review Board-approved protocol. Peripheral blood was collected into heparinized tubes by venous puncture. B cells were isolated by neutrophil functional analysis (28). Systemically healthy donors who provided B cells for cytokine analyses are summarized in supplemental Table S1.<sup>5</sup> In brief, the PD patients were characterized by periodontal aggressive periodontitis (27) but no other known disease. The PD patients included periodontal disease and diabetes patients responded differ-ent 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.

Statistical analyses

The Mann-Whitney U test was used for nonparametric comparisons of cytokine concentrations 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<sup>+</sup>/CD3<sup>+</sup>, CD19<sup>+</sup>/CD8<sup>+</sup>, and CD19<sup>+</sup>/CD27<sup>+</sup> 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<sup>+</sup> B cells from PD patients (Fig. 1D) with the prototypic TLR2 ligand Pam3CSK4 (Pam3). 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 <i>P. gingivalis</i> (34). We conclude that modest TLR2 elevation on B cells from PD patients confers significant increases in TLR2 ligand response.

Flow cytometry

B cells in whole blood were prepared and analyzed as published (18, 30). Intracellular IL-8 was labeled with FastImmune (BD Pharmingen). Alternatively, purified B cells from healthy donors were incubated for 24 h in the presence of the TLR9 ligand CpG (ODN 2006, 1 μg/ml) or IL-4 (10 ng/ml), alone or in combination for 72 h, before staining for surface TLR4 expression using standard procedures (30).

Biochemistry

B cells were isolated by negatively selecting magnetic beads and were >95% pure as assayed by flow cytometry with anti-CD19, 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′-TGGCCCATCGTGGCAA-3′; 5′-ACTTAT GACACCCTATCCCTTAC-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, IFN-γ, TNF-α, and GM-CSF (Invitrogen). TLR4 ligands have been described (31, 32).

4 Abbreviations used in this paper: PD, periodontal disease; ChIP, chromatin immunoprecipitation; DM, diabetes.

5 The online version of this article contains supplemental material.
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\(^+\)) 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 B cells from healthy donors (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. 1J). 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 single TLR ligands. The first series of analyses focused on cytokine secretion by B cells responding to the TLR2 ligand Pam3 (Fig. 2). 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.

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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 cytokine production was significantly higher in B cells from PD vs healthy donors, on average, to an equivalent level (Fig. 3E). PU.1 association with the TLR4 promoter increased (2) TLR2 ligand activates TLR4 (Fig. 3, B and C) make investigations 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 4A 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 (Pam 3) 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- and

**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 investigations 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 (Pam 3) 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- and
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, A–D and supplemental Fig. S4). Furthermore, LPS 1690 insignificantly changed Pam3-induced cytokine production (Figs. 4, G–I). Overall, data in Fig. 4 indicate that coengagement 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 supports 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 nor-gand-specific mechanisms. Finally, the similar response of B cells from healthy and PD donors indicates that B cell TLR9-mediated cytokine production plays no critical role in these patients.

**IL-1β production recovers 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 one vs two TLR ligands (as labeled) are highlighted with an asterisk (p < 0.05). The number of samples analyzed from DM patients was: 5, 8, 8, 5, 8. Number of PD samples analyzed are as listed in other figures.

**FIGURE 5.** TLR9 equivalently activates cytokine production by B cells from PD and healthy donors. A–D, B cells from PD patients were stimulated with the TLR9 ligand CpG ODN 2006 in the presence or absence of the TLR2 ligand Pam3, or the TLR4 ligand rLPS as indicated. Values of p for comparison of results from B cells from PD vs healthy donors (filled or open bars, respectively) were all >0.1. No significant difference was detected between TLR9 and TLR9 plus TLR2- or TLR9 plus TLR4-stimulated cells (p > 0.1). Cytokines measured were (A) IFN-γ, (B) TNF-α, (C) IL-10, and (D) IL-1β. Bar shows average of n = 6 (for IL-1β) or n = 5 (for IFN-γ, TNF-α, and IL-10) for CpG- or CpG plus rLPS-treated samples; n = 5 (for IL-1β) or n = 4 (for IFN-γ, TNF-α and IL-10) for CpG plus Pam3-treated samples.

**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, 31–53), 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 are particularly effective in activating a positive feedback loop to achieve robust IL-1β production in an autocrine/paracrine mechanism (S6). 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+/TLR4+ 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 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.

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