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Pathogen-Accelerated Atherosclerosis Occurs Early after Exposure and Can Be Prevented via Immunization

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Here we report on early inflammatory events associated with *Porphyromonas gingivalis*-accelerated atherosclerosis in apolipoprotein E knockout (ApoE$^{-/-}$) mice. Animals challenged with *P. gingivalis* presented with increased macrophage infiltration, innate immune marker expression, and atheroma without elevated systemic inflammatory mediators. This early local inflammatory response was prevented in mice immunized with *P. gingivalis*. We conclude that localized up-regulation of innate immune markers early after infection, rather than systemic inflammation, contributes to pathogen-accelerated atherosclerosis.

Despite evidence indicating that complications of atherosclerosis, such as myocardial infarction or coronary thrombosis, contribute to more than 50% of the deaths in the United States, approximately half of patients do not possess identified risk factors. Emerging evidence suggests that infection with specific pathogens may serve as an additional risk factor for atherosclerosis (11). It has been reported that the periodontal disease pathogen *Porphyromonas gingivalis* accelerates atheroma formation in an established murine model of atherosclerosis (7, 9, 10). Our group demonstrated that wild-type *P. gingivalis* but not a fimbria-deficient (*fimA*) mutant accelerates atherosclerosis in the apolipoprotein E knockout (ApoE$^{-/-}$) mouse model of atherosclerosis as detected 6 weeks after pathogen exposure (7). Interestingly, oral infection with both the wild type and the *fimA* mutant resulted in bacteremia and localization of the organisms to the aortic tissue; however, only the wild-type *P. gingivalis* strain up-regulated the innate immune receptors Toll-like receptor 2 (TLR2) and TLR4 in aortic tissue (7). While these studies established a role for *P. gingivalis* in the acceleration of atherosclerosis, as evidenced by late events in the atherosclerosis process, it was not known if this response occurred early after pathogen exposure. One recent report demonstrated that infection with cytomegalovirus (MCMV) promoted atheroma formation in the ApoE$^{-/-}$ murine animal model 2 weeks after infection with MCMV (18). Those investigators demonstrated both systemic and local immune responses 6 days following MCMV infection, identified by increased levels of gamma interferon and tumor necrosis factor alpha. To examine the early events associated with *P. gingivalis*-accelerated atherosclerosis in this study, we characterized the early local inflammatory response and atherosclerosis development in aortic tissue of ApoE$^{-/-}$ mice following *P. gingivalis* oral challenge. Our results indicate that mice infected with *P. gingivalis* presented with increased macrophage infiltration, innate immune marker expression, and atheroma without systemic inflammatory markers relative to uninfected mice. Furthermore, we demonstrate that mice immunized with heat-killed *P. gingivalis* prior to oral challenge fail to develop an early inflammatory response in the aorta or acceleration of atherosclerosis.

***P. gingivalis* rapidly accelerated atherosclerosis.** Five-week-old male ApoE$^{-/-}$ mice (Jackson Laboratories, Bar Harbor, Maine) were cared for in accordance with National Institutes of Health- and Boston University Institutional Animal Care and Use Committee-approved procedures, received standard chow diet and water ad libitum, and were randomly placed into three groups ($n = 10$ for each group). One group of ApoE$^{-/-}$ mice was challenged orally with *P. gingivalis* strain 381 five times a week for 3 weeks to mimic chronic exposure to *P. gingivalis*, as described previously (7, 9). A second group of animals was immunized subcutaneously two times a week for 3 weeks with 0.1 ml of heat-killed *P. gingivalis* 381 in sterile, pyrogen-free saline prior to oral challenge (6, 7). The third group of mice were not treated and served as age-matched controls (Fig. 1). A subset of similar groups of animals ($n = 6$) was followed for 6 weeks as appropriate controls for plaque accumulation in the established murine model of late events in the atherosclerotic process. All animals were monitored daily until sacrifice (24 h or 6 weeks after the final oral challenge) and appeared healthy throughout the course of this study. By a modification of the method of Paigen et al. (14), we examined cryosections of the aortic sinus for atherosclerotic plaque

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FIG. 1. Time schedule of experiments. Five-week-old male ApoE$^{-/-}$ mice were randomly assigned to an unchallenged control group, a group of animals challenged with *P. gingivalis*, or a group of immunized animals challenged with *P. gingivalis*. Immunized animals received 0.1-ml subcutaneous injections of heat-killed *P. gingivalis* 381 two times a week for 3 weeks prior to the oral challenge. Following antibiotic treatment, mice were orally challenged with *P. gingivalis* 381 five times a week for 3 weeks. Animals were sacrificed either 24 h or 6 weeks after the final oral challenge.

accumulation by oil red O staining. The average total lesion size and percentage of the lumen occupied by atheroma was determined by two independent observers, who were blinded to the identities of the individual groups. Analysis was completed by utilizing the first 10 μm of cryosection possessing all three leaflets of the aortic sinus and a second 10-μm cryosection 140 μm distal to the first section of each animal ($n = 4$ for each group) with a microscope coupled to a computer-assisted morphometry system (IPLabs; Scanalytics, Inc., Fairfax, Va.). Our result showed that ApoE$^{-/-}$ mice challenged with *P. gingivalis* demonstrated significantly more atherosclerotic plaque accumulation in the aortic sinus compared to the unchallenged ApoE$^{-/-}$ mice (Fig. 2B). As expected, the group of ApoE$^{-/-}$ mice which were immunized and challenged orally with *P. gingivalis* did not exhibit accelerated atheroma development and resembled unchallenged ApoE$^{-/-}$ mice (Fig. 2B). In addition, the levels of atherosclerotic plaque accumulation observed at 6 weeks were similar to those observed in our previous studies (data not shown; 3). This demonstrates that as early as 3 weeks after initial pathogen exposure, ApoE$^{-/-}$ mice chronically challenged with *P. gingivalis* present with increased atherosclerosis.

***P. gingivalis* elicits acute TLR, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) expression in the aortic arch.** TLRs, a group of molecular pattern receptors involved in pathogen recognition, have recently been associated with atherosclerosis (2, 3, 12, 19). Elevated TLR expression has been reported at sites of atheroma deposition in both humans and murine models (2, 7). The TLR family of cell surface receptors respond to a variety of microbial structures (15). TLR4 recognizes enteric lipopolysaccharide, while TLR2 recognizes peptidoglycan and lipopolysaccharide from *P. gingivalis* (17). After binding TLR ligands, a downstream cascade of signaling molecules, including the cytoplasmic adaptor molecule MyD88, is activated and recruited. ApoE$^{-/-}$ mice lacking MyD88 which were placed on a high-fat diet demonstrated reduced atheroma formation compared to ApoE$^{-/-}$ mice with functional MyD88 (2). Similarly, cell adhesion molecules including ICAM-1 and VCAM-1 have also been implicated in the development of atherosclerosis (13, 16). In order to determine if innate immune markers were expressed early after *P. gingivalis* challenge, we performed reverse transcription (RT)-PCR and immunohistochemical analysis for TLR2, TLR4, ICAM-1, and VCAM-1 in the aortic sinuses of ApoE$^{-/-}$ mice ($n = 4$ for each group). With specific primers (Table 1), RT-PCR revealed increased TLR2 and TLR4 (Fig. 3A), as well as ICAM-1 and VCAM-1 (Fig. 4A), expression compared with unchallenged controls or immunized ApoE$^{-/-}$ mice (Fig. 3A and 4A). Interestingly, animals immunized with a heat-killed *P. gingivalis* preparation prior to *P. gingivalis* challenge expressed less cell adhesion molecule and TLR-specific mRNA compared with nonimmunized orally challenged mice, and more resembled the uninfected controls (Fig. 3A and 4A). TLR2, TLR4, ICAM-1, and VCAM-1 expression in the aortic sinus was confirmed by immunohistochemistry (Fig. 3B and 4B). Cryosections were incubated with (i) rat anti-mouse TLR2 monoclonal antibody and isotype-matched control rat immunoglobulin G (IgG) (both kindly provided by Egil Lien, University of Massachusetts Medical School, Worcester), (ii) mouse anti-human TLR4 antibody and isotype-matched control mouse IgG2a (Biocarta, Carlsbad, Calif.) (7), (iii) rat anti-mouse ICAM-1 antibody and isotype-matched control rat IgG2a (Serotec, Kidlington, Oxford, United Kingdom), and (iv) rat anti-mouse VCAM-1 antibody and isotype-matched control rat IgG1 (Serotec). Immuno-

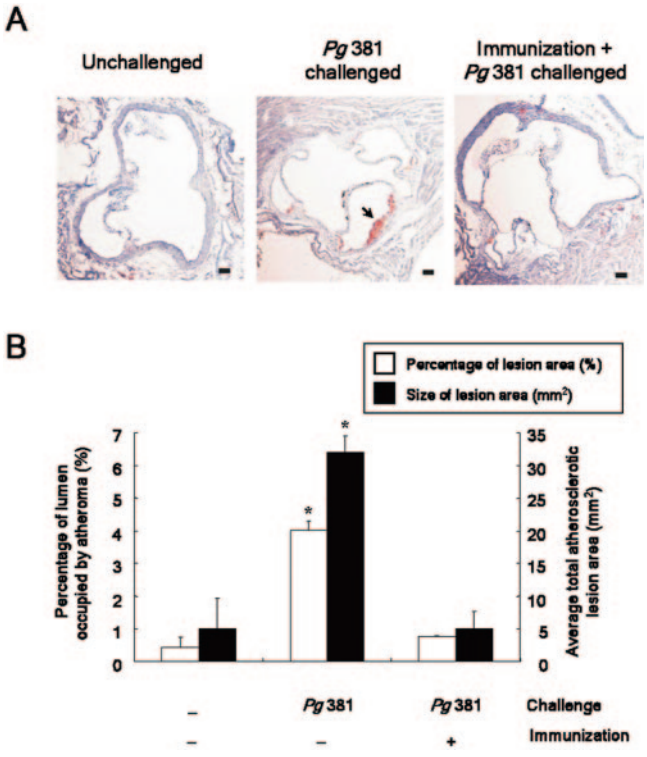

FIG. 2. Oral challenge of ApoE$^{-/-}$ mice with *P. gingivalis* (*Pg*) accelerates atherosclerotic plaque formation 24 h following challenge. (A) Oil red O-stained cryosections of the proximal aortas of 8-week-old mice. Scale bars = 100 μm. The arrow indicates a typical lipid-rich atherosclerotic area stained with oil red O. (B) Percentage of the total lumen of the aorta occupied by lesions (left *y* axis; open columns) and average total atherosclerotic lesion area (right *y* axis; filled columns). Data were evaluated as the mean ± the standard error of the mean. One-way analysis of variance with the Turkey-Kramer multiple-comparison test was performed to assess differences between groups, and $P < 0.05$ was considered statistically significant. *; $P < 0.05$ compared with unchallenged and immunized groups.

TABLE 1. Primers and amplification conditions used for RT-PCR

Gene product and primer	Sequence	AT[a] (°C)	Size (bp)	Cycle no.
TLR2				
Sense	5′-GAGCGAGCTGGGTAAAGTAGAAA-3′	58	528	35
Antisense	5′-AGCCGAGGCAAGAACAAAGA-3′			
TLR4				
Sense	5′-CAGTGGGTCAAGGAACAGAAGC-3′	58	540	30
Antisense	5′-GACAATGAAGATGATGCCAGAGC-3′			
ICAM-1				
Sense	5′-TGCGTTTTGGAGCTAGCGGACCA-3′	58	326	32
Antisense	5′-CGAGGACCATACAGCACGTGCAG-3′			
VCAM-1				
Sense	5′-CCTCACTTGCAGCCACTACGGGCT-3′	58	442	32
Antisense	5′-TTTTCCAATATCCTCAATGACGGG-3′			
β-Actin				
Sense	5′-TCATGAAGTGTGACGTTGACATCCGT-3′	57	285	32
Antisense	5′-CCTAGAAGCATTTGCGGTGCACGATG-3′			

[a] AT, annealing temperature.

enzyme staining was performed by the biotin-streptavidin-peroxidase method (DAKO, Carpinteria, Calif.). Our results demonstrate that ApoE$^{-/-}$ mice challenged with *P. gingivalis* presented with TLR2- and TLR4-specific staining (Fig. 3B). Similar to our RT-PCR data, TLR2 and TLR4 expression was not detected in unchallenged ApoE$^{-/-}$ mice or in ApoE$^{-/-}$ mice which were immunized and subsequently challenged with *P. gingivalis* (Fig. 3B). A similar pattern was observed for cell adhesion molecule expression. Elevated levels of ICAM-1 and VCAM-1 expression were observed in cryosections of the aortic sinuses of *P. gingivalis*-challenged mice; however, this was not observed in cryosections of unchallenged or immunized animals (Fig. 4B). Taken together, our data indicate that early innate immune activation, as evidenced by TLR and cell adhesion molecule regulation in the aortic sinus, occurs concurrently with atheroma deposition. Importantly, immunization with heat-killed bacteria was shown to inhibit the host inflammatory response, as well as prevent increased atheroma deposition.

***P. gingivalis* elicits macrophage recruitment in the aortic arch.** An influx of mononuclear cells, particularly macrophages, is indicative of the early atheroma (8). We observed enhanced staining for macrophages in cryosections of the aortic sinuses of ApoE$^{-/-}$ mice orally challenged with *P. gingivalis* by using rat anti-mouse Mac-3 IgG for macrophages and isotype-matched control purified rat IgG1 (BD PharMingen, San Diego, Calif.) (Fig. 4B). Tissue from the aortic arch of unchallenged ApoE$^{-/-}$ mice and mice which were immunized and orally challenged with *P. gingivalis* expressed low levels of macrophage-specific staining (Fig. 4B). Macrophage staining was localized primarily to the sites of atherosclerotic plaque in the aortic arch sinuses of ApoE$^{-/-}$ mice orally challenged with *P. gingivalis*.

***P. gingivalis* accelerates atherosclerosis without an elevated systemic host response.** The association of inflammation with the initiation and progression of atherosclerosis suggests that serum markers such as interleukin-6 (IL-6) and C-reactive protein may be useful in predicting an increased risk of coro-

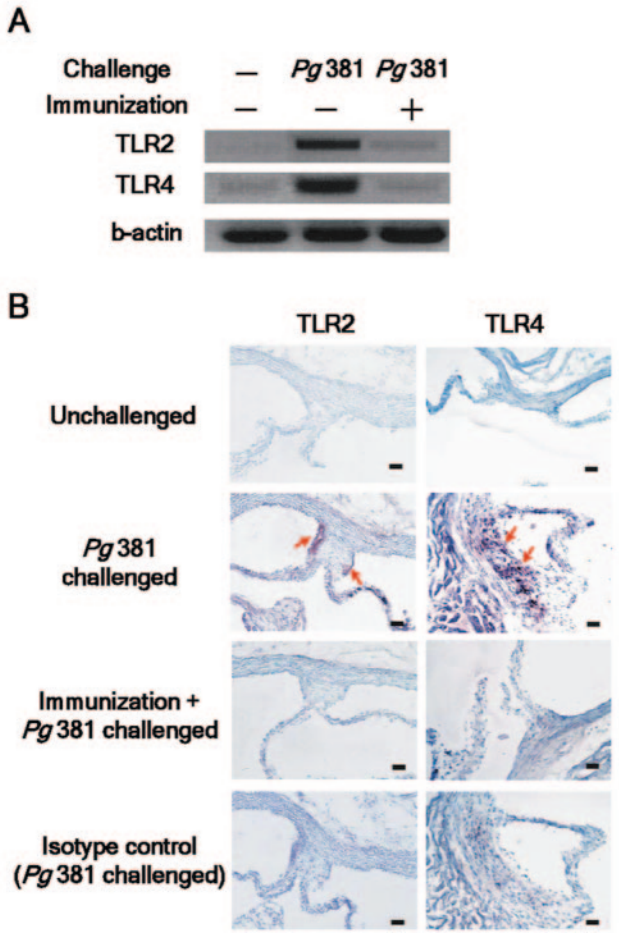

FIG. 3. ApoE$^{-/-}$ mice orally challenged with *P. gingivalis* (*Pg*) express increased TLR2 and TLR4 in aortic arch tissue 24 h following challenge. (A) RT-PCR amplification of TLR2 and TLR4 mRNAs from aortic arch tissue. (B) Immunohistochemical confirmation of TLR2 and TLR4 in aortic arch tissue. Red arrows indicate marker-positive stained areas. Scale bars = 50 μm.

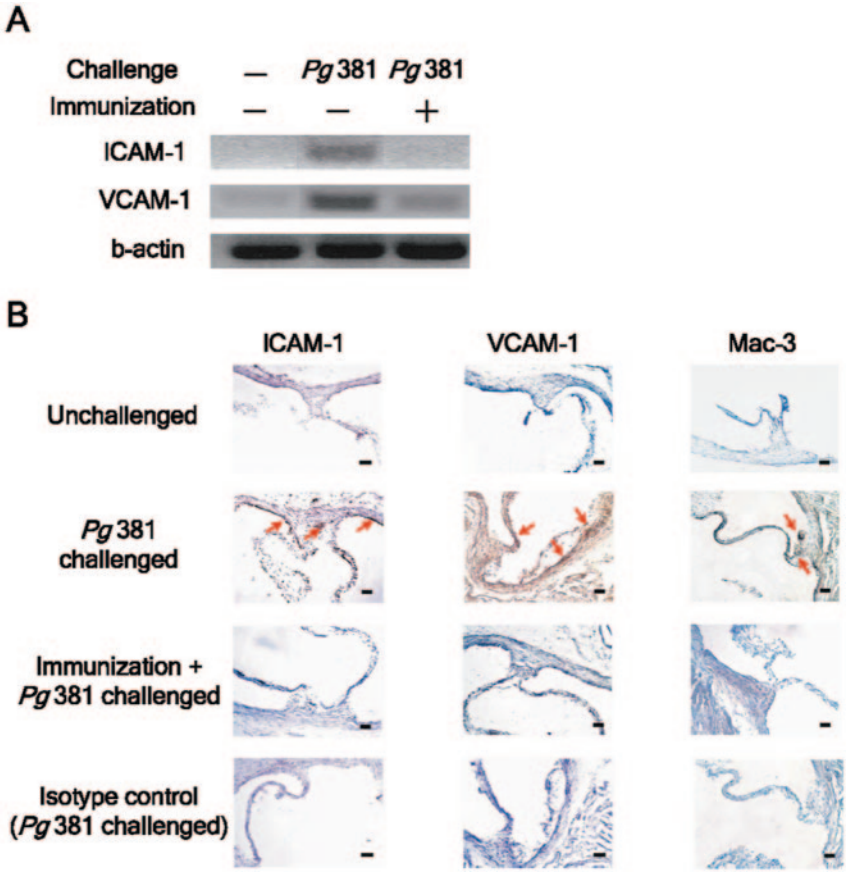

FIG. 4. Expression of ICAM-1, VCAM-1, and Mac-3 in the aortas of ApoE$^{-/-}$ mice orally challenged with *P. gingivalis* (*Pg*) 24 h following challenge. (A) RT-PCR amplification of ICAM-1 and VCAM-1 mRNAs. (B) Immunohistochemical confirmation of ICAM-1, VCAM-1, and Mac-3 in aortic arch tissue. Red arrows indicate marker-positive stained areas. Scale bars = 50 μm.

nary heart disease (15). At the time of sacrifice, serum was collected from each animal and examined for levels of IL-6 and serum amyloid A (SAA; the murine equivalent of human C-reactive protein) by enzyme-linked immunosorbent assay (Pierce Endogen, Rockford, Ill.). We observed that the levels of IL-6 and SAA in the sera of mice challenged with *P. gingivalis* were not significantly different than those of uninfected mice or mice immunized and subsequently challenged with *P. gingivalis* (Fig. 5). This was observed in samples obtained 1 h, 24 h, and 6 weeks after the final oral challenge. These observations suggest that oral infection with *P. gingivalis* does not result in a significant increase in the systemic inflammatory response.

Concluding remarks. In this study, we have demonstrated that shortly after initiation of oral infection, *P. gingivalis* elicits a local innate immune response in the aortic sinus which is characterized by up-regulation of TLRs and cell adhesion molecules and accelerates atherosclerosis in hyperlipidemic mice. Importantly, we have shown that innate immune activation and development of atherosclerosis are detectable shortly after bacterial infection and that these observations are readily prevented by immunization. The mechanism by which immunization prevented atherosclerosis and attenuated innate immune activation in the present study is not known. By employing diet-induced atherosclerosis models, it has been demonstrated that immunization with heat shock protein 65 accelerates atherosclerosis (5) and that both the cellular and humoral arms of the host response to heat shock protein 65 immunization play fundamental roles in stimulating atheroma deposition (4). Conversely Binder et al. (1) reported that *Streptococcus pneumoniae* vaccination reduced the extent of atherosclerosis in

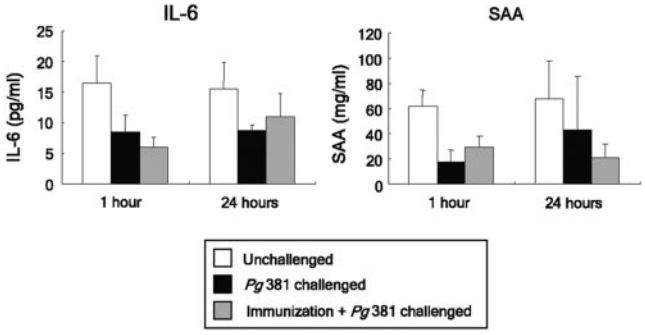

FIG. 5. Mice infected with invasive *P. gingivalis* (*Pg*) exhibited no difference in serum levels of IL-6 and SAA. Serum was collected from each animal at 1 h and 24 h after the final oral challenge, and levels of IL-6 and SAA were determined by enzyme-linked immunosorbent assay. No significant differences in IL-6 or SAA were observed between groups.

hyperlipidemic mice by eliciting cross-reactive antibodies that react with host oxidized low-density lipoprotein. However, in those studies it was not determined if *S. pneumoniae* infection accelerated atherosclerosis. Molecular mimicry and elicitation of cross-reactive antibodies may play a role in the protection afforded by immunization. Future investigations into this area are required to determine the exact mechanisms conveying protection by immunization with bacterial components.

In addition, we have demonstrated that *P. gingivalis* infection does not result in significant increases in the serum levels of IL-6 and SAA. We conclude from these results that systemic activation of inflammatory mediator expression by the host does not in itself contribute significantly to the observed increase in atheroma development following *P. gingivalis* infection. Interestingly, in contrast to our results, Lalla et al. (9) reported that oral challenge of ApoE$^{-/-}$ mice with *P. gingivalis* resulted in increased IL-6 in serum compared with unchallenged animals. Likewise, Li et al. (10) reported that intravenous infection with *P. gingivalis* resulted in increased SAA compared with unchallenged controls. The differences observed in those two studies, compared to our study, may reflect different routes of challenge, animal genotypes, numbers of animals examined, and time points used for assessment of IL-6 and SAA (5, 6). Future studies challenging mice deficient in IL-6 or SAA, in an ApoE$^{-/-}$ background, are required to clarify the role of these molecules in *P. gingivalis*-mediated acceleration of atherosclerosis. Our observations of ICAM-1 and VCAM-1 expression and macrophage infiltration early after *P. gingivalis* challenge demonstrate that *P. gingivalis* infection leads to localized activation of the aortic vascular endothelium. This local endothelial activation may lead to macrophage fatty streak formation and accelerated atherosclerosis. Further studies are needed to define the interaction of CAM-expressing vascular endothelial cells and macrophages and the temporal events that occur during these interactions. In summary, with *P. gingivalis* as a model organism, we have demonstrated that invasive bacterial infection elicits a local innate immune response via TLRs and up-regulation of cell adhesion molecules and that these events specifically accelerate atherosclerosis in hyperlipidemic mice. Importantly, we have shown that innate immune activation and atherosclerosis are detectable shortly after bacterial infection and that this was prevented by immunization. Taken together, these results indicate (i) that early innate immune activation occurs locally in the aortic arch in response to infectious challenge and is associated with pathogen-accelerated atherosclerosis and (ii) that immunization may be sufficient for prevention of pathogen-accelerated atherosclerosis.

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