Amyloid-DNA Composites of Bacterial Biofilms Stimulate Autoimmunity

Highlights
- Bacterial amyloid curli and DNA composites form within bacterial biofilms
- DNA accelerates the polymerization of bacterial amyloid curli
- Curli-DNA composites induce autoantibodies and type I interferon
- Infections with amyloid-expressing bacteria trigger autoimmunity

Authors
Paul M. Gallo, Glenn J. Rapsinski, R. Paul Wilson, ..., Roberto Caricchio, Stefania Gallucci, Çagla Tükel

Correspondence
ciukel@temple.edu

In Brief
Biofilms, multicellular bacterial communities, are associated with numerous infections including UTIs, rhinosinusitis, and periodontal disease. Tükel and colleagues show that bacterial amyloids and eDNA, components of biofilms, form immunogenic complexes that accelerate the progression of an autoimmune disease, SLE, via the generation of autoantibodies and type I interferon response.
Amyloid-DNA Composites of Bacterial Biofilms Stimulate Autoimmunity

Paul M. Gallo,1,2,6 Glenn J. Rapsinski,1,6 R. Paul Wilson,1 Gertrude O. Oppong,1 Uma Sriram,1,6 Mark Goulian,4 Bettina Buttaro,1 Roberto Caricchio,1 Stefania Gallucci,1,2,6 and Çagla Tükel1,6,*

1Department of Microbiology and Immunology
2Laboratory of Dendritic Cell Biology
3Division of Rheumatology, Department of Medicine
School of Medicine, Temple University, Philadelphia, Pennsylvania 19140, USA
4Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA
5Co-first author
6Co-senior author
*Correspondence: ctikel@temple.edu
http://dx.doi.org/10.1016/j.immuni.2015.06.002

SUMMARY

Research on the human microbiome has established that commensal and pathogenic bacteria can influence obesity, cancer, and autoimmunity through mechanisms mostly unknown. We found that a component of bacterial biofilms, the amyloid protein curli, irreversibly forms fibers with bacterial DNA during biofilm formation. This interaction accelerated amyloid polymerization and created potent immunogenic complexes that activated immune cells, including dendritic cells, to produce cytokines such as type I interferons, which are pathogenic in systemic lupus erythematosus (SLE). When given systemically, curli-DNA composites triggered immune activation and production of autoantibodies in lupus-prone and wild-type mice. We also found that the infection of lupus-prone mice with curli-producing bacteria triggered higher autoantibody titers compared to curli-deficient bacteria. These data provide a mechanism by which the microbiome and biofilm-producing enteric infections may contribute to the progression of SLE and point to a potential molecular target for treatment of autoimmunity.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disease in which both genetic and environmental triggers contribute to disease onset and to the production of autoantibodies to double stranded DNA (dsDNA) and nuclear proteins (Elkon and Stone, 2011; Morel, 2010; Moser et al., 2009). Infection is an important environmental trigger for lupus flares and a major cause of morbidity and mortality in SLE patients (Petri, 1998). In fact, 20%–55% of deaths in SLE patients are attributable to infections and up to 23% of hospitalizations are due to infectious complications (Barrera-Vargas et al., 2014; Fessler, 2002). Bloodstream infections, urinary tract infections, soft tissue infections, and pneumonia are common in SLE patients. Frequent Salmonella infections have been reported, especially those caused by Salmonella enterica serovar Typhimurium and serovar Enteritidis (Gerona and Navarra, 2009). Moreover, Salmonella behave more aggressively in SLE patients: instead of causing localized gastroenteritis, infection may result in bacteremia or complications in soft tissues with high mortality rates (Costa-Reis et al., 2013; Lim et al., 2001; Pablos et al., 1994; Tsao et al., 2002). Although bacterial infections are thought to contribute to SLE pathogenesis by inducing cell death and inflammation, the exact mechanisms by which bacteria contribute to SLE pathogenesis remain unknown.

Biofilms are bacterial communities embedded in an extracellular matrix (ECM), which protects bacteria from environmental stresses including antibiotics (López et al., 2010; O’Toole et al., 1999). Biofilms are formed on many biotic and abiotic surfaces including the mucosal surfaces of the human body and indwelling medical devices. Several infections are associated with biofilms including UTI, otitis media, and periodontal diseases (Bjamsholt, 2013), suggesting that the human immune system is exposed to biofilm components throughout life.

Amyloids are proteins with a conserved β sheet structure. Mammalian amyloids accumulate in tissues during various debilitating human diseases including Alzheimer’s disease (Schnabel, 2010). Several bacterial species actively produce amyloid proteins in biofilms including those of S. Typhimurium and E. coli. Amyloid fibers of the latter enteric bacteria are termed curli, which are encoded by the csg gene cluster composed of csgBAC and csgDEFG operons. Production and polymerization of bacterial CsgA protein leads to the generation of the bacterial amyloid curli (Barnhart and Chapman, 2006; Chapman et al., 2002; Römling et al., 1998; Wang et al., 2007). Curli fibers are expressed during various enteric infections including sepsis, gastroenteritis, and UTI (Bian et al., 2000; Humphries et al., 2003; Kai-Larsen et al., 2010). We have previously shown that curli fibers induce immune activation by triggering the Toll-like receptor (TLR) 2-TLR1 heterocomplex (Rapsinski et al., 2013; Tükel et al., 2005, 2009, 2010). In addition, many bacterial biofilms contain extracellular DNA (eDNA), which acts to stabilize the biofilm matrix (Whitchurch et al., 2002).

A number of reports have suggested that DNA complexed with a protein antigen can induce lupus-like disease (Desai et al., 1993; Lande et al., 2007). Human prototypic amyloidogenic
peptides, prion fragment and amyloid-β 1-42 can directly bind to DNA (Di Domizio et al., 2012b; Jiménez, 2010). Immunization with amyloid fibers complexed with nucleic acids, in the presence of the classic adjuvant CFA, can induce autoantibodies in nonautoimmune mice within 12 weeks (Di Domizio et al., 2012a). Although groundbreaking, the amyloid-DNA composites nonautoimmune mice within 12 weeks (Di Domizio et al., 2012b; Jime´ nez, 2010). Immunization with amyloid fibers complexed with nucleic acids, in the presence of the classic adjuvant CFA, can induce autoantibodies in nonautoimmune mice within 12 weeks (Di Domizio et al., 2012b; Jime´ nez, 2010). Immunization with amyloid fibers complexed with nucleic acids, in the presence of the classic adjuvant CFA, can induce autoantibodies in nonautoimmune mice within 12 weeks (Di Domizio et al., 2012b; Jime´ nez, 2010). Immunization with amyloid fibers complexed with nucleic acids, in the presence of the classic adjuvant CFA, can induce autoantibodies in nonautoimmune mice within 12 weeks (Di Domizio et al., 2012b; Jime´ nez, 2010). Immunization with amyloid fibers complexed with nucleic acids, in the presence of the classic adjuvant CFA, can induce autoantibodies in nonautoimmune mice within 12 weeks (Di Domizio et al., 2012b; Jime´ nez, 2010).

**RESULTS**

**Curli-DNA Composites in S. Typhimurium Biofilms**

Curli fibers are conserved among the members of Enterobactericeae family (Barnhart and Chapman, 2006). Here, we studied the biofilms of S. Typhimurium as a model organism, since Salmonella is an important cause of infection and morbidity in SLE patients (Gerona and Navarra, 2009). S. Typhimurium forms pellicle biofilms at the air–liquid interface similar to E. coli (Hung et al. 2013). When grown on microscope slides in 50 ml conical tubes for 72 hr, the biofilm thickness reached up to 33 μm (Figures 1A and S1A). Under the same experimental conditions, a csgBA mutant that could not express curli monomers failed to form a pellicle biofilm, indicating that curli is required for S. Typhimurium biofilm (Figures S1A and S1B). We monitored the kinetics of curli expression in the biofilms by flow cytometry using a S. Typhimurium strain carrying a plasmid in which P_csgBA promoter drives the expression of green fluorescence protein (GFP) (P_csgBA: gfp). Curli expression started within 48 hr after inoculation, as > 70% of the bacteria expressed GFP. By 72 hr, GFP was detected in virtually the entire population of the biofilm, suggesting that most bacteria express curli (Figure 1B). We also visualized curli fibers in biofilms by staining the pellicle with an amyloid specific stain, Congo red (CR) (Figure 1C).

We hypothesized that the nucleic acids found within the bacterial biofilms (Johnson et al., 2013; Whitchurch et al., 2002) could be derived from dying bacteria. To monitor cell death in the biofilm, we stained the bacteria in the pellicle with a live (Syto9) / dead (PI) stain. Approximately 10% of the bacteria in the biofilm were determined dead (Syto9 negative/PI positive) by 72 hr. Unexpectedly, a large fraction of the population—approximately 57% — showed positivity for both stains, suggesting that these cells were dying. However, this result was puzzling because the bacterial enumeration did not reveal a decrease in the bacterial numbers in the pellicle biofilm (Figures 1D and S1C). To further analyze this phenomenon, we stained the biofilm of a S. Typhimurium strain carrying the reporter P_csgBA::gfp with PI to visualize curli expression and DNA release. Green cells were surrounded by a red corona, demonstrating that eDNA was localized around live, curli-expressing cells and indicating that the high percentage of PI positivity in Figure 1D is not due to cell death. We also observed high DNA staining of channels within the biofilm (Figures 1E and 1F). Therefore, eDNA, either released by dying bacteria or actively released into the ECM, is an important component of the S. Typhimurium biofilm pellicle (Figure S1D).

We found the amyloid specific dye CR to be incompatible with nucleic acid dyes PI and DAPI but not with Toto-1. Therefore, we stained the S. Typhimurium biofilms using CR (red) and Toto-1 (blue) and showed that the amyloids and DNA colocalized (magenta) within S. Typhimurium biofilms (Figure 2A). Treatment of S. Typhimurium biofilms with DNase did not reduce the biofilm mass as dramatically as reported for P. aeruginosa biofilms, which do not contain amyloids (Whitchurch et al., 2002) (Figures 2B and 2C). Due to the resistance of amyloids to enzymatic breakdown, we hypothesized that the incorporation of eDNA into the curli fibers rendered eDNA resistant to DNase. To further investigate the interactions of curli and eDNA, we purified curli from S. Typhimurium biofilms (Collinson et al., 1991). It is important to note that the purification process of curli includes multiple cycles of DNase and RNase treatments, boiling in SDS, and overnight electrophoresis through a preparative SDS polyacrylamide gel to eliminate any contaminants. When we stained such purified curli individually with CR or nucleic acid-specific dyes, curli stained positive with PI and Hoescht 33258, indicating the presence of nucleic acids in the purified curli fibers (Figure 2D, center and right panel). Birefringence, a characteristic of amyloids stained with CR, was visible under polarizing microscopy (Figure 2D, left panel). Purified curli stained positive with ethidium bromide, a DNA-specific dye, confirming the presence of DNA in the fibers. Further treatment of purified curli with DNase or RNase did not remove the nucleic acids from the fibers unless fibers were broken down into its monomeric units by hexafluoroisopropanol (HFIP) and DMSO treatment (Figure S2). Using phenol-chloroform extraction, we were able to extract an average of 639 ng (SD = 200) of DNA per 100 μg of purified curli that had been extensively treated with DNase. Finally, purified curli was costained with Thioflavin T (ThT), another amyloid-specific stain, and Hoescht33258. Purified curli fibers stained positive with both dyes (Figure 2E). Thus, bacterial eDNA is protected from degradation by its close association with amyloid fibers.

**DNA Accelerates the Polymerization of Curli Fibers**

Nucleic acids promote the polymerization of a human amyloid precursor protein (Di Domizio et al., 2012b). Amyloid polymerization can be monitored using ThT, and it shows a sigmoidal polymerization curve with a lag phase followed by exponential growth of amyloid fibers, which eventually reaches a plateau as all monomers are consumed (Naiki et al., 1991). To determine the effects of DNA on curli polymerization, we utilized a synthetic...
peptide corresponding to the fourth and fifth repeats of the CsgA monomer of curli (CsgAR4-5), which is devoid of any DNA contamination, and we performed in vitro polymerization assays using ThT in the presence or absence of increasing concentrations of the synthetic oligonucleotide, CpG, and of Salmonella genomic DNA. Addition of DNA accelerated the polymerization of CsgAR4-5 by decreasing the lag phase of polymerization by an average 172 min with CpG DNA and 65 min with Salmonella genomic DNA. We think that the difference in the polymerization rates is due to the DNA fragment size in each DNA source. Since bacterial biofilms form in the gastrointestinal tract of humans (Bollinger et al., 2007; Macfarlane et al., 2011; Macfarlane and...
Figure 2. eDNA Forms Complexes with Curli in Biofilms Accelerating the Amyloid Polymerization Process and Limiting Its Degradation by DNase

(A) CLSM image of GFP expressing S. Typhimurium (green) biofilm grown for 72 hr and stained with CR (red) and TOTO-1 Iodide (blue).

(B) Crystal violet assay of S. Typhimurium biofilms grown in 96-well plates with or without 3 hr DNase treatment. Data shown is average and SE from 5 wells. Experiments were completed in triplicate.

(C) 3D reconstructions (upper) and side-view (lower) CLSM images of GFP expressing Salmonella biofilms with and without DNase treatment for 3 hr after biofilm was formed showing minimal change in biofilm thickness after treatment with DNase. White bars on side views represent 10 μm. Images are representative of three independent experiments.

(legend continued on next page)
Dillon, 2007; von Rosenvinge et al., 2013), we also asked whether this acceleration also occurred with eukaryotic DNA. We used salmon sperm DNA and found that it also accelerated curli polymerization by shortening the time in lag phase of polymerization by an average of 155 min (Figures 2F and 2G). These findings suggest a mechanism by which DNA provides structural stability to biofilms, by complexing with amyloids and promoting their polymerization. Furthermore, the fact that eukaryotic DNA also enhanced amyloid polymerization suggests that bacteria could incorporate eukaryotic DNA (e.g., released during tissue damage) into its biofilm structure. This process may generate highly immunogenic molecules because both amyloids and DNA can activate innate immune receptors (Hemmi et al., 2000; Tükel et al., 2009).

**Curli-DNA Composites Are Major Immune Stimulators in S. Typhimurium Biofilms**

Interactions of bacterial biofilms with the immune system are poorly characterized. Here, we sought to elucidate these interactions using an in vitro model of conventional dendritic cells (cDCs), the pivotal initiators of the immune response that are stimulated via pattern recognition receptors (PRRs) (Gallo and Gallucci, 2013; Iwasaki and Medzhitov, 2004). We incubated bone marrow-derived dendritic cells, expressing GFP, on S. Typhimurium biofilms. Biofilms were allowed to mature for 72 hr prior to addition of cDCs. The cDCs appeared to send dendrites into the biofilm (Figures 3A, S3A, and S3B) and phagocytose bacteria (Movies S1 and S2). Moreover, cDCs produced high amounts of proinflammatory cytokines including IL-6 and TNF-α (Figure 3B). These results indicate that cDCs can uptake antigens and be activated by the immigrant bacterial biofilm.

Lipid A and monomers of S. Typhimurium flagella are well-known immune activators that trigger TLR4-MD2-CD14 and TLR5, respectively (Gewirtz et al., 2001; Takeuchi et al., 1999). To determine the importance of LPS and flagellin in the stimulatory effect of biofilms, we established biofilms with a S. Typhimurium msbB mutant that expresses a tetra-acylated form of lipid A, which does not signal through the TLR4-MD2-CD14, and with a S. Typhimurium fliCfljB mutant that lacks flagella. After 48 hr, the biofilms formed by the mutant bacteria were comparable to the biofilms formed by wild-type S. Typhimurium. (Figures 3C and S3C). When cDCs were exposed to the biofilms, cDCs produced equally high amounts of IL-6 in response to the biofilms formed by wild-type S. Typhimurium, msbB mutants, and fliCfljB mutants (Figure 3B). Although there was a statistically significant difference in the amount of TNFα produced by cDCs in response to the msbB mutant biofilm when compared to the wild-type S. Typhimurium and the fliCfljB mutant biofilms, cDCs still produced high amounts of TNFα in response to the three biofilms (Figure 3B). Thus, although LPS and flagellin are important for the interaction of immune cells with planktonic S. Typhimurium, components other than LPS and flagellin exist in the S. Typhimurium biofilm that activate DCs.

Next, we sought to determine whether curli-DNA composites are involved in DC response to curli-containing biofilms. However, since a curli-deficient S. Typhimurium does not form biofilms (Figure S1B), we treated cDCs with a dose titration of purified curli fibers. To avoid contamination with LPS, we purified curli fibers from the msbB mutant and found that even nanogram quantities of curli induced significant amounts of IL-6 and IL-12 in cDCs. Interestingly, curli was very potent in inducing the Th1 cell-promoting cytokine IL-12 even when compared to the dose of LPS that showed the maximal cytokine response in cDCs (Figure 3D).

To determine the role of DNA in the stimulatory activity of curli-DNA composites from biofilms, we attempted to remove the DNA from the purified curli and found that we could not unless we dissociated curli into its monomers. However, this treatment damaged the DNA (Figure S2). To overcome this pitfall, we stimulated cDCs with the synthetic CsgAR4-5 peptide polymerized in the presence or absence of bacterial genomic DNA. DNA alone and CsgAR4-5 polymerized in the absence of genomic bacterial DNA induced minimal IL-6 and IL-12 production by cDCs. However, CsgAR4-5 polymerized in the presence of bacterial DNA induced significant amounts of IL-6 and IL-12, suggesting that curli and DNA synergize to activate innate immune cells (Figure 3E). The fact that the CsgAR4-5-DNA composites elicited much lower amounts of cytokines than the purified curli suggests that the natural aggregation of full-length curli may be required for its full activity.

**Curli-DNA Composites Stimulate the Type I Interferon Response**

Type I interferons (IFNs) are important stimulators of the innate and adaptive immune response and are pathogenic in SLE (Elkon and Stone, 2011). Type I IFNs can activate dendritic cells as an exogenous stimulus (Gallucci et al., 1999) and they mediate PRR-dependent activation of DCs through an autocrine feedback (Hoebbe et al., 2003). Here, we tested whether the curli-DNA composites purified from S. Typhimurium msbB biofilms stimulate DC activation and found that curli-DNA composites were potent inducers of type I IFNs and IFN stimulated genes (ISGs) in vitro in cDCs from lupus-prone NZBxW/F1 and non-lupus prone C57BL/6 mice (Figures 4A and 4B). Furthermore, in vivo injection of curli-DNA composites also resulted in the upregulation of the ISGs Isg15 and Irf7 in splenic DCs from young prediseased NZBxW/F1 lupus-prone mice (Figure 4C). These results indicate that curli-DNA composites are
potent danger signals that stimulate autoimmune-prone innate immune cells and may trigger autoimmunity by providing an adjuvant effect through the stimulation of the pathogenic IFN response.

**Curli-DNA Composites Promote Autoimmunity**

Next, we asked whether the naturally occurring curli-DNA composites found in bacterial biofilms could induce autoimmunity in the absence of any added adjuvant. Because we found that curli-DNA composites stimulate the production of type I IFNs (Figure 4), which accelerate disease onset in lupus-prone mice (Mathian et al., 2005), we hypothesized that exposure to curli-DNA composites may accelerate autoimmunity. We therefore injected 6-week-old, prediseased, NZBxW/F1 mice i.p. with PBS or 50 μg of curli-DNA composites once a week or three times a week and monitored autoantibody production. Mice injected with PBS remained negative for autoantibodies up to 20 weeks of age. Mice injected with curli-DNA composites rapidly produced anti-dsDNA and anti-chromatin autoantibodies, a hallmark of lupus autoimmunity (Figure 5A). As a protein control, we injected 50 μg of BSA i.p. into NZBxW/F1 age- and gender-matched mice and found that injections with BSA did not induce development of autoantibodies (Figure S4), suggesting specificity for the autoimmunogenicity of curli.

Other adjuvants, including TLR ligands, can accelerate murine lupus (Hang et al., 1983; Jørgensen et al., 2006). However, curli-DNA composites induce autoantibody production very quickly and consistently, with all mice becoming positive for anti-dsDNA within 2 weeks of the first injection. We confirmed lupus autoimmunity in curli-DNA treated mice by antinuclear antibody test (ANA). Curli-DNA treated mice became ANA positive (Figure 5B), with an initial perinuclear-ER staining pattern; this pattern then evolved into a nuclear staining pattern by 15 weeks of age, resembling the pattern expressed by aged, diseased Sle1,2,3 mice, another strain of mice that spontaneously develop lupus (Morel et al., 1997). Furthermore, when we injected curli-DNA composites into SvJ-129 mice, which do not develop lupus spontaneously but are genetically predisposed to autoimmunity (Bygrave et al., 2004), we found that these mice rapidly produced both anti-dsDNA and anti-chromatin autoantibodies (Figure S5), confirming that bacterial amyloid-DNA composites are sufficient to trigger autoimmunity.

We next analyzed the subclasses of autoantibodies that are induced by curli-DNA composites. We found that anti-dsDNA and anti-chromatin autoantibodies were mostly of immunoglobulin G (IgG) subclass 2a and 2b, as in diseased Sle1,2,3 lupus-prone mice (Figures 5C and 5D). Since IgG2a and IgG2b
subclasses are generally considered pathogenic in systemic autoimmunity (Clynes et al., 1998; Ehlers et al., 2006), our results indicate that curli-DNA composites promote the production of pathogenic autoantibodies.

**Curli-DNA Composites Induce Autoimmunity in Wild-Type Mice**

To test whether naturally occurring curli-DNA composites induce autoimmunity in mice not prone to lupus, we injected B6 mice i.p. with curli (50 μg per injection) three times a week and found anti-dsDNA and anti-chromatin autoantibodies as soon as 2 weeks after injection (Figure 6A), following the same kinetics seen in NZBxW/F1 and SvJ-129 mice. These results indicate that the direct exposure of the immune system to the danger signal curli-DNA composites is sufficient to break self-tolerance in non-autoimmune mice.

To investigate the effects of curli-DNA composites on adaptive immune cells in vivo, we injected mice i.p. with either 50 μg of curli-DNA composites or PBS and analyzed markers of activation in splenic populations 24 hr later. Injection of curli-DNA composites upregulated the expression of the costimulatory molecule CD86 on CD19+ B cells and the activation marker CD69 on both CD4+ and CD8+ T cells (Figure 6B), suggesting that curli-DNA composites stimulate polyclonal activation of T and B cells. It also caused an increase of CD11b+Ly6C+ inflammatory myeloid cells in the spleen (Figure S6). Moreover, curli-DNA composite injection induced DC activation in vivo, as splenic CD11c+ DCs upregulated both costimulatory molecules CD86 and CD80 (Figure 6C) and highly expressed the ISGs Irf7 and Isg15 (Figure 6D). Thus, bacterial amyloids, and in particular the curli-DNA composites, represent a class of danger signals that stimulate innate immune cells and induce polyclonal activation of adaptive immunity.

**Infection with Curli-Competent Bacteria Accelerates Autoimmunity**

We next asked whether curli-expressing bacteria, either commensal or pathogenic, promote autoimmunity in lupus-prone NZBxW/F1 mice. We injected young prediseased NZBxW/F1 mice i.p. with either wild-type (curli-expressing) or curli mutant commensal *E. coli* or virulent *S. Typhimurium*. Systemic route of infection (i.p.) was chosen to mimic the propensity of SLE patients to develop systemic infections including bacteremia. We injected *E. coli* once a week for 4 weeks or *S. Typhimurium* once every 2 weeks for 8 weeks. We spaced Salmonella...
Figure 5. Curli/DNA Composites Accelerate Systemic Autoimmunity in Lupus-Prone NZBxW/F1 Mice

(A) Anti-dsDNA and anti-chromatin autoantibodies were measured by ELISA in sera from NZBxW/F1 mice injected with PBS thrice a week, curli once a week (1x), or curli thrice a week (3x). Optical density (O.D.) indicates ELISA color change and the presence of anti-dsDNA or anti-chromatin autoantibodies. Error bars indicate SEM, n = 5 per group. The dotted horizontal line indicates cutoff for positivity, calculated as two standard deviations above the average of sera from naive C57BL/6 mice.

(B–D) Representative antinuclear antibody assay (ANA) of sera from mice as in Figure 5A at the indicated ages (B). Hep-2 cells were incubated with sera and FITC-conjugated anti-IgG Abs. Antibody isotype determinations of anti-chromatin (C) and anti-dsDNA (D) antibodies. Serum from 10 month-old-diseased Sle1,2,3 mouse was utilized as positive control and from naive C57BL/6 mouse was used as negative control. *p < 0.05.
injections every 2 weeks to prevent mouse death. We then stopped injections and let mice recover for at least 4 weeks. We found that mice infected with curli-competent bacteria, both E. coli and Salmonella, developed high titers of autoantibodies (Figures 7A and 7B), similar to those induced by isolated curli-DNA composites (Figure 5). Mice exposed to curli-deficient bacteria also developed autoantibody titers, albeit at lower amounts compared to curli-competent strains. This could be due to other danger signals expressed by the bacteria such as LPS, which was previously shown to trigger autoantibody production in mice (Fournié et al., 1974). The fact that higher amounts of autoantibodies were produced upon infections with curli-competent bacteria indicates that curli is a major autoimmunogen in these bacteria (Figures 7A and 7B). The predominant autoantibody isotype in response to infection was IgG2a, and IgG2b and IgG3 were also positive to a lesser extent (Figure 7C), suggesting that infection promotes the production of pathogenic autoantibodies to nuclear antigens. Furthermore, mice infected with curli-competent wild-type bacteria indicates that curli is a major autoimmunogen in these bacteria (Figures 7A and 7B). The predominant autoantibody isotype in response to infection was IgG2a, and IgG2b and IgG3 were also positive to a lesser extent (Figure 7C), suggesting that infection promotes the production of pathogenic autoantibodies to nuclear antigens. Furthermore, mice infected with curli-competent wild-type bacteria had significantly stronger ANA staining (Figure 7D). Interestingly, sera from mice infected with S. Typhimurium showed both nuclear and cytoplasmic staining, while mice infected with E. coli showed nuclear staining only, suggesting that different bacteria may diversify the autoantibody repertoire. After sacrificing the animals, we analyzed blood, spleens, and livers for colony forming units (CFUs) and found no evidence of persistent infection at the time of euthanasia (data not shown). Taken together, our results indicate that the exposure to curli or infection with biofilm-competent bacteria promotes the development of autoantibodies in susceptible mice.

DISCUSSION

Studies using amyloid specific dyes demonstrated that up to 40% of the bacterial biofilms contain amyloids (Larsen et al., 2007). However, it has been a challenge to identify novel bacterial amyloid proteins using basic amino acid alignment searches because these proteins do not share any sequence homology but they have a common quaternary structure. Although curli fibers were initially thought to be encoded by only the members of the Enterobactericeae family, gene homologs encoding curli were recently determined also in four phyla: Bacteroidetes, Proteobacteria, Firmicutes, and Thermodesulfobacteria (Dueholm et al., 2012; Hufnagel et al., 2013). Among these, Bacteriodesetes, Firmicutes, and Proteobacteria are the major phyla found in the gastrointestinal tract, but more work is needed to determine the abundance of amyloids in the gastrointestinal tract (Gill et al., 2006; Hooper et al., 2002; Turnbaugh et al., 2007). Besides curli fibers, amyloids are produced also by Staphylococcus aureus and Mycobacterium tuberculosis (Alteri et al., 2007; Chapman et al., 2002; Hufnagel et al., 2013; Schwartz et al., 2012).

Although it was known that amyloids and DNA are incorporated into the biofilm ECM (Whitchurch et al., 2002), little is known about their interactions. Here, we demonstrate that DNA released during biofilm formation is incorporated into curli fibers of S. Typhimurium and accelerates the fiber polymerization process. Because we found that only approximately 10% of the bacteria die in the biofilm population, the high number of live bacteria with a red DNA corona around them point to an active nucleic acid extrusion mechanism during S. Typhimurium biofilm development. Furthermore, the hollow areas lacking green bacteria but showing only the red amyloid stain suggest that in order to incorporate into curli fibers, eDNA must be present during the initial stages of amyloid production and polymerization.

Our results using eukaryotic DNA from salmon sperm also show that eukaryotic DNA can be incorporated into bacterial amyloid
fibers, which suggest that host DNA, released in the vicinity of a bacterial biofilm possibly during tissue damage, may be incorporated into the ECM through this mechanism. Moreover, neutrophils extrude large amounts of DNA called the neutrophil extracellular traps (NETs) as a mechanism to kill bacteria (Brinkmann et al., 2004). Recent studies have shown that P. aeruginosa can incorporate neutrophil-derived DNA into its ECM in order to form a thicker biofilm (Walker et al., 2005). Together with our results, these findings suggest that bacteria may take advantage of eukaryotic DNA in the vicinity to enhance its ECM.
DCs are the sentinels of the immune system, which recognize pathogen-associated molecular patterns (PAMPs) (Iwasaki and Medzhitov, 2004) and damage-associated molecular patterns (DAMPs) (Gallo and Gallucci, 2013) during infection or tissue damage and initiate the adaptive immune response (Merad et al., 2013). We found that DCs are highly activated by biofilms and by curli-DNA composites in particular. We have also reported that the amyloid component of curli fibers stimulates the macrophages through the TLR2-TLR1 complex (Rapsinski et al., 2013). We found that DCs are highly activated by biofilms and by curli-DNA composites in particular. We have also reported that the amyloid component of curli fibers stimulates the macrophages through the TLR2-TLR1 complex (Rapsinski et al., 2013; Tükel et al., 2010). These results indicate that curli fibers are PAMPs, able to stimulate the innate immune system. Although studies on eDNA and its sensors are limited, bacterial dsDNA can stimulate TLR9 (Hemmi et al., 2000) and cytosolic STING-dependent DNA sensors, resulting in a type I interferon response (Ishikawa et al., 2009). The fact that curli-DNA composites are more immunostimulatory than curli or DNA alone suggests that different immune receptors might synergize to recognize these novel molecular patterns in bacterial biofilms.

As many other PAMPs, curli-DNA composites strongly induce the expression of type I IFNs and the IFN-stimulated response in DCs in vitro and in vivo. A type I IFN signature is upregulated in PBMCs of SLE patients (Baechler et al., 2003; Bennett et al., 2003; Crow et al., 2003; Feng et al., 2006; Han et al., 2003) and in DCs from lupus-prone mice (Sirram et al., 2012), and administration of exogenous type I IFNs accelerates autoimmunity in lupus-prone mice (Mathian et al., 2005). Therefore, the results that curli-DNA composites accelerate the onset of autoimmunity in lupus-prone mice can be partially explained by the strong induction of type I IFNs in DCs as well as by the polyclonal activation of the adaptive immune system, as curli upregulated activation markers in splenic T and B cells.

SLE manifests with the production of antinuclear antibodies, including those directed against DNA, ribonucleaseprotein complex (RNP), and nucleosomes (Tan, 2012). Our findings that naturally occurring bacterial curli-DNA composites, which can contain both bacterial and eukaryotic DNA, efficiently trigger type I IFN stimulation and autoantibody production point to a unique bacterial stimulator of autoimmunity. The fact that eukaryotic DNA complexes with the bacterial amyloid curli brings up the possibility of the exposure of the lupus-prone immune system to autoAgs by the curli-DNA composites in the presence of the adjuvant effects caused by TLR triggering and type I IFN stimulation. Self-Ags can be released by NETs or by necrotic cells during tissue damage induced by bacterial infections. Moreover, defects in phagocytosis and clearance of apoptotic cells are associated with lupus (Nagata et al., 2010) and may allow self-DNA from postapoptotic cells to be incorporated into biofilms and become immunogenic.

SLE has a multifactorial pathogenesis in which the genetic make-up and environmental triggers are considered the major players (Liu and Davidson, 2012). Among the environmental triggers, infections have been proposed as either the initiators of autoimmunity or the triggers of flares, the sudden increase in disease severity (Petri, 1998). Several studies have tested this hypothesis, providing conflicting results: for example, lupus-prone MRL/pr mice bred in germ-free conditions develop lupus-like disease (Maldonado et al., 1999), suggesting that genetics trump environmental factors. However, the fact that SLE has a concordance of 30%–40% in identical twins (Connolly and Hakonarson, 2012), clearly indicate that the environmental triggers are required in human disease. Consistent with the idea that bacterial infections contribute to lupus pathogenesis, we found that infection with biofilm-competent, curli-producing bacteria exacerbates autoimmunity in lupus-prone mice. These results are possible due to the direct exposure to curli itself but also to the fact that curli-expressing bacteria are better protected from the immune response—thereby increasing exposure and causing a greater immune activation.

How bacterial infections contribute to lupus pathogenesis and flares remains inconclusive. Our study suggests that biofilm-derived curli-DNA composites can accelerate autoimmunity through the induction of type I IFNs, the activation of DCs, and the polyclonal stimulation of T and B cells. The fact that also the commensal strain of E. coli could accelerate autoimmunity, and that Salmonella and E. coli induced different patterns of autoantibodies, highlights the need to address how the interaction with pathogens and microbiota affects autoimmunity in genetically susceptible individuals.

Our results—that curli-DNA composites can trigger autoimmunity even in nonautoimmune-prone mice—raise the question of why autoimmunity is not more frequent in the human population that is often exposed to biofilm-forming bacteria. A working hypothesis is that in non-lupus-prone individuals, or murine strains, natural infections do not normally result in the systemic release of curli-DNA composites in amounts equivalent to those we injected in this study; while in susceptible individuals, genetically determined dysfunctions may increase the systemic exposure to curli-DNA composites during chronic infections. The more frequent outcome of Salmonella infection in bacteremia and complications in soft tissues in SLE patients (Lim et al., 2001; Pablos et al., 1994; Tsao et al., 2002), and the recent identification of increased levels of circulating endotoxin in SLE patients (Shi et al., 2014), support this hypothesis. In addition, because inflammatory cytokines can decrease intestinal mucosal barrier function (Turner, 2009), the high levels of cytokines present in predisease stages of lupus (Connolly and Hakonarson, 2012; Sirram et al., 2012) might compromise mucosal barrier function and allow the biofilm material produced by microbiota at mucosal sites to access the immune system, act as a danger signal, and trigger lupus onset.

Our study highlights a role of bacterial amyloid-DNA composites in stimulating the innate and the adaptive immune system and suggests that chronic biofilm-producing bacterial infections may represent an important environmental contributor to SLE pathogenesis.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**
S. Typhimurium strain IR715 is a fully virulent, nalidixic acid-resistant strain derived from the ATCC strain 14028 (Stojilkovic et al., 1995). An unmarked deletion of csgBA mutant of S. Typhimurium strain 14028 (Nishimori et al., 2012), S. Typhimurium EHW26, a 3ΔCjfΔB mutant, and S. Typhimurium RPW3 that contains a mutation in msbB were previously described (Raffatellu et al., 2005). E. coli Nissle 1917 was first described by Alfred Nissle (Nissle, 1957).

**Biofilms and CLSM**
Bacterial strains were grown in LB no-salt media at 30 °C to promote biofilm formation. DNA was visualized in biofilms by staining with 1 mg/ml propidium...
from infection for 4 and 8 weeks after the last injection (for every 2 weeks for 8 weeks (4 injections total). Mice were allowed to recover and age-matched female NZBxW/F1 mice at 104 CFU per injection about once per week for 4 weeks (4 injections total). SCID mice were injected i.p. with PBS, or 50 μl PBS either once a week or three times a week. Serum was collected to monitor autoantibody production.

**Lupus and Infections**

*E. coli* and *S. Typhimurium* were grown in biofilm-promoting conditions. Live wild-type *E. coli* or its csgA mutant was injected i.p. into 8-week-old female NZBxWF1 mice at 10^5 CFU per injection once a week for 4 weeks (4 injections total). Live *S. Typhimurium* or its isogenic csgBA mutant was injected i.p. into age-matched female NZBxWF1 mice at 10^6 CFU per injection about once every 2 weeks for 8 weeks (4 injections total). Mice were allowed to recover from infection for 4 and 8 weeks after the last injection (for *S. Typhimurium* and *E. coli*, respectively).

The rest of the Experimental procedures are in the Supplemental Information.

**Statistical Analysis**

Data were analyzed using Prism software (GraphPad). One-sample t test or two-tailed Student’s t test were used as appropriate. A p value of < 0.05 was considered significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures, Supplemental Experimental Procedures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.06.002.

**AUTHOR CONTRIBUTIONS**

Co-first authors P.M.G. and G.J.R. contributed equally to this work. G.J.R. performed the experiments related to the initial discovery of nucleic acids in curli fibers. G.J.R. also performed biofilm experiments, amyloid polymerization experiments, and confocal microscopy. P.M.G. performed the experiments of activation of dendritic cells in vitro, in vivo treatment of mice with antibody ELISAs, and ANA assay. P.M.G. and R.P.W. conducted the experiments for the in vivo activation of lupus with infections. U.S. conducted ELISAs. M.G. constructed the crystal violet assay. S.G. and C.T. designed and oversaw all experimental work and wrote the manuscript, which was further edited by all authors. All authors approved the final manuscript.

**ACKNOWLEDGMENTS**

We would like to thank Drs. A.J. Baumler, P.L. Cohen, P.J. Piggot, and P. Matzinger for reading the manuscript and providing constructive critiques.

Work in C.T.’s laboratory was supported by the NIH, NIAID 1R03AI107434 and 1R21AI105370. Work in S.G.’s laboratory was supported by the NIH, NIAID RO1-AI076423, the FCCC-Temple University Nodal grant, and the Lupus Research Institute, Innovative Research Grant. R.C. was supported by NIH, NIAMS RO1-AR061569. P.M.G. was partially supported by the Lupus Foundation’s Goldie Simon Preceptorship Award.

Received: April 24, 2014

Revised: December 8, 2014

Accepted: April 6, 2015

Published: June 16, 2015

**REFERENCES**


