Antibody Repertoire Development in Fetal and Neonatal Piglets. IX. Three Pathogen-Associated Molecular Patterns Act Synergistically to Allow Germfree Piglets to Respond to Type 2 Thymus-Independent and Thymus-Dependent Antigens

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Antibody Repertoire Development in Fetal and Neonatal Piglets. IX. Three Pathogen-Associated Molecular Patterns Act Synergistically to Allow Germfree Piglets to Respond to Type 2 Thymus-Independent and Thymus-Dependent Antigens

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Newborn piglets maintained germfree (GF) cannot respond to either thymus-dependent (TD) or type 2 thymus-independent Ags (TI-2) unless colonized with bacteria. We show here that pathogen-associated molecular patterns (PAMPs), including muramyl dipeptide (MDP), LPS, and a B-class CpG oligonucleotide (CpG-B), can substitute for gut flora in the induction of neonatal immunoresponsiveness. These PAMPs alone or in combination had little effect on serum IgG and IgA levels, but CpG-B and MDP elevated total IgM levels 3- to 7-fold above that seen in colonized controls after booster immunization. Although only CpG-B could alone stimulate immunoresponsiveness, co-administration of LPS or MDP resulted in a 5-fold increase in the IgG response to both immunogens. Co-administered MDP did not promote secondary IgG responses to either Ag but instead pronounced secondary IgM responses to the epitopes of both immunogens. LPS co-administered with CpG-B may promote class switch recombination or cause differentiation of previously switched cells that become responsive after exposure to CpG-B. Primary and secondary IgG responses also equally recognize the epitopes of the TI-2 and TD immunogens, whereas IgM responses favored the TI-2 epitope. Because PAMPs alone may result in Abs to 2,4,6-trinitrophenyl and FLU without immunization, it suggests they alone cause differentiation of B cells of the preimmune repertoire. The finding that both bacterial PAMPs and colonization are capable of stimulating Ab responses in both immunized and nonimmunized piglets suggests that PAMPs derived from host flora may play a major role in awakening adaptive immunity in neonates. The Journal of Immunology, 2005, 175: 6772–6785.

It is generally recognized that colonization of the gastrointestinal (GI) tract significantly influences the development of the neonatal immune system (1–9). Colonization of the gut by commensal bacteria is necessary for proper nutrition and metabolism by the host (9, 10), but its effect on immunological development may also have relevance to oral tolerance, allergy, and inflammatory bowel disease (11–13). Bacteria that colonize the GI tract may exert their influence by providing a source of foreign Ags and/or a source of bacterial products that cause maturation of naïve APCs and B cells. Because bacterial Ags do not cause development of the gut-associated immune system in rabbits (14), it suggests that other non-Ag features of bacteria are more important.

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2 Address correspondence and reprint requests to Dr. John E. Butler, Department of Microbiology and Interdisciplinary Immunology Program, 3-501 BSF, 51 Newton Road, University of Iowa, Iowa City, IA 52242; E-mail address: John-buter@uiowa.edu
3 Abbreviations used in this paper: GI, gastrointestinal; CSR, class switch recombination; PRRR, pattern recognition receptor; PGN, peptidoglycan; DC, dendritic cell; MDP, muramyl dipeptide; NOD, nucleotide-binding oligomerization domain; ODN, oligodeoxynucleotide; pDC, plasmacytoid DC; GF, germfree; TD, T dependent; TI-2, type 2 T independent; FLU, fluorescein; KHL, keyhole limpet hemocyanin; TNF, 2,4,6-trinitrophenyl; PC, phosphorylcholine; HSA, human serum albumin; PSA, porcine serum albumin; MLN, mesenteric lymph node.
TLR2 and nonacylated MDP that stimulates through NOD2. Because MDP is chemically synthesized, its use in experimental studies is desirable because PGNs from different sources may have different effects (35, 36) and using synthesized MDP avoids the complicating effects of other bacterial components that may contaminate bacterially derived PGNs.

In addition to LPS and MDP, nonmethylated DNA from prokaryotic organisms (37) and simple eucaryotic organisms (38) also acts as an adjuvant for mammalian B cell responses (39). Nonmethylated bacterial DNA can be administered as synthetic oligodeoxynucleotides (ODNs) bearing the CpG motif (37) called CpG ODN. The use of synthetic CpG ODN, as with MDP, avoids ambiguity caused when bacterial DNA preparations are contaminated with other bacterial products such as LPS. CpG ODN is recognized by TLR9, an endosomal/endoplasmic reticulum receptor found in both B cells and plasmacytoid DCs (pDCs; Refs. 40–43). Three major forms of CpG ODN, A-class and B-class (sometimes called D and K, respectively; Ref. 44) and C-class CpG, are recognized and these have differential activity in pDCs and B cells (45–47). Class B oligos have phosphorothiate backbone with multiple TCG motifs, while class A oligos have mixed phosphodiester-phosphorothioate backbones with multiple TCG motifs, at one or both ends. Class A oligos preferentially stimulate TNF-α and IL-8 release from pDCs, while class B oligos triggers pDC maturation and sustained type I IFN release (48). Memory B cells express higher levels of TLR9 and may be more responsive to CpG-B simulation than naive B cells, which, however, respond synergistically if stimulated through their BCR, which up-regulates their TLR9 expression (19). pDCs have an absolute requirement for TLR9 and the adapter MyD88 (49). The role of TLRs in mediating the effects of bacterial products and CpG ODN has been reviewed recently (37, 50, 51).

We have shown previously that colonization of germfree (GF) piglets 1 elevates serum Ig levels 10- to 30-fold depending on the isolate, 2) preferentially influences diversification of the mucosal immune system (52), and 3) is required for piglets to mount serum Ab responses to both T-dependent (TD) and type 2 T-independent (TI-2) immunogens given i.p. (2). Furthermore, viral infection has a similar effect presumably because of stimulation through TLRs 3, 7, or 8 (53). Because it has been observed more than two decades ago that GF isolator piglets were much more responsive to bacterial Ags if contaminated with LPS (K. Petzoldt, unpublished observations), we wondered to what extent the ability to respond to TD and TI-2 immunogens was dependent on living bacteria vs the direct or indirect adjuvant effect of various nonliving bacterial products, e.g., PAMPs. In mice, TD immunogens have an absolute requirement for class II-mediated peptide presentation to helper T cells (54), whereas the requirement for responses to TI-2 Ags is more controversial (55). Although cognate T cell interaction may not be needed for responses to TI-2 immunogens, cytokines derived from T cells and accessory cells are essential (55–58). Because GF piglets respond to neither type of immunogen, their APCs and B cells apparently remain immature until stimulated through their PRRs.

It is important to realize that Cesarian-derived piglets differ from newborn mice in having never been exposed to maternal immunoglobulins and other regulatory factors in utero (59, 60). This may explain why GF isolator piglets do not respond to TD or TI-2 immunogens (2), whereas >95% of GF mice do respond to TI-2 immunogens (61). We hypothesized that the difference between the response capabilities of fetal/neonatal piglets and rodent pups reflects the developmental differences described above in that GF mice may have received certain positive developmental stimuli in utero so their B cells or APCs are less naive. Because their precocial nature allows them to be maintained in isolators or in a specific pathogen-free environment separate from their birth mothers (62–65), newborn piglets represent an ideal immunological tabula rasa. Newborn organisms that are able to run, walk, and forage immediately after birth are called precocial. This includes most invertebrates, lower vertebrates, jackrabbits but not cotton-tails, or lab rodents but all large farm animals.

Much has been published about the effects of various PAMPs on cells in vitro. However, the value of these reports would be increased if similar events could be demonstrated in vivo. The tabula rasa of the newborn piglet offers an opportunity for such in vivo confirmation. This is the first report on the in vivo effects of PAMPs in a totally naive mammal and shows that CpG-B, but not LPS or MDP, allows piglets to respond to TI-2 and TD immunogens. However, both MDP and LPS act synergistically to enhance IgM and IgG responses to both immunogens. These findings indicate that PAMPs can substitute for colonization and may explain colonization-induced immunoresponsiveness of newborns to TI-2 and TD immunogens.

Materials and Methods

Experimental design of isolator piglet studies

All piglets were recovered by cesarean derivation as described by Miniatis and Jol (63) and maintained in GF isolators (2, 52). The capacity of the isolator facility required that the studies be done using multiple experimental groups, which had the advantage of mixing unrelated, outbred animals so major differences would not reflect simple genetic variation. However, animals from the same litters were placed in pairs into different treatment groups. As previously and diagrammatically described (2), piglets in nine groups (A–I; Table I) were colonized within the first 2 days of life with Escherichia coli GF and given PAMPs (see below). Those in appropriate groups were immunized i.p. with a mixture of 3 mg of fluorescyl (FL)-keyhole limpet hemocyanin (KLH) and 3 mg of 2,4,6-triitrophenyl (TNP)-Ficoll on day 7 and most groups were boosted with a similar dose three weeks later (Table I). FL-KLH and TNP-Ficoll were prepared and administered as described previously (2). In previous studies, doses up to 19 mg were tested, and the amounts used here were considered optimal. Weekly blood samples were collected 1 day before immunization or i.p. injection of bacterial products (see below). These were used for measurement of Ig levels and IgM, IgG, and IgA Ab responses.

Measurement of serum Ig and specific Abs

Total serum levels of porcine IgM, IgG, and IgA and serum Abs to fluorescein (FLU) and TNP were measured as described previously (2). The number of determinations per week per group is given in Table I. DNP and phosphorylcholine (PC) conjugates were prepared as described previously (66, 67). Briefly, haptens were conjugated to either electrophoretically pure porcine serum albumin (PAA) or human serum albumin (HSA) purchased from Sigma-Aldrich. LPS for immunoassays include that obtained from Sigma-Aldrich. LPS for immunooassays include that obtained from Sigma-Aldrich (see “Source and administration of bacterial products”) and LPS prepared from E. coli G58-1 using previously described methods (68). Ab activity was measured by ELISANALYSIS (69) and, in most cases, expressed as ELISA units relative to a reference standard. Specific activity expresses the ratio of ELISA units of activity per microgram of the isotype in question (Table II; Ref. 70). An increase in specific activity after boost was used as one criterion for determining whether a secondary response occurred. However, data on specific activity only allow comparisons to be made among treatment groups and not between Ags, e.g., FLU vs TNP. Therefore, we determined the relative response to different Ags by direct titration. This procedure involves the comparison of whole curve titration plots using TNP-PAA, DNP-PAA, FLU-PAA, PC-HSA, LPS of E. coli G58-1, whole bacteria, fibrinogen, B-lactoglobulin, PSA, and HSA as Ags (see Figs. 5–7). PC, whole bacteria, and fibrinogen were used to test for polyclonal activation, B-lactoglobulin was used to test for Ab to dietary protein, and DNP is a chemical analog of TNP, was used to test for fine specificity. In the case of nonimmunized piglets, the alkaline phosphatase-driven enzymic reaction was allowed to proceed for 12 h in the dark, whereas the usual reaction time was 1 h.
Bacterial colonization. The system of weekly administration of PAMPs was adopted to provide continuous immune activation, simulating the effects of continued orally. The system of weekly administration was always done 1 day after blood collection, i.e., blood was collected on day 6, and Ag and/or the bacterial products was given on day 7. PAMPs were given i.p. so that the amount entering the piglet could be controlled, a value not easily controllable when the ligands are given orally. The system of weekly administration of PAMPs was adopted to provide continuous immune activation, simulating the effects of continued bacterial colonization.

Source and administration of bacterial products
MDP was purchased from Sigma-Aldrich and was given at 0.5 mg/wk. CpG ODN 2006, a B-class CpG ODN (referred to as CpG-B), was obtained from Coley Pharmaceutical Group, contained no detectable endotoxin, and was routinely administered weekly i.p. at a dose of 2 mg alone or together with MDP and LPS. Initial studies were conducted to establish the final dosage. E. coli LPS (L-4391; Sigma-Aldrich) was administered at 3 mg/wk; preliminary titration studies indicated this was the maximum dosage that does not produce endotoxin shock in newborn piglets. Intraperitoneal administration was always done 1 day after blood collection, i.e., blood was collected on day 6, and Ag and/or the bacterial products was given on day 7. PAMPs were given i.p. so that the amount entering the piglet could be controlled, a value not easily controllable when the ligands are given orally. The system of weekly administration of PAMPs was adopted to provide continuous immune activation, simulating the effects of continued bacterial colonization.

Spectratypic analyses
Spectratyping was performed as described previously (71). Briefly, spectratyping is length analysis of CDR3 and is done by PCR amplification of the CDR3 region using a primer set spanning from FR3 to JH (FR4). The amplification is accompanied by the use of 32P-nucleotides and the product is separated on a standard polyacrylamide sequencing gel.

Statistical evaluation
Mean differences were statistically evaluated whenever appropriate, using Student’s two-tailed t test. Statistical outcomes are indicated on figures and in figure legends. The number of animals and measurements per week on which statistical analyses are based is given in Table I.

Results
GF piglets receiving CpG-B or CpG-B+MDP have a serum Ig profile that favors IgM after boosting
Fig. 1 compares the levels of total IgG, IgM, and IgA in sera of GF piglets, colonized piglets, and GF piglets given PAMPs. Results show that IgG and IgA levels are 3- to 10-fold higher in colonized piglets than any group receiving PAMPs. In contrast, weekly administration of CpG-B or CpG-B + MDP raised IgM levels on week 5 3- to 7-fold higher than in GF control (group A) or colonized piglets (group B). Levels in piglets receiving CpG + MDP (group H) were significantly greater than in piglets receiving only CpG (group C). LPS alone did not elevate the level of any isotype above that of the control (group A). Whereas colonization results in an early (primary) increase in IgM by week 2 that then shifts to IgG, such a profile was only seen in GF piglets when LPS was coadministered with CpG (group F), although the increase in IgG on week 5 was only a trend. Thus, CpG-B and CpG-B + MDP given to GF animals preferentially support late IgM synthesis in GF piglets, while colonization or LPS given together with CpG-B favor early IgM production that later shifts to IgG synthesis.

Table I. Piglet groups and their treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Animal</th>
<th>Determinations per week*</th>
<th>PAMP</th>
<th>Immunization</th>
<th>Booster</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>GF only</td>
<td>4</td>
<td>17 12</td>
<td>None</td>
<td>FLU-KLH</td>
<td>FLU-KLH</td>
</tr>
<tr>
<td>B</td>
<td>G58–1 colonized</td>
<td>4</td>
<td>14 14</td>
<td>None</td>
<td>FLU-KLH</td>
<td>FLU-KLH</td>
</tr>
<tr>
<td>C</td>
<td>GF + CpG</td>
<td>6</td>
<td>23 20</td>
<td>CpG</td>
<td>FLU-KLH</td>
<td>FLU-KLH</td>
</tr>
<tr>
<td>D</td>
<td>GF + CpG</td>
<td>3^b</td>
<td>13 13</td>
<td>CpG</td>
<td>FLU-KLH/</td>
<td>FLU-KLH</td>
</tr>
<tr>
<td>E</td>
<td>GF + CpG</td>
<td>2</td>
<td>7 7</td>
<td>CpG</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>F</td>
<td>GF + CpG + LPS</td>
<td>8</td>
<td>32 30</td>
<td>CpG + LPS</td>
<td>FLU-KLH</td>
<td>FLU-KLH</td>
</tr>
<tr>
<td>G</td>
<td>GF + CpG + LPS</td>
<td>5</td>
<td>23 18</td>
<td>CpG + LPS</td>
<td>FLU-KLH</td>
<td>FLU-KLH</td>
</tr>
<tr>
<td>H</td>
<td>GF + CpG + MDP</td>
<td>4</td>
<td>14 15</td>
<td>CpG + MDP</td>
<td>FLU-KLH</td>
<td>FLU-KLH</td>
</tr>
<tr>
<td>I</td>
<td>GF + LPS</td>
<td>2</td>
<td>19 16</td>
<td>LPS</td>
<td>TNP-Ficoll</td>
<td>TNP-Ficoll</td>
</tr>
</tbody>
</table>

* Number of measurement per week for all animals in each treatment group. Values on left are for Ig determination and those on the right for Ab measurement (SpAbI). The difference reflects the failure to detect Ab responses in some samples from animals tested.

Table II. Specific activity of colonized or CpG-B treated piglets on weeks 4 and 5^c

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>IgM anti-TNP</th>
<th>IgG anti-TNP</th>
<th>IgM anti-FLU</th>
<th>IgG anti-FLU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>B</td>
<td>Colonized</td>
<td>209 118</td>
<td>9.4 5.2</td>
<td>10.4 29.4^c</td>
<td>1.1 0.8</td>
</tr>
<tr>
<td>C</td>
<td>CpG-B</td>
<td>139 276^b</td>
<td>183^c 167</td>
<td>2.4 12.0^b</td>
<td>65^c 46</td>
</tr>
<tr>
<td>D</td>
<td>CpG-B, twice</td>
<td>267 723^b</td>
<td>0 127^b</td>
<td>0 1.9</td>
<td>0 10.4^b</td>
</tr>
<tr>
<td>E</td>
<td>CpG-B control</td>
<td>104 110</td>
<td>0 96^b</td>
<td>0 0</td>
<td>5.2^c 32^b</td>
</tr>
<tr>
<td>F</td>
<td>CpG-B + LPS</td>
<td>314 506^b</td>
<td>278^c 319</td>
<td>8 19^b</td>
<td>25^c 86^b</td>
</tr>
<tr>
<td>G</td>
<td>CpG-B + LPS, nonboost</td>
<td>692^c 545</td>
<td>194^c 123</td>
<td>13.3 3.4</td>
<td>11^c 5</td>
</tr>
<tr>
<td>H</td>
<td>CpG-B + MDP</td>
<td>113 211^b</td>
<td>489^c 540</td>
<td>1.8 6.2^b</td>
<td>152^c 193</td>
</tr>
</tbody>
</table>

^c Results are presented for all treatment groups giving ELISA activity above that of control group A (Table I; Figs. 1–3). Data calculated as the net ELISA response after subtraction of values for control group A.

^b Significantly higher on week 5 (postboost) than on week 4 (preboost).

^Significantly higher than for colonized piglets.
CpG-B alone but not LPS or MDP stimulates immunoresponsiveness in GF piglets

Fig. 1 shows that CpG-B was the only PAMP studied that alone effects Ig levels. The response profiles in Figs. 2 and 3 also show that CpG-B was the only PAMP studied that was alone capable of stimulating immunoresponsiveness in GF piglets. However, responses were not statistically above GF controls until later in the treatment when significant secondary IgM responses to both immunogens were seen (Fig. 3, A and B). When administered only at the time of immunization and boost (group D), secondary IgM but not IgG responses were seen (Fig. 3). The absence of a secondary IgG response suggests that CpG-B does not cause CSR in vivo (Figs. 2, C and D, and 3, C and D).

Coadministration of LPS or MDP with CpG-B differentially enhances immunoresponsiveness in GF piglets

Although neither LPS (group I; Table I) nor MDP (data not shown) alone were able to stimulate IgM or IgG responses above the levels seen in the GF control (group A; Figs. 2 and 3), coadministration of CpG-B + LPS yields an IgM response profile to the TD immunogen (FLU) that best resembles that of colonized piglets (Fig. 2B). However there was only a delayed IgG response to TNP (TI-2 Ag) and no secondary response (Figs. 2A and 3A). When MDP was coadministered with CpG-B, pronounced secondary IgM responses to both immunogens were observed and that for TNP exceeded the secondary IgM response obtained with CpG-B alone (Fig. 2A). CpG-B + MDP and CpG-B + LPS yielded a delayed IgG response to both immunogens that was significantly greater than seen with CpG-B alone (Figs. 2, C and D, 3, C and D) but as with IgM, boosting the animals on week 4 did not yield a secondary response to the TI-2 epitope TNP (Fig. 3C). Whereas CpG-B + LPS or CpG-B + MDP both result in secondary IgM responses to FLU (Fig. 3B), only CpG-B + LPS results in a secondary IgG response to the TD immunogen (Fig. 3D). Nonboosted GF piglets given CpG-B + LPS gave strong IgM responses to both immunogens but predictably no secondary IgM response (Fig. 3, A and B). The relative magnitude of the IgM and IgG responses of group G (nonboosted) and group F (boosted) differ. The preference toward IgM responsiveness in group G presumably reflects genetic differences because they came from different litters.

The pronounced enhancement by CpG-B + MDP of secondary IgM responses (Fig. 3, A and B) and enhanced but delayed IgG responses, with no booster effect (Figs. 2, C and D, 3, C and D), suggests that MDP does not cause CSR but drives expansion of Ag-specific IgG cells. This differed from the augmentation property of LPS when given with CpG-B, which did cause secondary IgM and IgG responses to the TD immunogen (Figs. 2D and 3D) but not to the TI-2 Ag (Fig. 3).

PAMP-treated GF piglets produce IgG Abs with higher specific activity than colonized littermates

Specific activity is the ratio of ELISA activity to the amount of total Ig of the corresponding isotype. An increase in specific activity reflects an increase in the proportion of Ab that specifically recognizes the immunizing Ag and/or its affinity (66, 70). Table II shows that among GF piglets treated weekly with PAMPs, the
preboost IgM-specific activity was not higher than in piglets colonized with benign E. coli. However, all GF groups that gave significant secondary IgM responses (Fig. 3) also had significantly higher specific activity (Table II). This was not seen in group G that was not boosted or group E that was not immunized. In contrast to IgM, IgG-specific activity to both Ags was much higher in all GF piglets treated weekly with PAMPs than in colonized piglets. This was even the case for IgG on week 5 in nonimmunized piglets (group E; Table II).

Among colonized piglets, a postboost increase in specific activity was only seen for IgM responses to FLU (Table II). CpG alone did not result in postboost increases in IgG-specific activity unless CpG-B was given only at immunization and boost (group D). Animals given CpG-B + LPS (group F) had elevated postboost IgG
FIGURE 3. Primary, preboost, and postboost IgM and IgG responses of all treatment groups (see Table I). A and B present the IgM response data while C and D present the IgG response data. *, Postboost ELISA activity statistically greater at the 5% level than preboost activity, suggesting a secondary immune response. Values of $p$ in A range from $1.6 \times 10^{-3}$ to $5.6 \times 10^{-3}$, in B from $1.8 \times 10^{-2}$ to $5.6 \times 10^{-3}$, and in D from $2 \times 10^{-3}$ to $2.5 \times 10^{-3}$. 
anti-FLU but not anti-TNP. Among GF piglets receiving CpG-B + MDP, only IgM-specific activity was higher after boosting (Table II).

*Elevated specific activity in PAMP-treated GF piglets was not reflected in the selective clonal expansion of B cells in the mesenteric lymph node (MLN)*

Fig. 1 shows that serum IgG and IgA levels in GF piglets given CpG-B or CpG-B + LPS or CpG-B + MDP are only one-tenth of that seen in colonized piglets yet IgG Ab activity to TNP and FLU is 5- to 20-fold higher on week 5 than in colonized piglets (Fig. 2). This suggests that a much higher proportion of the IgG Abs in GF animals given PAMPS are specific for the immunizing Ags than in colonized animals. This is also reflected in higher specific activities (Table II). This was also seen in the IgM response to TNP in GF animals receiving CpG-B or CpG-B + LPS (Table II). We wondered if the increased specific activity was due to selective expansion of individual B cell clones in the MLN because animals were immunized i.p. However, when the total Ig transcripts from the MLN were analyzed spectratypically, the repertoire in GF piglets receiving CpG-B + MDP was polyclonal just as in colonized animals and GF controls (Fig. 4). Selection to alter the polyclonal pattern was only apparent in one colonized piglet and in the virus-infected piglet used as a positive control. When only IgG transcripts were spectratyped, the same results were obtained (data not shown).

*Colonization and PAMP exposure produce similar specificity profiles to TD and TI-2 epitopes in primary and secondary responses*

Figs. 5 and 6 provide examples of the IgM and IgG response profiles of colonized and PAMP-treated GF piglets, respectively. The primary IgM response in colonized piglets (Fig. 5) favors the TI-2 epitope, LPS, and the TNP analog DNP, whereas the primary IgG response equally favors the TD and TI-2 epitopes. The secondary responses to FLU and TNP of both isotypes were dwarfed by responses to Ags of the colonizing bacteria (G58-1) and its LPS, but both secondary IgM and IgG responses equally favored the TD and TI-2 epitopes. In PAMP-treated GF piglets (Fig. 6), the primary and secondary IgM response strongly favored the TI-2 epitope TNP, whereas the primary and secondary IgG responses equally favor both epitopes (Fig. 6). Not surprisingly, we observed no significant response to LPS prepared from G58-1 to whole G58-1 E. coli or LPS from Sigma-Aldrich in noncolonized GF piglets. The response profiles of piglets receiving CpG-B + MDP or CpG-B + LPS (data not shown) were identical to those for piglets receiving CpG-B only (Fig. 6). Neither GF nor colonized animals recognized the control hapten (PC) or the hapten carriers used in the ELISA (PSA or HSA; data not shown).

**PAMPs elicit weak natural Abs predominately to TNP**

Previous studies indicated that colonization alone results in serum Abs to TNP and FLU in nonimmunized piglets (2). Therefore, we wondered to what extent responses to the epitopes of TD and TI-2 immunogens were dependent on exposure to PAMPS alone. Fig. 7 shows the primary response titration profiles to several Ags in nonimmunized, control GF piglets (group E). CpG-B-treated GF piglets preferentially recognized TNP with IgM Abs when given only CpG-B, which is also reflected in their IgM anti-TNP-specific activity (Table II). Interestingly, the IgG-specific activity of those nonimmunized piglets on week 5 to both TNP and FLU was also significantly elevated above that of week 4 even though no Ag was given (Table II). In preliminary data from a nonimmunized group not shown in Table I, IgG responses to TNP were only seen when LPS was coadministered. In all cases, these natural Ab responses were much weaker than in immunized piglets (see inset, Fig. 7). Weak IgM responses to FLU and fibrinogen were seen in all animals, and IgG responses of similar magnitude to these Ags were also seen in piglets receiving CpG-B + LPS but not those receiving CpG-B alone. Virtually no responses were seen to other Ags.
including PC, E. coli, or LPS. For that reason the average response to all other test Ags was pooled and is represented as “other.”

**Discussion**

Observations in our own and other laboratories have shown that colonization of the GI tract is important for maturation of the mammalian adaptive immune system (1–3, 5, 6, 10, 52, 72). It has become well-established that stimulation through PRRs of the innate immune system can stimulate adaptive immune responses (73) and that exposure to bacterial products is necessary for the maturation of dendritic/macrophage APCs (51, 74–77). The best understood PRRs are the TLRs, but the cytoplasmic NOD receptors are also important (32–34). PRRs recognize specific bacterial and viral molecular patterns, e.g., LPS (TLR4), PGNs (TLR2, TLR6, and TLR11), and unmethylated CpG motifs (TLR9).
TLR6, NOD1, and NOD2), bacterial DNA (TLR9), and viral RNA (TLR3, TLR7, and TLR8). Because we have shown previously that intestinal colonization elevates serum Ig levels 10- to 100-fold in newborn piglets (52) and is required for allowing immune responses in isolator piglets to TD and TI-2 Ags (2), we wondered whether certain bacterial PAMPs would have the same effect as exposure to living microorganisms. If such ligands could substitute for colonization, it would suggest that colonization acts through PRRs and therefore might be used in vaccines to awaken the adaptive immune system of the neonate. Up to this point, most information available on the role of PAMPs has been derived from in vitro studies that cannot always account for the complexities of the in vivo environment. Therefore, we choose the GF piglet model to test the in vivo effect of three PAMPs: bacterial DNA in the form of CpG ODN, a bacterial ligand characteristic of Gram-negative (LPS), and one (MDP) representing an epitope of PGNs found in both Gram-negative and Gram-positive bacteria. Although the selection of this model was discussed in the introduction, suffice it

**FIGURE 6.** Specificity response profiles for the primary (week 2; A and B) and secondary (week 5; C and D) IgM and IgG responses of a representative GF isolator piglet given weekly doses of CpG-B and immunized and boosted with TNP-Ficol and FLU-KLH. Legend the same as Fig. 5. When the response profiles for CpG-B + LPS or CpG-B + MDP were studied, they were identical to those for CpG-B only piglets (data not shown).
here to reiterate that piglets receive no maternal IgG in utero and is the only described model in which an adaptive immune response is absent without colonization (2). Nevertheless, the piglet model currently suffers from the lack of transgenic and knockout pigs, mAb with which to monitor cell phenotype or TLR expression, and reliable cell lines for in vitro studies. Thus, we can describe here only an in vivo phenomenon that cannot yet be tested at the cellular and molecular level. Despite this major shortcomings, our data show that: 1) PAMPs or colonization are required for differentiation of Ag-specific B cells of the preimmune repertoire to become Ab-secreting cells; 2) PAMP-induced differentiation of TNP-specific B cells does not require Ag but is greatly augmented by its coadministration; 3) naive porcine B cells or DCs apparently express TLR9 but probably not TLR4 or the NOD2 receptor; 4)
LPS or MDP are unable to awaken the naive adaptive immune system but can greatly augment the stimulatory effect of bacterial DNA; 5) LPS and MDP appear to cause CSR or selective expansion of Ag-specific IgG cells of the preimmune repertoire after their exposure to CpG-B; and 6) the PAMPs studied act synergistically and differentially. Separate data are not presented for piglets receiving only MDP because they behave like LPS only piglets.

Surprisingly, our in vivo studies using LPS alone, a well-known mouse B cell mitogen in vitro, did not significantly raise serum Ig levels above those of GF controls (Fig. 1) and did not stimulate IgM or IgG responses to TNP or FLU when used at the highest concentration (2 mg/ml) that did not produce endotoxin shock (Figs. 1–3). We interpret this to mean that naive, Ag-specific IgM+ B cells of the newborn piglet are unresponsive to LPS, e.g., they lack TLR4 unless first stimulated with bacterial DNA (see below). The differentiation of such cells to secrete IgM, even in the absence of Ag (Fig. 7), suggests they do express TLR9. This is consistent with observations that TLR9-bearing B cells undergo proliferation and produce IgM in response to CpG (19, 39). Perhaps the LPS responsiveness of mouse B cells studied in vitro is because: 1) they are more mature due to prior encounter with PAMPs (61); 2) maternal regulatory factors transmitted in utero up-regulate TLR4; 3) changes in TLR4 expression are caused by their in vitro recovery; or 4) mouse B cells studied in vitro are not representative of those in the gut or the peritoneum. Regarding the latter, intestinal or mesenteric B cells, DCs, or epithelia may lack TLR4 expression to avoid LPS-induced gut inflammation following colonization (78, 79). First, efforts to compare the outcome of these studies with those in mice are complicated because chemically synthesized PAMPs (excluding LPS) were not always used in earlier mouse studies; thus, contaminations by other natural products could have compromised these earlier studies (80–82). Second, there are few data on TLR expression in swine, although both TLR4 and TLR9 have been cloned (gi: 58696571 and 47522745). Shimosata et al. (83) showed that TLR9 was present on both B cells and DCs of jejunal Peyers patches and was expressed in the MLN. Corresponding data on TLR4 expression are not available. A formal test of this hypothesis depends on an analysis of TLR4 and TLR9 expression on individual cell types in the newborn piglet using both transcript analysis and TLRSpecific mAb. This is hampered by the lack of porcine cell lines for in vitro studies and the lack of mAb that can allow recovery of B cell and DCD subsets.

Weekly administration of bacterial DNA (as CpG-B) resulted in a 3-fold higher serum IgM level after booster immunization than was seen in colonized piglets, whereas LPS given alone marginally raised IgG and IgM levels on week 5 above those in GF controls. MDP alone had no effect (data not shown) (Fig. 1). However, coadministration of MDP doubled IgM levels and also increased serum IgG, although the levels were still 5-fold lower than in colonized piglets (Fig. 1). Serum IgA profiles resembled those of IgG but showed a consistent “primary” increase on week 2. Although CpG-B + LPS failed to raise total IgM levels after challenge in contrast to CpG-B given alone or CpG-B + MDP, coadministration of LPS resulted in a pronounced secondary IgG response to FLU, the TD Ag, but not TNP (TI-2 immunogen; Fig. 3 and Table II). This suggests that IgG+ cells of the preimmune repertoire become responsive to LPS after exposure to CpG-B. This phenomenon is also apparent by the preferential Ag response stimulated in nonimmunized piglets (Fig. 7). Perhaps IgM+ and IgG+ B cells of newborn piglets resemble those of humans (19) in lacking expression of TLR4, TLR6, or NOD2 but rapidly up-regulate TLR4 and NOD2 expression after stimulation through TLR9. Alternatively, LPS may cause CSR to IgG as shown with studies on mouse B cells (17, 84, 85) that express TLR4 (19). It is important to emphasize that CSR occurs in the fetal piglet in the absence of regulatory maternal Abs or environmental Ag (86), so newborn animals do have IgG+ and IgA+ cells at birth. In contrast to LPS, recognition of MDP, presumably through up-regulation of NOD2 after CpG-B contact, accelerates B cell proliferation and differentiation of both IgG- and IgM-secreting plasma cells but does not result in a secondary IgG response (Fig. 3, C and D). The effect observed when CpG-B was administered alone is consistent with the work of Liu et al. (84) and He et al. (87) in confirming that CpG-B acts directly on B cells that promotes IgM production without CSR. Current in vitro data are lacking on the synergistic effect of MDP on PGNs on CpG-stimulated B cells. However, it has been shown that CpG-B can cause germline transcription of Cγ genes, thus positioning cells for a second signal, perhaps through LPS but not MDP. This could presumably lead to CSR and the secretion of IgG (87). Overall, our data indicate differential synergy in the action of MDP and LPS. Synergy between MDP and LPS has been shown previously, but bacterial DNA was not included in these studies (88).

The outcome of the in vivo studies presented here may not result from the action of PAMPs directly on B cells but rather through their indirect action on DCs or other cell types. pDCs in mice and humans also express TLR9 (40, 42, 43). Some studies with human B cells indicate that CpG-B is unable to stimulate naive B cells and that only memory B cells are responsive (19, 89). The involvement of DCs in the response to CpG-B + LPS could also explain our findings because LPS-stimulated myeloid DCs up-regulate B cell-activating factors such as B cell activation factor of the TNF family (90), causing prolonged B cell survival and CSR (91). The in vivo phenomenon we describe in this article cannot be easily addressed at the cellular and molecular level in swine given the paucity or reagents and the poor characterization of the swine immune system by mouse standards. Therefore, identifying the cells bearing the functional TLR involved and their changes in expression that can explain the phenomenon described is simply impractical at this point.

In all cases in which GF piglets received PAMPs, primary and secondary IgM responses strongly favored the epitope of the TI-2 immunogen (TNP), whereas IgG responses equally favor FLU (TD immunogen) and TNP (Fig. 6). This outcome is logical because the vast majority of the B cells in newborn piglets express IgM (92, 93) and 90% transcribe IgM (86). The epitope specificity of the primary and secondary IgM and IgG responses to the epitopes of the TI-2 and TD immunogens in colonized piglets is the same as for PAMP-treated piglets (Fig. 5). Therefore, PAMPs given to GF piglets mimic the pattern seen in colonized piglets, supporting the hypothesis that colonization-induced adaptive responses depend on the PAMP supplied by the gut flora. This is a supposition lacking experimental evidence because in this study PAMPs were given i.p. Providing PAMP by gavage is a second phase study, is fraught with logistical problems that are expensive to resolve, and financially irresponsible to address until there is clear evidence that PAMPs can awaken the immune system of the naive piglet.

Except for the secondary IgM response to FLU, colonized piglets respond poorly to the model TI-2 and TD Ag used (Figs. 2 and 3). This prompted an analysis of the immune response to the colonizing E. coli and its LPS. These studies show that colonized piglets also gave pronounced responses to the Ags of the colonizing E. coli G58-1 and LPS extracted from G58-1 that dwarfed the antihapten response (Fig. 5). Thus, Ab responses to these and other bacterial Ag presumably explain the much higher serum IgG and IgA levels of colonized piglets (Fig. 1) and especially their lower IgG-specific activity to FLU and TNP (Table II).
conflicts with that of Duchmann et al. (94) that responses to commensal bacteria are absent in normal individuals, raising the possibility that swine are different or that tolerance to normal gut flora does not become established in 5 wk. Antibacterial responses were not seen in GF piglets given only PAMPs (Fig. 6). This included no response to LPS even though the Cpg-B + LPS groups received 2 mg LPS/wk. The absence of Ab to LPS in piglets receiving it (Figs. 6 and 7) indicates it does not act as an immunogen unless generated from colonizing bacteria. Finally, the two immunogens generally behaved as described in mice and as we have shown previously for swine (2).

We have previously shown that nonimmunized, colonized piglets (2) make Abs to both TNP and FLU. We observed the same for those receiving Cpg (Fig. 7). We interpret this to mean that naive, Ag-specific B cells of the preimmune repertoire are stimulated to differentiate to plasma cells by either PAMPs or colonization. Although expansion of IgM-producing cells may be favored because of their higher frequency (92, 93), it cannot explain the increase in IgG-specific activity (Table II). It is well known that the preimmune repertoire encodes Abs that recognize many environmental and self-Ags and do so with high connectivity and promiscuity (95–97). Surprisingly, there was little response to PC, generally recognized as a germline-encoded specificity, but rather the response to TNP was pronounced. What seems peculiar is that few IgM B cells of the preimmune repertoire recognize the phenanthrinering structure of FLU unless they are IgG-bearing cells (Fig. 7). The apparent high frequency of TNP- or DNP-specific cells in the preimmune repertoire may partially explain why they have been popular epitopes for model immunogens and often recognized by plasmacytomas induced in mice and rats (98). This is supported by spectratypic analyses (Fig. 4). Because the B cell repertoire of GF piglets and fetuses is polyclonal (Ref. 71; Fig. 4), the higher specific activity of the secondary IgG response to TNP and FLU given Cpg-B + LPS (Table II) apparently results from the expansion of many and not just a few clones that can recognize these aromatic haptons. Of course, the polyclonal spectratype might simply reflect that few B cells are Ag specific, and their selection is dwarfed by the overall preimmune repertoire. The much lower IgG response of colonized piglets vs their PAMP-treated littermates (Figs. 2 and 3; Table II) may suggest that IgG responses to TNP and FLU are diluted by IgG responses to the Ags of colonizing bacteria. Alternatively, colonization may stimulate development of immune homeostasis that subsequently suppresses responses stimulated in GF animals by PAMPs alone.

Investigators accustomed to reviewing mouse studies may note the small number of animals used (Table I). This is an economic issue that did not comprise any of the major conclusions of the study. These include the observation that Cpg-B alone can substitute for colonization by causing differentiation of preimmune B cells to secreting Abs to TNP and TD Ags and that MDP augments the response to many of the same effects as gut colonization.

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**Disclosures**

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