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B cells do not take up bacterial DNA: An essential role for antigen in exposure of DNA to TLR9

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Running Title: B cell uptake of bacterial DNA depends on antigen.

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Abstract

Murine dendritic cells (DC) and macrophages respond to bacterial CpG DNA through TLR9. Although it is frequently assumed that bacterial DNA is a direct stimulus for B cells, published work does not reliably show responses of purified B cells. Here we show that purified splenic B cells did not respond to *E. coli* DNA with induction of CD86, despite readily responding to single stranded (ss) phosphodiester CpG oligodeoxynucleotides (ODN). This was due to a combination of weak responses to both long and double stranded (ds) DNA. B cell DNA uptake was greatly reduced with increasing DNA length. This contrasts with macrophages where DNA uptake and subsequent responses were enhanced with increasing DNA length. However, when DNA was physically linked to hen egg lysozyme (HEL), HEL-specific B cells showed efficient uptake of DNA, and limited proliferation in response to the HEL-DNA complex. We propose that, in the absence of other signals, B cells have poor uptake and responses to long dsDNA to prevent polyclonal activation. Conversely, when DNA is physically linked to a B cell receptor (BCR) ligand, its uptake is increased, allowing TLR9-dependent B cell activation in an antigen-specific manner. We could not generate fragments of *E. coli* DNA by limited DNaseI digestion that could mimic the stimulatory effect of ss CpG ODN on naïve B cells. We suggest that the frequently studied polyclonal B cell responses to CpG ODN are relevant to therapeutic applications of phosphorothioate-modified CpG-containing ODN, but not to natural responses to foreign or host ds DNA.

Introduction

Unmethylated CG dinucleotides (CpG motifs) in DNA are recognised as foreign, and stimulate innate immune responses through toll-like receptor 9 (TLR9).[1,2] Mouse B cells[1], macrophages[3] and dendritic cells (DCs)[4] are reported to respond to CpG DNA. TLR9 is a member of a subset of the TLR family (TLR3, TLR7, TLR8 and TLR9), which recognise foreign nucleic acids and are generally located on intracellular membrane compartments. If cells are to respond to extracellular DNA via intracellular TLR9, DNA uptake mechanisms are clearly required. Thus DNA uptake is a point at which TLR9 responses can be regulated or restricted. In myeloid cells, TLR9 localises to the endoplasmic reticulum and moves to meet DNA-containing endosomes.[5-8] In resting B cells, TLR9 colocalised with early endosomal markers.[9] Ligation of the B cell receptor (BCR) led to recruitment of TLR9 to an intracellular MHCII loading compartment, to which the BCR delivers antigen.[9] This facilitates the TLR9-mediated recognition of DNA within antigen complexes.

The role of mouse B cell TLR9 in promoting autoantibody production has been intensively studied.[10-12] BCR-mediated uptake of complexes containing DNA results in synergistic signalling involving both the BCR and TLR9, which promotes B cell proliferation.[10,12] Efficient cellular activation by antigen-TLR ligand complexes is thought to explain the fact that in the autoimmune condition systemic lupus erythematosus (SLE) many autoantibodies target DNA, or proteins which associate with DNA and RNA. The targeted delivery of DNA to TLR9 via BCR-mediated uptake may cause accumulation of enough poorly stimulatory self DNA to allow a TLR9 response. In addition, in SLE there can be elevated circulating interferon-α which further enhances responses to weak TLR9 agonists in B cells.[11]

Studies on TLR9 now predominantly use phosphorothioate-stabilised CpG ODN, and frequently assume that these synthetic single stranded ODN will mimic the biological effect of bacterial or viral DNA. The nuclease resistance of phosphorothioate ODN means that they are more potent *in vivo* than bacterial DNA,[13] and they also show higher cellular uptake than ODN with a natural backbone.[14] Furthermore, we found that the reported species differences in the optimal flanking sequences of the CpG motif required for TLR9-dependent responses actually only applies to phosphorothioate ODN.[15] Phosphorothioate ODN also show a degree of CpG-independent activation of B cells.[16,17] DNA structure, on the other hand, can also affect TLR9 responses. Increasing DNA length improved macrophage DNA uptake and subsequent responses.[18] Double stranded DNA was also more potent in macrophage activation.[18] Therefore, studies aiming to understand normal biological responses to CpG DNA should use naturally occurring DNA and not exclusively synthetic ODN.

Bacterial DNA has a much higher frequency of stimulatory CpG motifs than mammalian DNA,[19] and there is a common perception that bacterial DNA is a strong polyclonal B cell mitogen. Whilst, mouse naïve B cells are viewed as responding polyclonally to TLR9 ligands, human naïve B cells are proposed to have antigen-specific TLR9 responses[20] as their expression of TLR9 is low until they are stimulated through the BCR.[21,22] There is no doubt that synthetic phosphorothioate-stabilised CpG ODN are a powerful polyclonal stimulus for B cells,[23-25], but two reports using highly purified mouse B cells failed to find direct stimulation of proliferation and antibody production by bacterial DNA.[26,27] Another study suggested that human B cells were insufficiently sensitive to *E. coli* DNA for it to elicit polyclonal responses in a physiological setting.[28] Polyclonal B cell activation can occur during infections, but an overwhelming response is not to the benefit of the host. Work presented here demonstrates that natural DNA alone does

not contribute to polyclonal activation, because naïve B cells are not able to take up bacterial DNA. B cell TLR9 appears to be engaged by natural DNA only when the DNA is associated with antigen taken up through the BCR, and will therefore only enhance antigen-specific responses. Our work clarifies the common misconception that bacterial DNA is a polyclonal mitogen for mouse B cells, and provides insight into likely B cell TLR9 functions in the host response to pathogen challenge.

Results

WEHI231 cells respond to CpG ODN but not *E. coli* DNA.

Our previous work showed that in macrophages and dendritic cells, DNA uptake and subsequent response to CpG motifs increased with DNA length.[18] However, increasing the length of DNA did not enhance uptake in L929 fibroblasts or two B cell lines.[18] We therefore questioned whether B cells have efficient responses to long bacterial DNA. WEHI231 is an immature B cell line known to respond to CpG-containing ODN.[29,30] WEHI231 cells were treated with a 44 base CpG phosphodiester ODN or *E. coli* DNA for 24h and supernatants were assayed for production of IL-6. IL-6 production was readily detectable in response to 3μg/ml of the CpG-containing ODN LO, but unexpectedly, no IL-6 was detected from treatment with up to 50μg/ml *E. coli* DNA (Figure 1A). The two most obvious structural differences between ODN and bacterial DNA are length and strandedness. To assess the effect of strandedness, the stimulatory ODN LO was made ds. This completely prevented activity at concentrations up to 50μg/ml. Thus in this cell line, ds DNA is apparently non-stimulatory. However, strandedness is unlikely to be the only factor preventing responses to *E. coli* DNA, as boiled *E. coli* DNA was similarly inactive (Figure 1A). In contrast to these findings in WEHI231 cells, *E. coli* DNA and dsLO both readily stimulated IL-6 production from bone marrow-derived macrophages (BMM) as expected (Figure 1B). In BMM, similarly to the RAW264 cell line,[18] ds ODN were more potent than ss ODN, perhaps because of improved uptake[18] and stability.

Purified splenic B cells have limited responses to double-stranded and long DNA.

To confirm whether the non-responsiveness of WEHI231 cells to *E. coli* DNA was reflective of primary B cells, we purified murine splenic B cells. B cells were negatively selected by depleting all CD43 positive cells from mixed splenocytes, leaving a population of marginal zone and

follicular B cells that were more than 95% CD19 positive (Figure 2A). The response of B cells to

E. coli DNA and ODN was measured by upregulation of the co-stimulatory marker CD86. This

flow cytometric assessment allowed us to be sure that the population as a whole were responding,

whereas an ELISA assay for cytokine may detect activation of a subpopulation or a

contaminating population. In addition, upregulation of CD86 is likely to be a sensitive measure

for a response to CpG DNA, since the requirements for induction of B cell CD86 expression are

less stringent than those for proliferation; purified naïve B cells were reported to respond to CpG

ODN with induction of CD86, although they required collaborating signals such as CD40L or T

cell products in order to proliferate.[22,31] Similarly to WEHI231 cells, purified splenic B cells

responded to ss CpG ODN, but not *E. coli* DNA (Figures 2B and C). Responses to the ss ODN

occurred in a polyclonal manner, as can be seen from raw flow cytometry data (Figure 2B)

therefore data is presented as mean fluorescence intensity for CD86 staining for the entire

population. DNA strandedness and length were considered as explanations for the inactivity of *E.*

coli DNA on primary B cells.

Although the effect was not as absolute as in the WEHI231 cell line, double strandedness was

clearly unfavourable for activation of splenic B cells. A response to the ds phosphodiester ODN

1668 was only detected at 10μM (equivalent to 60μg/ml of active strand for 1668) (Figure 2D).

No activity of the GC inversion of 1668 was observed, as expected for responses mediated by

TLR9. The complementary sequence to 1668 (1668R), which does not contain a canonical CpG

motif (G/AACGTT), was similarly inactive. Apart from strandedness, length was also a factor

since the 22mer 1668 was much more potent than the 44mer LO in upregulating CD86 in splenic

B cells (Figure 2E). This was not due to suboptimal flanking sequences in LO, since in

macrophages the direct reverse was seen, with LO being dramatically more active than the

shorter 1668.[18] The poor B cell responses to the 44mer ODN LO declined still further when the ODN was made ds (Figure 2E). Thus, both double strandedness and DNA length are likely to contribute to the lack of activity of *E. coli* DNA on purified B cells. As a corollary of this, it could be argued that B cell responses to *E. coli* DNA should emerge if the DNA was sheared and denatured. The length of the DNA appeared to be critical as freshly boiled *E. coli* DNA was also inactive in primary B cells (Figure 2F). However even when DNA was cleaved to a size range of 20-250bp by limited DNaseI digestion (Supplementary Figure 1) and denatured, it was still unable to measurably activate primary B cells (Figure 2F). This is probably largely due to the limited range of DNA size to which B cells respond (i.e. effective responses to 22 base CpG ODN but poor responses once DNA reaches 44 bases shown in Figure 2E). DNaseI digestion of DNA, which introduces single strand nicks and double strand breaks, could not necessarily yield sufficient concentration of DNA within the appropriate size range. In addition, the frequency of CpG motifs in the degraded *E. coli* DNA is not so great as in the 1668 ODN, adding to the inefficiency of B cell recognition of CpG sequences within natural DNA.

Limited uptake of long DNA limits B cell responses.

A limiting step in macrophage responses to TLR9 ligands is DNA uptake, and increased uptake of long ODNs by BMM correlated with their greater potency.[18] We examined whether restrictions on DNA uptake were affecting the pattern of response of purified B cells. B cells were incubated with Cy3-labelled ODN for 1h and DNA uptake was measured by flow cytometry. B cell uptake of the shorter ODN 1668 was up to 4-fold greater than the uptake of LO (Figure 3A). Thus, the low uptake of longer ODNs would limit B cell responses to these agonists.

The possibility that poor uptake is responsible for the lack of activity of *E. coli* DNA in B cells was investigated by comparing the uptake of linearised plasmid DNA and short ODN in B cells and BMM. Plasmid DNA was used in order to have DNA of a defined length and degree of labelling per molecule, rather than using *E.coli* DNA which is partially sheared. B cell and BMM uptake of the short ODN 1668 was readily detectable. BMM uptake of ODN 1668 was linear with concentration up to 10μg/ml, consistent either with pinocytotic uptake, or uptake by a receptor which is either abundant or of low affinity, and therefore not saturated at the concentrations used (Figure 3B). Consistent with our previous results in macrophages showing length-dependent uptake,[18] plasmid DNA accumulated efficiently in BMM, but uptake was barely detectable in primary B cells (Figure 3C). In B cells the major route for taking up foreign material is via the BCR. Previous studies have suggested that optimal B cell activation by CpG DNA requires linkage to antigen.[10,23] We next determined whether in our hands DNA conjugation to antigen was a requirement for B cell responses to plasmid DNA.

B cell responses to DNA:antigen complex

Complexes were prepared by biotin-labelling both HEL and plasmid DNA, with addition of neutravidin. HEL was labelled with approximately one biotin per molecule, and plasmid with an estimated 30 biotin residues. Figure 4A shows the formation of complexes assessed by agarose gel electrophoresis, when neutravidin and HEL were bound in increasing amounts to plasmid DNA. The fastest migrating supercoiled plasmid form was retarded upon formation of the protein-DNA complex, and some formation of much larger complexes is indicated by increasing amounts of plasmid in the well. Mixing of DNA and HEL without addition of neutravidin caused no alteration in DNA migration.

We sought to confirm that a complex of DNA and antigen gives efficient B cell proliferation using our prepared complexes. A pure population of HEL-specific splenic B cells was isolated from $SW_{HEL}RAG2^{-/-}$ mice and labelled with CFSE. Cells were stimulated with DNA:HEL complex or DNA mixed with HEL at a molar ratio of 1:10, and harvested after 2, 3 and 4 days of culture to examine cell division by dilution of CFSE label. Cells which did not stain with propidium iodide were selected as the viable population for analysis of proliferation (Supplementary Figure 2). There was no division of cells treated with HEL alone, HEL:neutravidin complex, DNA alone, DNA:neutravidin complex or HEL mixed with DNaseI-digested DNA to control for impurities (data not shown). At day 2 of treatment with DNA mixed with HEL there was a slight shoulder on the CFSE profile, showing very limited replication (Figure 4B). Cells treated with the DNA:HEL complex showed a clearer indication of a population of cells which had undergone one round of division at day 2 (Figure 4C). Only a minority of cells survived and responded to these treatments, although the majority of the cells proliferated in response to the phosphorothioate-stabilised CpG ODN 1668S (Figure 4D and Supplementary Figure 2). By day 3, clear proliferation could be seen in the small number of surviving cells in the sample treated with DNA:HEL complex (Figure 4E), but insignificant numbers of cells treated with the DNA+HEL mixture survived (Supplementary Figure 2). These results contrast with the robust replication and expansion of cell numbers seen when cells were exposed to the ODN 1668S (Figure 4E and Supplementary Figure 2). In summary, there was clear evidence for proliferation of a limited number of cells exposed to DNA:HEL complex, but this was not sustained and most cells were dead after 4 days.

The relatively poor ability of the HEL:DNA complex to sustain survival and proliferation was surprising given the potent response to a single agent, the phosphorothioate CpG ODN. We

considered whether the biotinylation of the plasmid DNA could affect its ability to stimulate TLR9. The activities of plasmid and biotinylated plasmid were compared using a macrophage cell line with stably integrated NF-κB-dependent reporter.[19] Supplementary Figure 3 shows that biotinylation did not affect TLR9 recognition of plasmid CpG motifs. DNase digestion confirmed that the macrophage response was due to plasmid DNA and not a contaminant.

B cells take up DNA in complex with antigen

No previous work has assessed the impact of linking antigen to bacterial DNA on its cellular uptake. We examined this using complexes prepared with plasmid labelled with both biotin and Cy3 (Figure 4A). HEL-specific splenic B cells were exposed to a fixed amount of labelled plasmid, with increasing amounts of HEL either directly linked to the plasmid, or just mixed with the plasmid. The uptake of DNA-HEL complexes into primary HEL-specific B cells was strongly dependent on the number of HEL molecules linked to each plasmid molecule (Figure 5). A large improvement in uptake was seen when the ratio of DNA to HEL was increased from 1:1 to 1:3, and uptake reached a maximum at a ratio of 1:20. The uptake of DNA alone or DNA linked to neutravidin at a 1:30 ratio ("D:N") was undetectable. Unexpectedly, mixing HEL and DNA, without addition of neutravidin to link the two, gave low but detectable DNA uptake, correlating with the small amount of proliferation seen under these treatment conditions (Figure 4B). This low level of uptake suggests either that BCR ligation and signalling induces a mechanism allowing limited uptake of long DNA, or that HEL and DNA interact independently of the biotin-dependent association. As a control, B cells were also isolated from C57BL/6 mice, and these showed no uptake of HEL:DNA complexes. Uptake of Cy3-labelled 1668 ODN is shown to confirm that the C57BL/6 B cells were viable. This data suggests that murine B cells only

internalise long ds DNA if it is presented in combination with an antigen that can engage the BCR.

Discussion

DNA or DNA-containing complexes must undergo endocytic uptake in order to activate TLR9, since this receptor is localized in endosomal compartments. Expression of the costimulatory marker CD86 on purified primary follicular and marginal zone B cells was induced by phosphodiester CpG ODN but not *E. coli* DNA (Figure 2C). This was explained by lack of appreciable uptake of long DNA molecules. These results contrast with the widespread perception that B cells respond polyclonally to bacterial DNA. The ability of B cells to take up oligonucleotides but not long DNA molecules contrasts with macrophages, where long DNA is taken up more efficiently and is a more potent stimulus.[18] However, plasmid DNA was internalised by B cells when complexed with an antigen recognized by the BCR. BCR-mediated uptake of antigen:DNA complexes evidently overcomes the limitations on DNA length for uptake; B cells can take up particles up to at least 0.5μm diameter linked to antigen.[32] Long foreign DNA within DNA-antigen complexes is likely to enhance activation of B cells in an antigen-specific manner, and have no non-specific polyclonal effect. The polyclonal activation of B cells widely reported with phosphorothioate-modified CpG ODN may have no relevance to the normal B cell response to infection.

In parallel with the greatly improved uptake of DNA when complexed to HEL, we found that such complexes were able to elicit proliferation of HEL-specific B cells, whereas DNA or HEL alone had no effect. Similar results have been seen with HEL and CpG ODN bound to beads, where there was no uptake or response unless HEL and CpG ODN were bound on the same

beads.[32] Furthermore, Uccellini *et al.*[11] found that with physiologically relevant concentrations of DNA, bacterial and other DNAs longer than 50bp only promoted B cell proliferation when physically linked to an antigen recognised by the BCR. Our work has shown that this is explained by the lack of uptake of uncomplexed long bacterial DNA. The same study[11] showed a minor amount of proliferation in B cells from C57BL/6 mice in response to *E. coli* DNA alone. This was attributed to the low but finite frequency of cells in the normal repertoire that possess BCRs directly recognising DNA, since it was not seen in B cells with transgenic BCR[11]. A number of previous reports have shown mouse B cell proliferation in response to bacterial DNA,[1,33-35] and some may have similarly been measuring responses of cells with DNA-specific BCRs. Most studies have also been done on unpurified or partially purified splenocytes that may contain other B cell subsets with greater responses to bacterial DNA. Alternatively, bacterial DNA may trigger the production of cell surface or secreted molecules from other cell types, which then directly activate B cells or alter the threshold for B cell activation. A previous study showed production of IgM by mixed splenocytes but not purified B cells in response to bacterial DNA, suggesting a role for paracrine factors.[36] A further complication is the lack of control for purity of bacterial DNA in some studies.

The importance of BCR-mediated uptake of DNA-containing complexes and delivery of DNA to TLR9 has been studied extensively in the context of SLE,[10-12,23] and the requirement for TLR9 in these responses has been established.[11,23] This process is viewed as a way that chromatin debris, with a low frequency of CpG motifs, can accumulate to sufficient levels to stimulate TLR9 and contribute to autoantibody production. The lack of general B cell uptake of purified DNA we observed here has not been previously reported. It suggests that BCR-mediated antigen-DNA uptake is not only required for B cell responses to poorly stimulatory self DNA but is also

necessary for effective responses to a strong TLR9 ligand, bacterial DNA. *In vivo* other routes may also contribute to enhanced B cell DNA uptake and responses. Recent work found that complex formation between DNA and the cationic antimicrobial peptide LL-37 increased B cell responses to *E. coli* DNA.[37]

We observed that coadministration of unlinked HEL and DNA increased B cell uptake of long DNA, but not to the same extent as HEL linked to DNA. One explanation for our observations is that HEL associates with DNA and mediates DNA uptake. HEL is a basic protein with a pI of 10.7, and an ability to bind DNA under physiological conditions.[38] Our findings suggest that the DNA-binding ability of HEL should be taken into account in studies investigating requirements for B cell activation with HEL-specific BCR. This is especially relevant since even self DNA from spent culture medium has been shown to contribute to activation of B cells via TLR9.[10] Another possibility is that BCR ligation stimulates the cell to increase DNA uptake. Published work showed no effect of BCR ligation on the uptake of FITC-dextran,[39] suggesting that an increase in non-specific pinocytosis is unlikely to explain the effect of HEL on DNA uptake. However, a recent study has shown that stimulation of B cells with a protein immune complex recognised through the BCR acted synergistically with unlinked CpG DNA of 600bp to promote some B cell proliferation.[40] Hence it is possible that BCR ligation can specifically enhance DNA uptake, in order to allow B cells that have recognised antigen to actively survey their environment for PAMPs.

Despite efficient uptake of the HEL-DNA complex, HEL-specific B cells proliferated in only a limited fashion in response to the plasmid-containing complex. A small proportion of cells formed B cell blasts, and these lost viability by the fourth day of treatment. It is possible that

linkage of HEL to a whole bacterial genome would give a more potent stimulus due to uptake of a greater total length of DNA. Treatment with a phosphorothioate CpG ODN alone gave a more robust proliferative response, but these cells also started losing viability by Day 4. This is consistent with earlier work showing the proliferative response to CpG ODN was not so sustained as the response to other polyclonal activators.[41] Experiments *in vivo* have shown that particulate complexes of antigen and CpG ODN elicited a short lived response with differentiation of B cells to extrafollicular plasma cells peaking at day 3 after stimulation.[32] The full proliferation and differentiation of human naïve B cells is proposed to require three signals - BCR ligation, T cell help and TLR stimulation.[42] With BCR ligation and either TLR stimulation or T cell help, cells initiated proliferation but viability was not maintained.[42] It is likely that inputs from a number of sources such as antigen avidity, TLR ligation, T cell help, and cytokine environment can all contribute to pushing B cells over the activation threshold. The *in vivo* contribution of T cell-independent B cell activation via BCR and TLR signalling to generation of autoantibodies or protective antibodies early in infection remains to be fully established, but some work shows that generation of extrafollicular autoantibody producing cells required TLR stimulation but did not require T cells.[43]

The most common TLR9 ligands used in the literature are phosphorothioate-modified ODN. These are powerful stimuli, and their study is relevant to therapeutic concerns. However results obtained with these stabilised ODN, and even with phosphodiester ODN, cannot be inferred to reflect responses to natural DNA. In an infection, B cells are likely to encounter DNA either as long molecules within viruses, or as a complex with bacterial proteins, released from damaged bacteria. Antigenic particulate material is presented to B cells on the surface of macrophages lining the subcapsular sinus in the lymph nodes.[44,45] Some degradation of bacterial DNA in the

extracellular environment via DNaseI could be expected. Here we used limited DNaseI digestion with or without denaturation, in order to see if degraded bacterial DNA would be able to activate B cells like ODN. We were unable to detect any effect of the partially degraded DNA, probably due to the very limited DNA size range taken up by B cells and the lower frequency of CpG motifs in bacterial DNA than in CpG ODN. Degradation of DNA allowing autonomous B cell responses to free bacterial DNA is thus unlikely to occur *in vivo*. We therefore question the biological relevance of responses to short single stranded phosphorothioate-modified ODN which are powerful B cell activators in the absence of other stimuli. In addition, we observed that B cell responses were not efficiently elicited by dsDNA ODN (Figure 2). Many workers routinely boil bacterial DNA for eliciting TLR9 responses, but there is unlikely to be a mechanism *in vivo* which would cause large scale denaturation of extracellular foreign DNA.

This work has clearly shown that TLR9 responses in B cells are critically controlled by low uptake of long DNA. This barrier can be overcome if DNA is part of a complex recognised by the BCR. Thus in natural infections, foreign DNA may promote antibody responses to closely associated foreign antigens. The often-studied phenomenon of B cell activation by short CpG ODN may have little biological relevance, except in the context of therapeutic applications.

Methods

Cell Culture

RPMI 1640 containing 10% FCS (Gibco), 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin was used for culture of primary macrophages. FCS was screened for low basal activation of macrophages and optimal CpG DNA responses. Primary B cells were grown in the above medium with addition of 10mM HEPES buffer, 0.1mM non-essential amino acids, 1mM sodium pyruvate and 50μM 2-mercaptoethanol. WEHI231 culture medium also contained 10mM HEPES buffer, 1mM Sodium Pyruvate and 50μM 2-mercaptoethanol. WEHI231 cells were a gift from David Tarlinton (Walter and Eliza Hall Institute, Melbourne, Australia). Bone marrow-derived macrophages (BMM) were differentiated from the bone marrow of C57BL/6 mice as described previously.[46] B cells were obtained from spleens of C57BL/6 mice or $SW_{HEL}RAG2^{-/-}$ mice. SW_{HEL} mice, which express a HEL-specific BCR[47] were originally provided by Robert Brink (Garvan Institute of Medical Research, Sydney). These were crossed with $RAG2^{-/-}$ mice to provide pure populations of B cells with HEL-specific BCR. All animal work was approved by University of Queensland or WEHI animal ethics committees and carried out according to institutional guidelines. Splenocytes were isolated as described previously.[18] For DNA uptake studies, B cells were purified from splenocytes by negative selection using an antibody against CD43. Briefly, red blood cells were lysed in lysis buffer (150mM ammonium chloride, 10mM potassium hydrogen carbonate and 1mM disodium EDTA) and remaining cells incubated with CD43 MACS microbeads (Miltenyi Biotech) and then passed twice over an LD separation column. B cell purity was 95-98% as determined by CD19 staining. CD43- cells contain marginal zone and follicular B cells but not B1 or plasma cells[48] or a sub-population of memory B cells.[49] For B cell proliferation studies, cells were isolated by MACS with a preliminary percoll gradient step to enrich for small resting follicular B cells, as described.[41]

DNA and ODN

The following ODN were synthesized with a phosphodiester backbone and were purchased from Geneworks (Adelaide, Australia).

1668 5′-TCCATGACGTTCCTGATGCT-3′,[50] 1668R is complementary to 1668,

LO 5′-GCCTTAGGGACTAGCTCATAACGTTCCTGATGCTGTAGAGTAGG-3′, 1668-GC 5′-TCCATGAGCTTCCTGATGCT-3′. 1668S ODN has the same sequence as 1668, but has a phosphorothioate-modified backbone. Where use of dsODN is noted, ODN were made ds by addition of a complementary strand in PBS, heating at 60°C for 5min, followed by slow cooling to room temperature. *E. coli* strain B DNA (Sigma) was purified using phenol chloroform extraction and ethanol precipitation followed by four extractions using Triton-X114 to remove LPS, phenol chloroform extraction and ethanol precipitation as described previously.[19] Purity of DNA was confirmed by lack of macrophage stimulatory activity of DNaseI-digested DNA (Supplementary Figure 3). pBluescriptSK+ plasmid DNA was prepared using a Qiagen Endo-free maxiprep kit. Lack of LPS contamination of DNA was confirmed by examining stimulation of the NF-κB responsive reporter gene in ELAM9 cells,[19] by complete DNaseI-digests of DNA samples. For denatured *E. coli* DNA, samples were placed on a 100°C heating block for 10min and then placed directly on ice before addition to cells. Small sized fragments of *E. coli* DNA were generated by limited digestion with DNaseI, then purified by phenol-chloroform extraction and ethanol precipitation (Supplementary Figure 1). Plasmid and genomic DNA from *E.coli* lacks the CpG methylation which interferes with TLR9 recognition. The two major bacterial methylases are Dam, which methylates adenine in GATC sequences and Dcm which methylates the second cytosine in CCA/TGG sequences, and these do not affect TLR9-mediated responses.[18]

DNA Uptake

The ODNs 1668 and LO were synthesized with a 5′ Cy3 label (Geneworks, Adelaide, Australia). Plasmid pBluescript SK+ was prepared by Qiagen endo-free maxiprep kit (Qiagen, GmbH, Germany). Plasmid was linearised with endonuclease EcoRV and purified by Triton X-114 extraction as for *E. coli* DNA above. Linearised plasmid was labeled with Cy3 using a label IT tracker kit (Mirus, Madison, WI, USA). Efficiency of labeling of ODN and plasmid was estimated by measuring A260 and A550 and using a molar absorption coefficient of 150 000 for Cy3 at 550nm. 200 000 B cells/well were plated in 24 well plates in 500µl of complete medium, treated with various concentrations of labeled DNAs for the indicated times then harvested and washed with PBA (PBS + 0.1% sodium azide and 0.1% BSA). Cells were then incubated with 100µg/ml DNaseI (Sigma) for 10 min at room temperature to remove any DNA bound to the cell surface. Cells were washed with PBA and then analysed by flow cytometry (FACS-calibur or FACScanto, Becton Dickinson). Results were corrected for a small difference in Cy3 labeling efficiency for different ODN.

ELISA

$1x10^6$ B cells/well were plated in 500µl in 24-well plates and treated with DNA for 24h. ELISA was performed using paired antibodies against mouse IL-6 (BD PharMingen) as described previously.[51]

Cell surface marker staining

150 000 B cells/well were plated in 200µl in 48-well plates and treated with DNA for 24h. Cells were harvested, washed in PBS, blocked in 1% FCS/PBA and then stained with either rat anti-mCD19 (BD PharMingen), rat anti-mCD86 (eBioscience) or matching isotype controls for 1h.

Cells were then washed twice with PBA and then analysed by flow cytometry with either a BD FacsCalibur or BD LSRII. These instruments have different dynamic ranges which affect the numerical output for fluorescence levels, but not the relative fluorescence levels between samples. Mean fluorescence intensity (MFI) is expressed in arbitrary units and absolute values are of no consequence. Analysis was performed using CellQuest (Version 3.1) (Becton Dickinson).

Preparation of HEL-DNA complexes

Complexes were prepared by covalent linkage of biotin to both HEL and DNA, and addition of neutravidin. For uptake studies, pBluescriptSK+ (pBS) was labeled with both biotin and Cy3 using labelIT kits (Mirus). The extent of Cy3 labelling was assessed by measuring A550, and determined to be approximately 33 labels per plasmid. A similar amount of labeling by biotin was anticipated from use of equivalent amounts of labelling reagent. For proliferation studies, pBS was labeled only with biotin, at an estimated 20 labels per plasmid. TLR9 stimulatory activity of the biotinylated DNA was confirmed by using the NF-κB dependent reporter cell line ELAM9 as described previously.[19] HEL was labeled with biotin using amine-reactive N-hydroxysuccinimide biotin (Sigma), at a molar ratio of HEL:NHS-biotin of 1:1.7. The labelling was done for 3h in 50mM MES, pH6 to give preferential modification of the N terminal amine. HEL was determined to be more than 95% labeled, as determined by pull-down on streptavidin-sepharose. Trial binding reactions using neutravidin (Pierce), which should have four biotin-binding subunits, showed a maximum of 2-3 active binding sites per neutravidin tetramer. For preparation of complexes, biotin-pBS was mixed with biotin-HEL at various molar ratios in PBS with 0.5mg/ml BSA, prior to addition of neutravidin, which was added equimolar with the HEL. For uptake studies, the molar ratios of DNA:neutravidin:HEL ranged from 1:1:1 to 1:30:30. For

B cell proliferation experiments, the molar ratio of DNA:neutravidin:HEL was 1:10:10. Control reactions omitting either one or two components of the complex were also prepared.

Analysis of B cell proliferation

Follicular B cells were isolated from $SW_{HEL}RAG2^{-/-}$ mice and stained with 10μM CFSE for 10min at 37°C. Cells were plated in 96 well plates at 20 000 cells per well, and incubated with HEL-DNA complexes or control 1668S for four days. Except for the 1668S-treated sample, each day 75% of the medium was replaced to ensure results were not compromised by DNA degradation. After 2, 3 and 4 days incubation, cells were harvested and stained with propidium iodide for flow cytometric analysis. Viable cells were gated for analysis of CFSE dilution.

Acknowledgments

Supplementary Information

Supplementary information is available at the Immunology and Cell Biology website.

References

1   Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R *et al.* CpG Motifs in Bacterial-DNA Trigger Direct B-Cell Activation. Nature 1995; 374: 546-49.

2   Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H *et al.* A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408: 740-45.

3   Stacey KJ, Sweet MJ, Hume DA. Macrophages ingest and are activated by bacterial DNA. J. Immunol. 1996; 157: 2116-22.

4   Sparwasser T, Koch ES, Vabulas RM, Heeg K, Lipford GB, Ellwart JW *et al.* Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur J Immunol 1998; 28: 2045-54.

5   Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF *et al.* TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol 2004; 5: 190-8.

6   Leifer CA, Brooks JC, Hoelzer K, Lopez J, Kennedy MN, Mazzoni A *et al.* Cytoplasmic targeting motifs control localization of toll-like receptor 9. The Journal of biological chemistry 2006; 281: 35585-92.

7   Brinkmann MM, Spooner E, Hoebe K, Beutler B, Ploegh HL, Kim YM. The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. The Journal of cell biology 2007; 177: 265-75.

8   Ivanov S, Dragoi AM, Wang X, Dallacosta C, Louten J, Musco G *et al.* A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. Blood 2007.

9   Chaturvedi A, Dorward D, Pierce SK. The B cell receptor governs the subcellular location of Toll-like receptor 9 leading to hyperresponses to DNA-containing antigens. Immunity 2008; 28: 799-809.

10 Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature 2002; 416: 603-7.

11 Uccellini MB, Busconi L, Green NM, Busto P, Christensen SR, Shlomchik MJ *et al.* Autoreactive B cells discriminate CpG-rich and CpG-poor DNA and this response is modulated by IFN-alpha. J Immunol 2008; 181: 5875-84.

12 Viglianti GA, Lau CM, Hanley TM, Miko BA, Shlomchik MJ, Marshak-Rothstein A. Activation of autoreactive B cells by CpG dsDNA. Immunity 2003; 19: 837-47.

13 Stacey KJ, Blackwell JM. Immunostimulatory DNA as an adjuvant in vaccination against Leishmania major. Infect Immun 1999; 67: 3719-26.

14 Sester DP, Naik S, Beasley SJ, Hume DA, Stacey KJ. Phosphorothioate backbone modification modulates macrophage activation by CpG DNA. J Immunol 2000; 165: 4165-73.

15 Roberts TL, Sweet MJ, Hume DA, Stacey KJ. Cutting edge: species-specific TLR9-mediated recognition of CpG and non-CpG phosphorothioate-modified oligonucleotides. J Immunol 2005; 174: 605-8.

16 Monteith DK, Henry SP, Howard RB, Flournoy S, Levin AA, Bennett CF *et al.* Immune stimulation--a class effect of phosphorothioate oligodeoxynucleotides in rodents. Anticancer Drug Des 1997; 12: 421-32.

17 Liang H, Nishioka Y, Reich CF, Pisetsky DS, Lipsky PE. Activation of human B cells by phosphorothioate oligodeoxynucleotides. J Clin Invest 1996; 98: 1119-29.

18 Roberts TL, Dunn JA, Terry TD, Jennings MP, Hume DA, Sweet MJ *et al.* Differences in macrophage activation by bacterial DNA and CpG-containing oligonucleotides. J Immunol 2005; 175: 3569-76.

19   Stacey KJ, Young GR, Clark F, Sester DP, Roberts TL, Naik S *et al.* The molecular basis for the lack of immunostimulatory activity of vertebrate DNA. J Immunol 2003; 170: 3614-20.

20   Lanzavecchia A, Sallusto F. Toll-like receptors and innate immunity in B-cell activation and antibody responses. Curr Opin Immunol 2007; 19: 268-74.

21   Bourke E, Bosisio D, Golay J, Polentarutti N, Mantovani A. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood 2003; 102: 956-63.

22   Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood 2003; 101: 4500-4.

23   Busconi L, Bauer JW, Tumang JR, Laws A, Perkins-Mesires K, Tabor AS *et al.* Functional outcome of B cell activation by chromatin immune complex engagement of the B cell receptor and TLR9. J Immunol 2007; 179: 7397-405.

24   Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. J. Immunol. 2000; 164: 944-52.

25   Goeckeritz BE, Flora M, Witherspoon K, Vos Q, Lees A, Dennis GJ *et al.* Multivalent cross-linking of membrane Ig sensitizes murine B cells to a broader spectrum of CpG-containing oligodeoxynucleotide motifs, including their methylated counterparts, for stimulation of proliferation and Ig secretion. Int. Immunol. 1999; 11: 1693-700.

26   Brummel R, Lenert P. Activation of marginal zone B cells from lupus mice with type A(D) CpG-oligodeoxynucleotides. J Immunol 2005; 174: 2429-34.

27   Spies B, Hochrein H, Vabulas M, Huster K, Busch DH, Schmitz F *et al.* Vaccination with plasmid DNA activates dendritic cells via toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. J Immunol 2003; 171: 5908-12.

28   Wagner M, Poeck H, Jahrsdoerfer B, Rothenfusser S, Prell D, Bohle B *et al.* IL-12p70-dependent Th1 induction by human B cells requires combined activation with CD40 ligand and CpG DNA. J Immunol 2004; 172: 954-63.

29   Macfarlane DE, Manzel L, Krieg AM. Unmethylated CpG-containing oligodeoxynucleotides inhibit apoptosis in WEHI-231 B lymphocytes induced by several agents: evidence for blockade of apoptosis at a distal signalling step. Immunology 1997; 91: 586-93.

30   Yi AK, Krieg AM. CpG DNA rescue from anti-IgM-induced WEHI-231 B lymphoma apoptosis via modulation of I kappa B alpha and I kappa B beta and sustained activation of nuclear factor-kappa B/c-Rel. J Immunol 1998; 160: 1240-5.

31   Gantner F, Hermann P, Nakashima K, Matsukawa S, Sakai K, Bacon KB. CD40-dependent and -independent activation of human tonsil B cells by CpG oligodeoxynucleotides. Eur J Immunol 2003; 33: 1576-85.

32   Eckl-Dorna J, Batista FD. BCR-mediated uptake of antigen linked to TLR9 ligand stimulates B-cell proliferation and antigen-specific plasma cell formation. Blood 2009; 113: 3969-77.

33   Messina JP, Gilkeson GS, Pisetsky DS. Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. J Immunol 1991; 147: 1759-64.

34   Messina JP, Gilkeson GS, Pisetsky DS. The influence of DNA structure on the in vitro stimulation of murine lymphocytes by natural and synthetic polynucleotide antigens. Cell Immunol 1993; 147: 148-57.

35   Yi AK, Chace JH, Cowdery JS, Krieg AM. IFN-gamma promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides. J Immunol 1996; 156: 558-64.

36   Brummel R, Roberts TL, Stacey KJ, Lenert P. Higher-order CpG-DNA stimulation reveals distinct activation requirements for marginal zone and follicular B cells in lupus mice. Eur J Immunol 2006; 36: 1951-62.

37   Hurtado P, Peh CA. LL-37 promotes rapid sensing of CpG oligodeoxynucleotides by B lymphocytes and plasmacytoid dendritic cells. J Immunol 2010; 184: 1425-35.

38   Lin KC, Wey MT, Kan LS, Shiuan D. Characterization of the interactions of lysozyme with DNA by surface plasmon resonance and circular dichroism spectroscopy. Appl Biochem Biotechnol 2009; 158: 631-41.

39   McGovern EM, Moquin AE, Caballero A, Drake JR. The effect of B cell receptor signaling on antigen endocytosis and processing. Immunol Invest 2004; 33: 143-56.

40   Avalos AM, Latz E, Mousseau B, Christensen SR, Shlomchik MJ, Lund F *et al.* Differential cytokine production and bystander activation of autoreactive B cells in response to CpG-A and CpG-B oligonucleotides. J Immunol 2009; 183: 6262-8.

41   Turner ML, Hawkins ED, Hodgkin PD. Quantitative regulation of B cell division destiny by signal strength. J Immunol 2008; 181: 374-82.

42   Ruprecht CR, Lanzavecchia A. Toll-like receptor stimulation as a third signal required for activation of human naive B cells. Eur J Immunol 2006; 36: 810-6.

43   Herlands RA, Christensen SR, Sweet RA, Hershberg U, Shlomchik MJ. T cell-independent and toll-like receptor-dependent antigen-driven activation of autoreactive B cells. Immunity 2008; 29: 249-60.

44   Carrasco YR, Batista FD. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. Immunity 2007; 27: 160-71.

45   Junt T, Moseman EA, Iannacone M, Massberg S, Lang PA, Boes M *et al.* Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. Nature 2007; 450: 110-4.

46   Costelloe EO, Stacey KJ, Antalis TM, Hume DA. Regulation of the plasminogen activator inhibitor-2 (PAI-2) gene in murine macrophages. Demonstration of a novel pattern of responsiveness to bacterial endotoxin. Journal of leukocyte biology 1999; 66: 172-82.

47   Phan TG, Amesbury M, Gardam S, Crosbie J, Hasbold J, Hodgkin PD *et al.* B cell receptor-independent stimuli trigger immunoglobulin (Ig) class switch recombination and production of IgG autoantibodies by anergic self-reactive B cells. J Exp Med 2003; 197: 845-60.

48   Wells SM, Kantor AB, Stall AM. CD43 (S7) expression identifies peripheral B cell subsets. J Immunol 1994; 153: 5503-15.

49   Driver DJ, McHeyzer-Williams LJ, Cool M, Stetson DB, McHeyzer-Williams MG. Development and maintenance of a B220- memory B cell compartment. J Immunol 2001; 167: 1393-405.

50   Yi AK, Hornbeck P, Lafrenz DE, Krieg AM. CpG DNA rescue of murine B lymphoma cells from anti-IgM-induced growth arrest and programmed cell death is associated with increased expression of c-myc and bcl-xL. J Immunol 1996; 157: 4918-25.

51   Sweet MJ, Leung BP, Kang D, Sogaard M, Schulz K, Trajkovic V *et al.* A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. J Immunol 2001; 166: 6633-9.

Figure Legends

Figure 1. The B cell line WEHI231 does not respond to long or ds CpG DNA. WEHI231 cells (A) or BMMs (B) were treated with indicated concentrations of either *E. coli* DNA, boiled *E. coli* DNA, ss CpG ODN (LO), or ds CpG ODN (dsLO) for 24h and then supernatants were assayed by ELISA for IL-6 production. Results are the average of duplicate treatments and error bars show the range of the duplicates. Results are representative of 2-3 independent experiments.

Figure 2. Purified splenic B cells do not respond to *E. coli* DNA. **A.** Purity of negatively selected B cells as determined by CD19 expression. Isotype control staining – filled histogram, CD19 staining - thick line. **B-F.** Purified B cells were incubated with indicated concentrations of DNA for 24h. Cells were then harvested, stained for the presence of cell surface marker CD86 and analysed by flow cytometry. MFI indicates mean fluorescence intensity of cells stained for CD86. The higher baseline values for MFI in panel F are of no consequence, and are due to use of a flow cytometer with different dynamic range (see methods). Relative changes in MFI are similar between instruments. In C-F, results show the average of duplicate treatments. Error bars, which may fall within the size of the symbol, indicate the range of the values within the experiment. Results are representative of three to four independent experiments for C-E and two experiments for F. **B.** B cells respond to 22 base ODN 1668, but not *E. coli* DNA. Overlays of flow cytometry data, filled histogram – stained untreated control, grey line – 50µg/ml *E. coli* DNA, black line – 10µM 1668. **C.** Quantification of response to 22 base ODN 1668 and *E. coli* DNA. **D.** Effect of double strandedness on response to 1668. Cells were incubated with either 1668, ds1668, the complement of 1668 (1668R) or the GC inversion of 1668 (1668-GC). **E.** Short ODN are more immunostimulatory on B cells than long ODN. Cells were treated with 22 base CpG ODN (1668), 44 base CpG ODN (LO), ds 44 base CpG ODN (dsLO) or the GC inversion of 1668

(1668-GC). **F.** Shearing with DNaseI (sEC), boiling (bEC) or both treatments (bsEC) of *E. coli* (EC) DNA does not make it stimulatory on primary B cells. DNA was treated with 2 different concentrations of DNaseI (0.02 or 0.03 U/µl) for 20 minutes, and the degree of digestion is shown in Supplementary Figure 1.

Figure 3. B cells have poor uptake of long DNA. Uptake of Cy3-labelled DNA was analysed by flow cytometry. DNA uptake was uniform and dose-dependent in the populations and is expressed as mean fluorescence intensity (MFI). **A.** B cells were treated with the indicated concentration of Cy3-labelled ODNs, either 22 bases (1668) or 44 bases (LO) for 1h. Results were adjusted to account for slightly different efficiencies of labelling of ODN. **B.** BMM and purified B cells were treated with the indicated concentrations of Cy3-1668 for 2h. **C.** BMM and B cells were treated with the indicated concentrations of Cy3-linearised plasmid for 2h. Results are the average of duplicate treatments and the error bars indicate the range of the values within the experiment. Results are representative of three (A) or four experiments (B and C).

Figure 4. Antigen-DNA complexes induce limited proliferation of B cells. **A.** The formation of Cy3 fluorescently labelled plasmid DNA-HEL complexes was assessed by the effect on plasmid migration in an agarose gel. Samples were DNA alone (D), DNA mixed with HEL at 1:30 molar ratio (D+H), or DNA-neutravidin-HEL conjugate (D:H). The ratios of DNA to HEL are indicated, and neutravidin was added in equimolar amounts to HEL in order to allow complex formation. The arrow indicates the supercoiled plasmid band which shows retarded migration with increasing amounts of bound HEL. **B-E.** Proliferation of HEL-specific B cells in response to HEL:DNA complex was measured by CFSE dilution. Cells were left untreated, or stimulated with either HEL mixed with DNA (HEL + DNA), HEL-DNA conjugate or 1µM 1668S. The

HEL-DNA conjugate was prepared at a molar ratio of DNA:neutravidin:HEL of 1:10:10. The final concentration of HEL in incubations was 33ng/ml, and DNA was 0.45μg/ml. DNA was not Cy3-labelled in these experiments. Results shown are CFSE dilution within the viable cell population analysed by flow cytometry, and represent all the treatments where there was any degree of proliferation. **B.** Effect of HEL mixed with DNA after 2 days incubation. **C.** Effect of HEL-DNA conjugate after 2 days incubation. **D.** Effect of 1668S after 2 days incubation. **E.** Effect of HEL-DNA conjugate and 1668S after 3 days incubation, compared to untreated sample from day 2.

Figure 5. Linking plasmid DNA to antigen allows B cell DNA uptake. Uptake of HEL-DNA complexes by HEL-specific B cells. Cells were incubated for 1.75 hours with HEL-neutravidin (H:N), DNA alone (D), DNA-neutravidin (D:N) or the DNA-HEL conjugate (D:H) or DNA mixed with HEL (D+H) at various DNA:HEL molar ratios as indicated. The concentration of plasmid DNA in all incubations was 0.45 μg/ml. Cy3-labelled 1668 phosphodiester ODN (CpG) was used at 3μM as a control. Cy3-DNA uptake was measured by flow cytometry, and expressed as mean fluorescence intensity (MFI). Bars indicate the average of duplicate treatments and the error bars show the range of the values. Data is representative of 3 separate experiments.
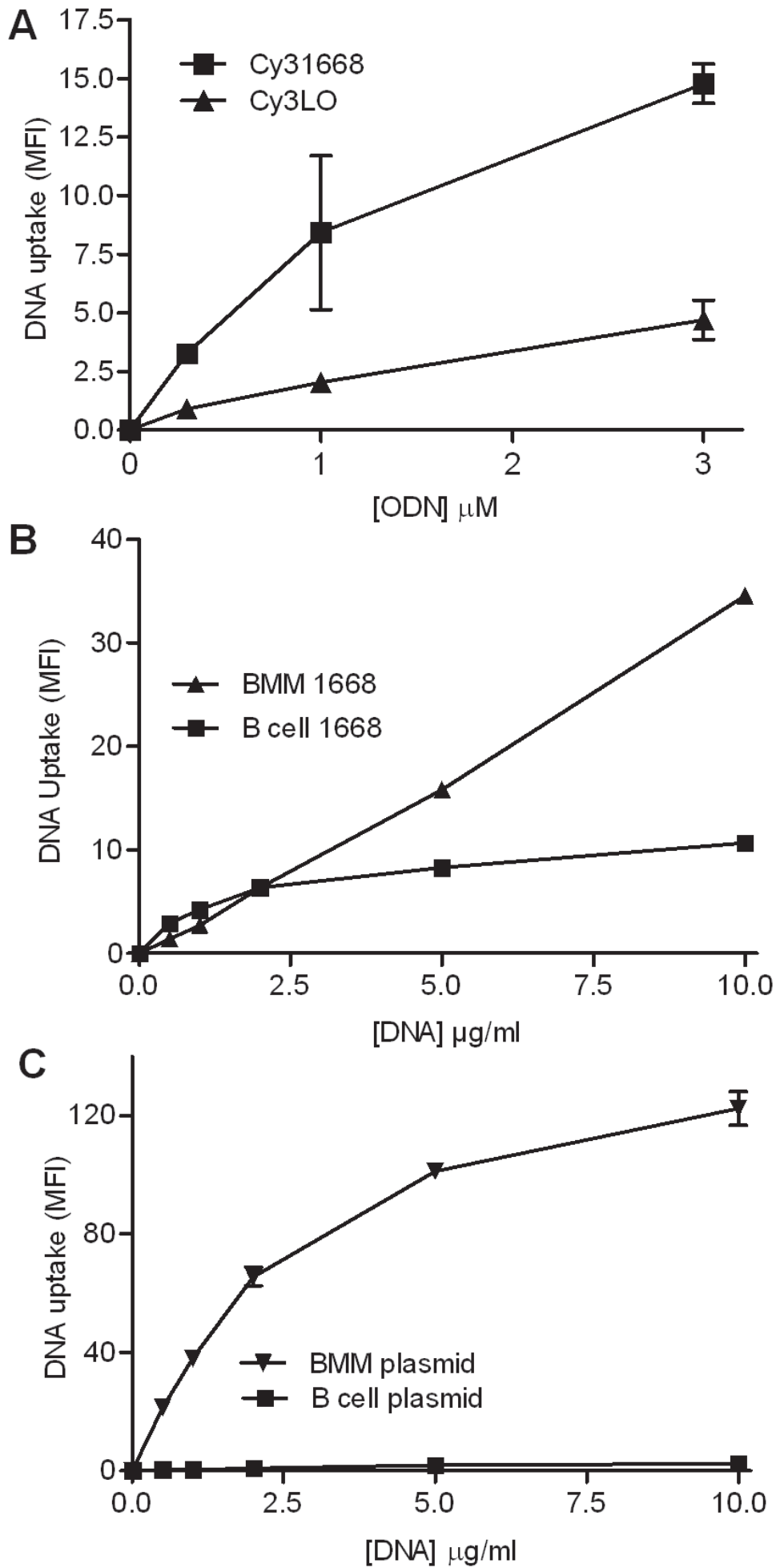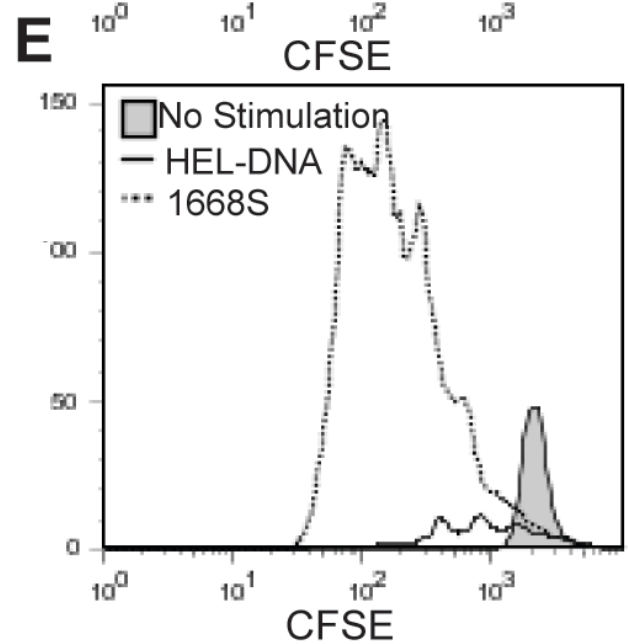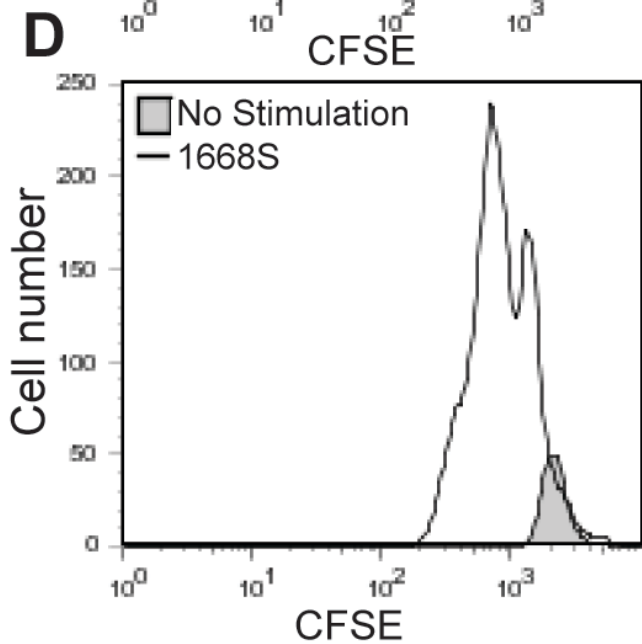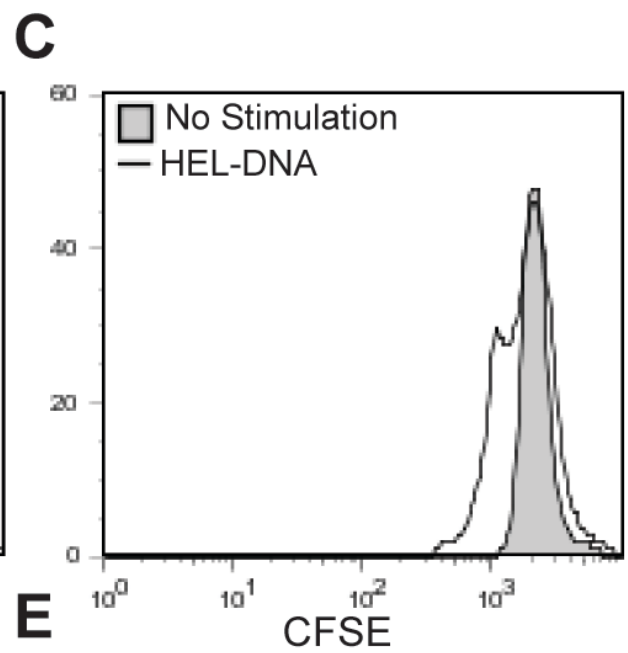
2

A

Cell Number — CD19

B

Cell Number — CD86

C

CD86 expression (MFI)
Untreated / E.coli DNA 50µg/ml / 1668 10µM / 1668 3µM

D

CD86 expression (MFI) vs [DNA] µM
- 1668
- 1668-GC
- ds1668
- 1668R

E

CD86 expression (MFI) vs [DNA] µM
- 1668
- 1668-GC
- LO
- dsLO

F
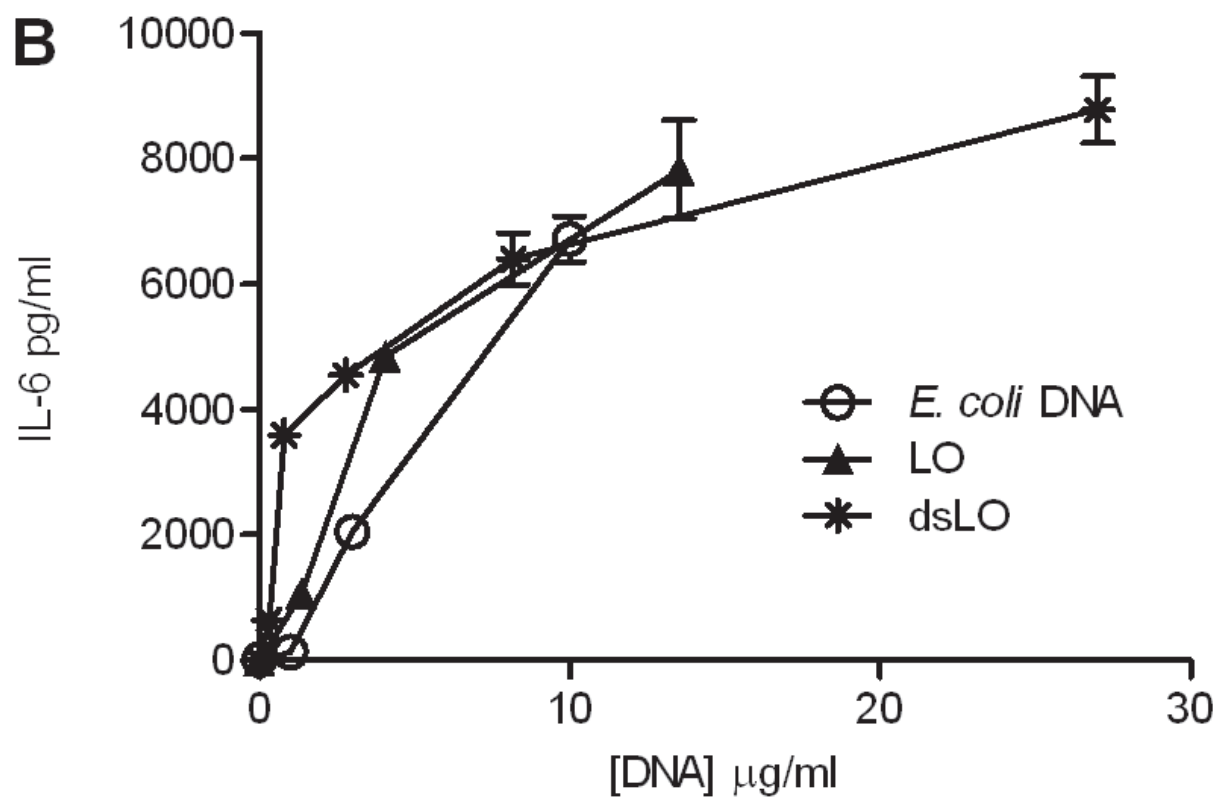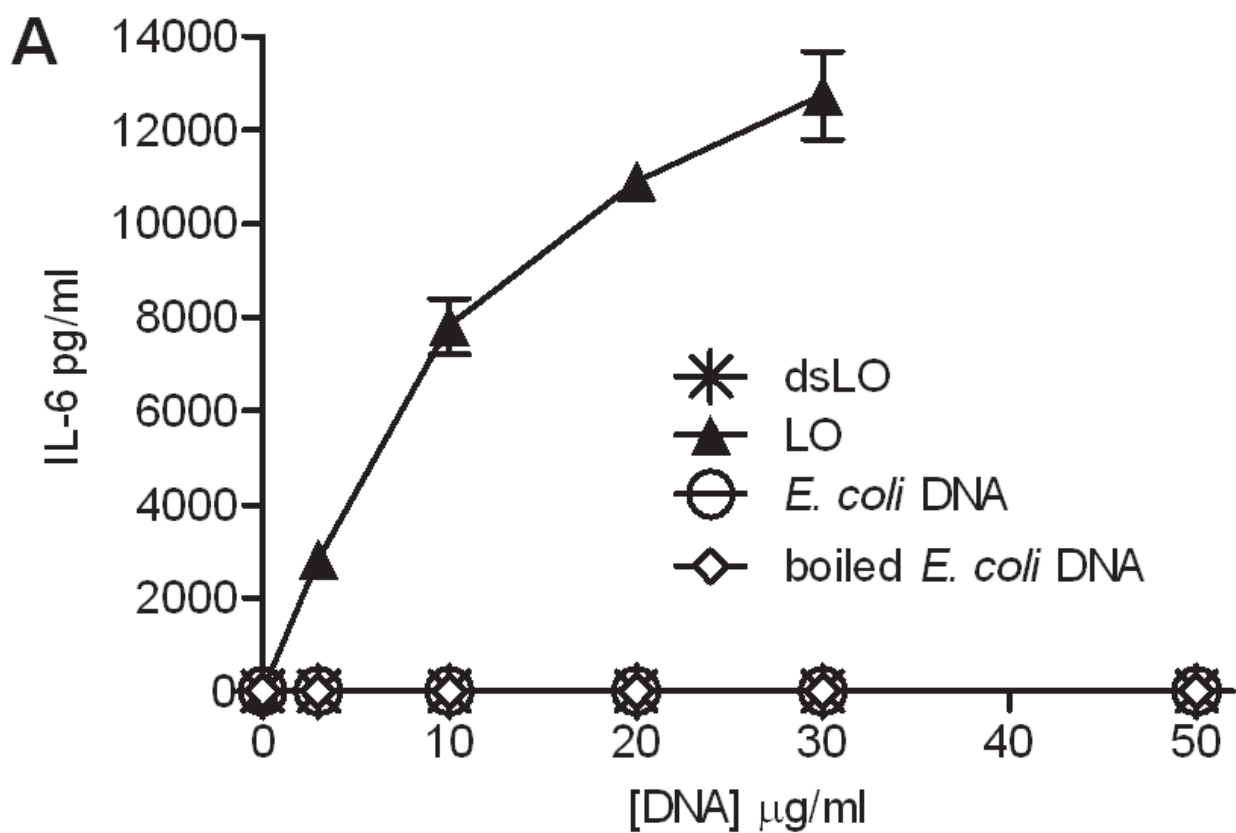
CD86 expression (MFI) vs [DNA] µg/µl
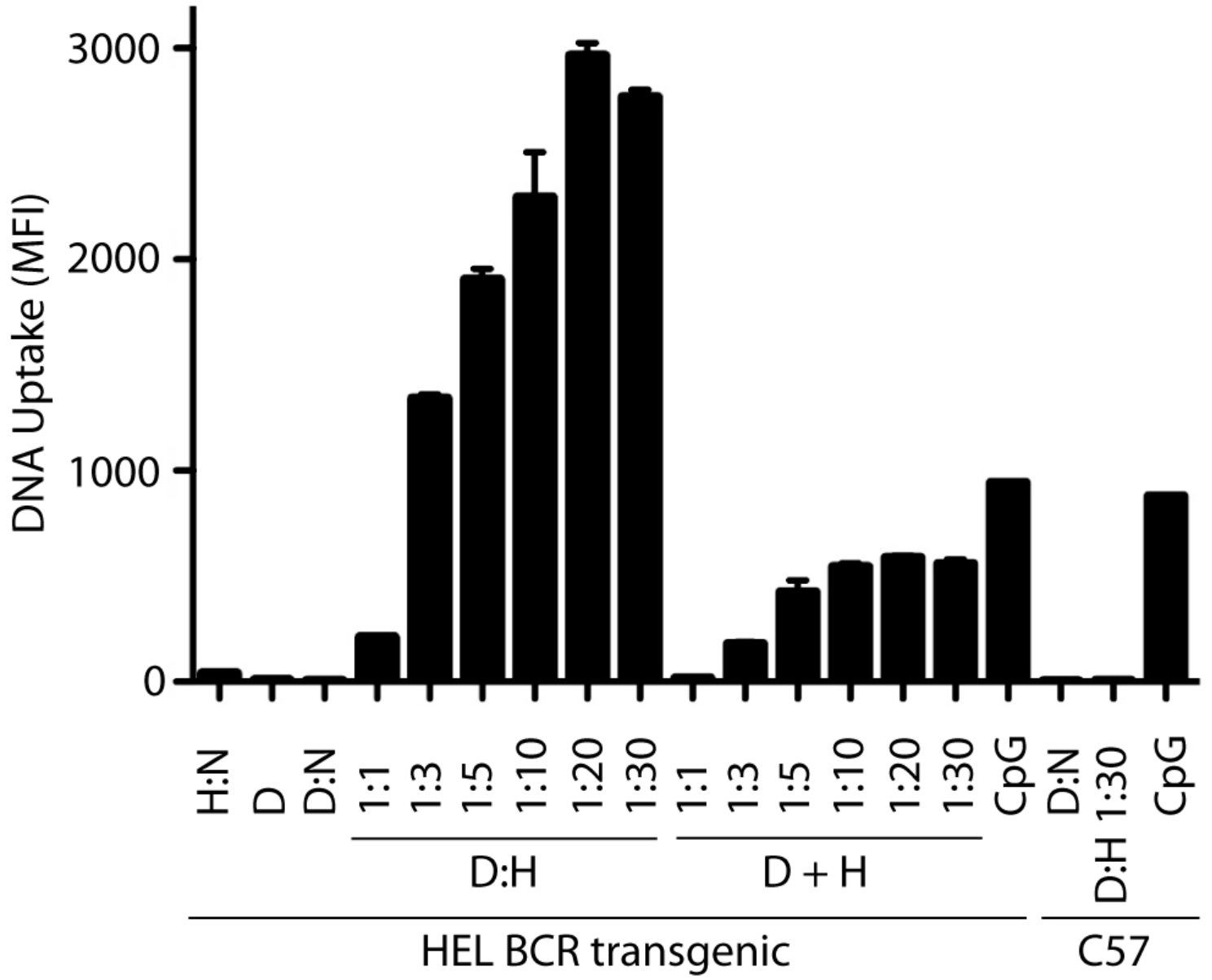- EC DNA
- sEC 0.02
- sEC 0.03
- 1668
- bEC
- bsEC 0.02
- bsEC 0.03
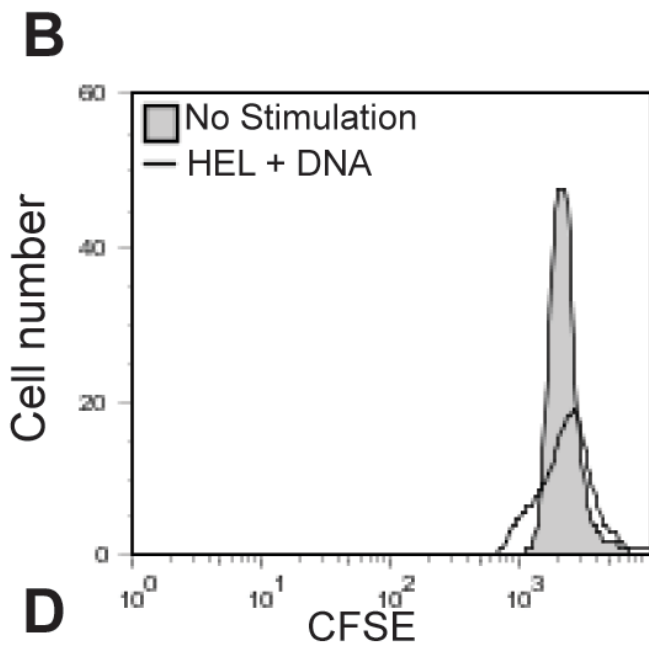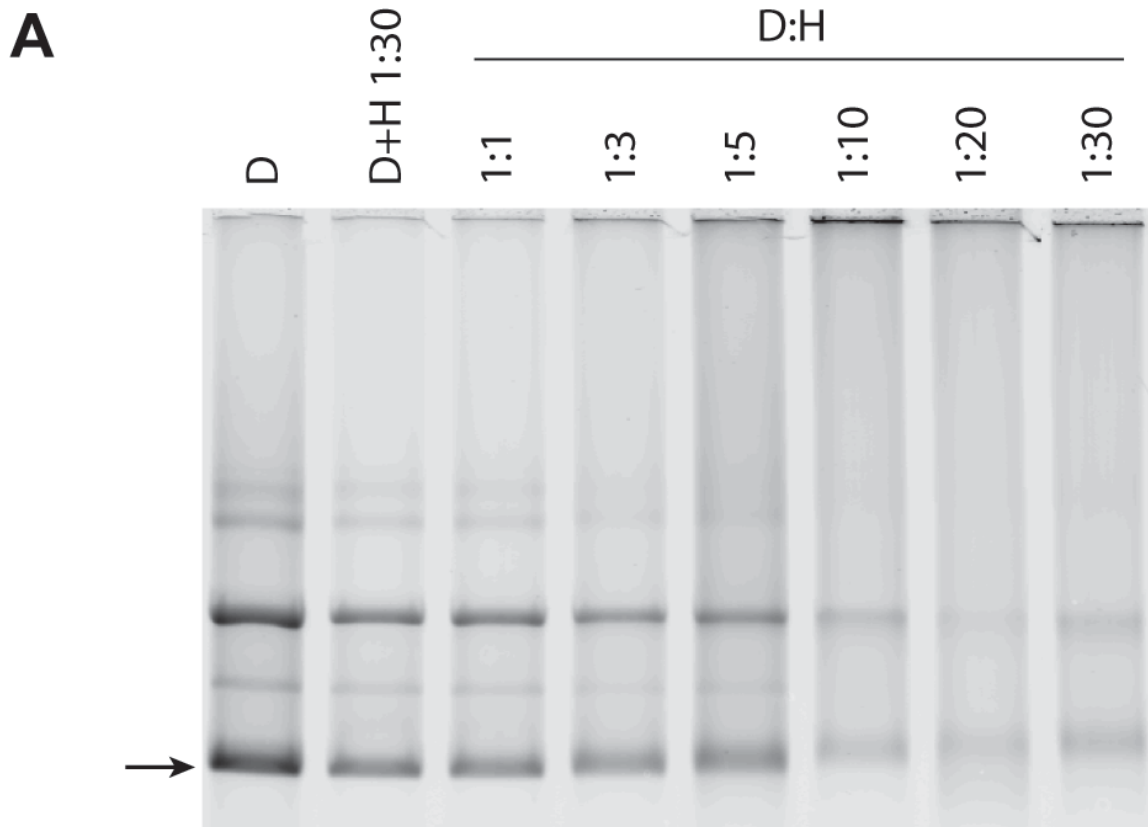
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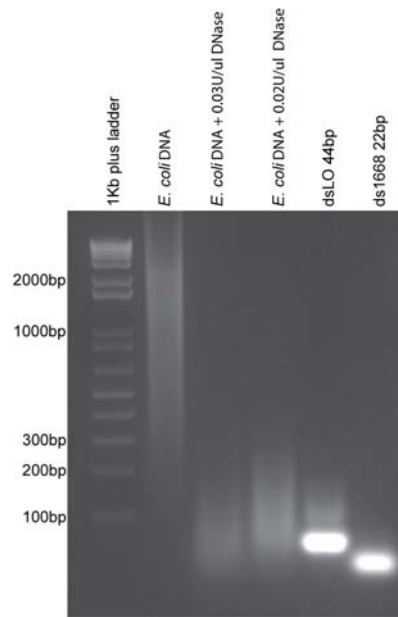
Figure S1. Controlled digestion of *E. coli* DNA with DNase I. *E. coli* DNA was incubated with 0.02 or 0.03 units/µl of DNase I for 20min, then purified by phenol chloroform extraction and ethanol precipitation. Samples were analysed on a 2% agarose gel, and compared to undigested *E. coli* DNA and ds ODN of 44bp and 22bp. DNase digestion resulted in DNA products between approximately 20 and 250 bp in size.
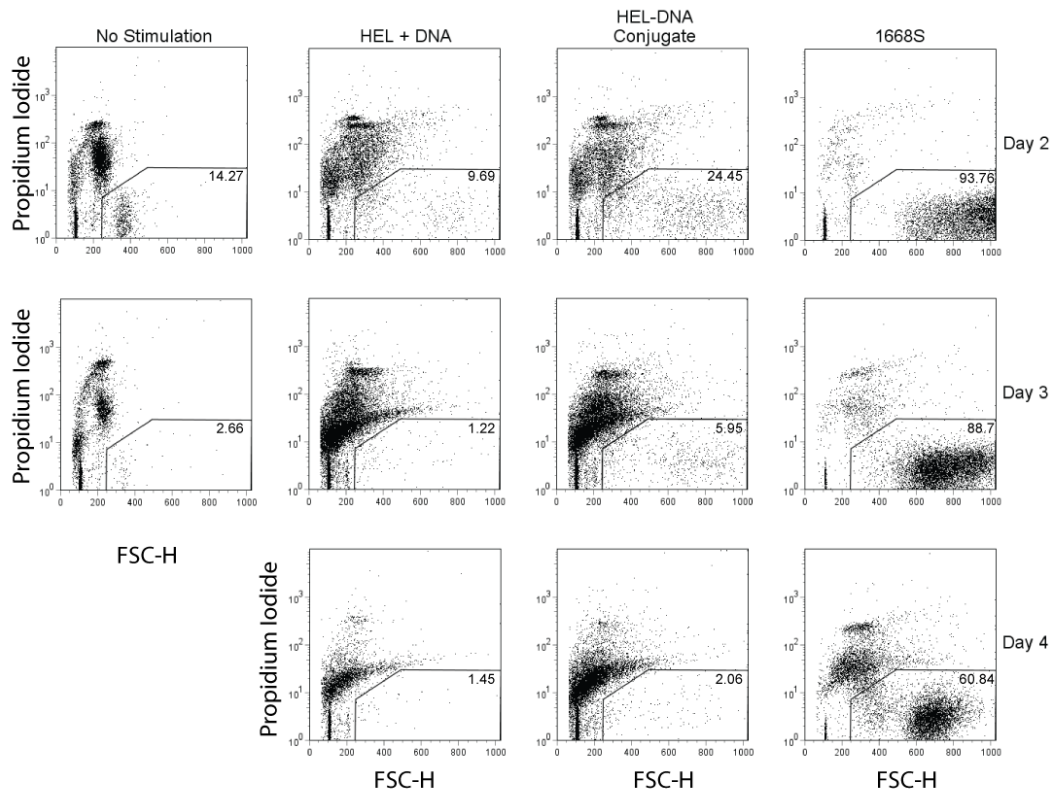
Figure S2. Antigen-DNA complexes induce limited survival of B cells. In the experiment depicted in Figure 4B, cells were stained with propidium iodide (PI) and assessed on a plot of PI staining (y axis) vs forward scatter (x axis) to identify viable cells. Cells which were PI negative were gated as the viable population for analysis of CFSE dilution in Figure 4B. The sharp line at low FSC and low PI staining is due to beads which were added for quantification of cell number. As in Figure 4B, cells were either left untreated, or stimulated with either HEL alone, HEL-neutravidin complex, DNA alone, HEL mixed with DNA (HEL + DNA), HEL mixed with DNaseI-digested DNA, HEL-DNA conjugate or 1668S. Results are shown here for those samples which had enhanced cell survival and proliferation relative to untreated cells. The treatments in other samples had no detectable effect. The percentage of total cells falling in the gated viable region is indicated on each graph.
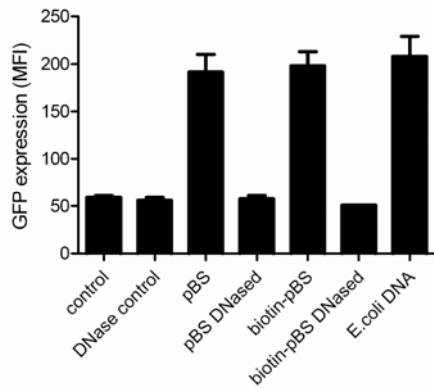
Figure S3. Biotinylation of plasmid DNA does not affect its TLR9-stimulatory activity. Biotinylated plasmid, as used to form DNA-HEL complexes in Figure 4B, was compared for TLR9-mediated activation to unmodified plasmid, using macrophage cells with a stably integrated NF-κB responsive GFP reporter (ELAM9 cells[19]). Cells were incubated with 2µg/ml pBluescript (pBS), biotin-pBS or *E. coli* DNA for 6h. Digestion of plasmid with DNaseI confirmed the lack of stimulatory contaminants. The DNase control contained buffer and DNaseI but no DNA. GFP expression was analysed by flow cytometry. Bars show the average of duplicate treatments and the error bars show the range of the values.