A threshold level of TLR9 mRNA predicts cellular responsiveness to CpG-ODN in haematological and non-haematological tumour cell lines

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Abstract:

The human toll like receptor 9 (TLR9) detects differences between microbial and host DNA, based on unmethylated deoxycytidyl deoxyguanosine dinucleotide (CpG) motifs, leading to activation of both innate and adaptive immune mechanisms. The synthetic TLR9 agonist, CpG-ODN, can substitute for microbial DNA in these responses, and is in clinical trials as an immunomodulatory agent in diseases as diverse as infections, cancer and allergic disorders. Human TLR9 is expressed on cells of haematopoietic origin (principally plasmacytoid dendritic cells and B cells), but has also been described as being expressed on a number of other cell types. In order to clarify the expression and function of TLR9 in a range of cells of both haematopoietic and non-haematopoietic origin, we investigated the level of expression of TLR9 mRNA, and the ability of the cells to respond to CpG-ODN by upregulation of cell surface markers, cytokine production, cellular proliferation and activation of NFκB. Our data show that the cellular response to CpG-ODN depended on a threshold level of expression of TLR9. TLR9 was widely expressed amongst B cell tumours (with the exception of myeloma cell lines), but we did not find either threshold levels of expression of TLR9 or responses to CpG-ODN in several myeloma or myeloid tumour cell lines or any non-haematological tumour cell lines tested in our study. TLR9-positive cells varied significantly in their responses to CpG-ODN, and the level of TLR9 expression beyond the threshold did not correlate with the magnitude of the response to CpG-ODN. Finally, CpG-ODN induced NFκB activation and increased cellular proliferation in Hek293 cells that had been stably transfected with hTLR9, but did not affect the expression of surface markers or synthesis of IL-6, IL-10 or TNF-α. Thus both haematological and non-haematological cells expressing appropriate levels of TLR9 respond to CpG-ODN, but the nature of the TLR9-mediated response is dependent on cell type.

Keywords:
Toll like receptor; TLR9; CpG-ODN; reverse transcriptase polymerase chain reaction; NFκB

Introduction:

The immune system has developed defence mechanisms against infectious micro-organisms, based on the identification of pathogen associated molecular patterns (PAMPs). Crucial to mediating these effects is the family of toll like receptors (TLRs). Of these, TLR9 detects differences in the chemical structure between microbial and host DNA, principally by recognising unmethylated deoxycytidyl deoxyguanosine dinucleotide (CpG) motifs in bacterial or viral DNA [1,2]. In humans, TLR9 is expressed predominantly in B-cells and plasmacytoid dendritic cells (pDC) [3-7]. However, studies have reported human TLR9 (hTLR9) expression in other immune cells, such as activated neutrophils, natural killer cells, eosinophils and T lymphocytes [5, 8-10]. TLR9 expression has also been reported in some non-immune human cell types, such as pulmonary epithelium, keratinocytes and intestinal epithelial cells, and in lung and intestinal tumour cells [11-15].

Like the other microbial nucleic acid-sensing TLRs (TLR3, 7 and 8), TLR9 is expressed within the intracellular compartment. Prior to exposure to microbial DNA, TLR9 is retained within the endoplasmic reticulum [16]. Expression of the functional receptor requires trafficking to endolysosomes, which is facilitated by the chaperone molecule UNC93B1 [17], and requires cleavage of the TLR9 ectodomain [18]. Whilst both full-length and cleaved forms of TLR9 are able to bind microbial DNA, only the cleaved form is capable of recruiting MyD88 on activation [18], initiating the TLR signalling cascade.

Identifying the expression and function of TLR9 in immunological and non-immunological cell types, including both normal and tumour cells, is central to understanding the range of biological effects mediated by TLR9, and the potential of CpG-ODN as immunotherapeutic agents. The aims of this work were to quantify TLR9 expression in a panel of human tumour cells of haematopoietic and non-haematopoietic origin, and to correlate the levels of expression with responsiveness to stimulation with CpG-ODN. Using a semi-quantitative RT-PCR method, we show that TLR9 mRNA was widely expressed amongst B cell tumours (with the exception of myeloma cell lines), but was weakly or not expressed in several myeloid tumour cell lines, nor in any non-haematological tumour cell lines tested. We then studied the ability of the cells to upregulate the expression of several immunologically important cell surface markers

(CD54, CD80, CD86, MHC class II), to secrete cytokines (IL-6, IL-8, IL-10 and TNF-α), and to increase cellular proliferation in response to the TLR9 ligand CpG-ODN. The cellular response to CpG-ODN depended on a threshold level of expression of TLR9, and correlated with activation of NFkB and increased cellular proliferation, but TLR9-positive cells varied significantly in other responses to CpG-ODN.

Materials and Methods:

Cells and cell lines used in the study.

Cells and cell lines used in the study are described in Table 1, and were maintained in growth medium (RPMI-1640 or Iscove's Modified Dulbecco's Medium) supplemented with 10% Foetal Bovine Serum (FBS), streptomycin (100μg/ml), penicillin (100U/ml), and 2mM L-glutamine (all from Sigma-Aldrich, UK), at 5% CO_2, in a humid environment at 37ºC. Normal human peripheral blood B cells were isolated from mononuclear cells (PBMCs) from heparinised blood of volunteer healthy adult donors by positive selection, using 20μl of $CD19^+$ magnetic microbeads (Miltenyi Biotech, Bisley, UK) per $1x10^7$ total cells, according to the manufacturer's instructions, and were used as positive controls of TLR9 expression and function. *Ex vivo* B-CLL cells and mantle cell lymphoma cells (TH044 and FC029) were isolated in a similar way from the blood of patients with these conditions. Purity of the isolated B-cell populations was analysed by flow cytometry using anti-CD20 FITC (Beckman Coulter, UK), and was routinely greater than 95%. Studies involving samples from patients had the approval of the Local Research Ethics Committee. All of the other tumour cells used in the study were established cell lines generated from patients with a variety of haematological and non-haematological malignancies.

Transfection of Hek293 cells with hTLR9.

Human embryonal kidney cell line, Hek293 (provided by Dr. Sek Chow, University of Leicester, UK), was transfected with two hTLR9-containing plasmids (pDisplay.HA/TLR9 and pClneo.TLR9; the gift of Dr Cynthia Leifer, USA [19]), or with the empty plasmids as controls, using Effectene gene transfection reagent (Qiagen, UK) according to the manufacturer's instructions. Briefly, cells were plated in six-well plates (Nunclon™ Surface, Nunc products, UK) at $5x10^5$ cells/well, and transfection was carried out on the following day with 0.4μg/well of the linearised plasmid, to yield cell lines

Hek293.HA/TLR9 and Hek293.TLR9 respectively. The cells were cultured for 24 hours at 37ºC and 5% CO_2, washed with phosphate buffered saline (PBS), and then transferred to growth medium containing 0.5mg/ml G418 (Geneticin, Sigma-Aldrich, UK) for one to two weeks to select for stable transfectants, before freezing in aliquots in liquid nitrogen. Transfected cells were maintained in culture in 0.3mg/ml G418 for a maximum of 5 weeks, before being replaced with freshly thawed cells. Expression of TLR9 mRNA was determined by RT-PCR for each batch used.

Semi-quantitative RT-PCR for hTLR9 expression:

Total cellular RNA was extracted using RNeasy Mini Kits (Qiagen, UK) according to the manufacturer's instructions, and cDNA was prepared by the Superscript™ first strand synthesis system (Invitrogen, UK), according to the manufacturer's instructions. TLR9 mRNA expression was determined by semi-quantitative RT-PCR using the LightCycler system (Roche Diagnostics, Mannheim). β-actin was used as a control PCR, and for normalisation of TLR9 expression. The PCR were carried out using the following primer combinations:

hTLR9-FW2 5'-ACAACAACATCCACAGCCAAGTGTC-3';

hTLR9-RW2 5'-AAGGCCAGGTAATTGTCACGGAG-3' [20].

β-actin-FW 5'-GCTCGTCGTCGACAACGGCTC-3';

β-actin-RW 5'- CAAACATGATCTGGGTCATCTTCTC-3'.

TLR9 primers yielded a PCR product of 250bp, while β-actin primers gave a 353bp product. All primers were obtained from MWG (MWG, Ebensburg, Germany). Amplification of cDNA was carried out in the presence of SYBR green (Quanti Tect SYPR® Green PCR Kit, Qiagen, UK). TLR9 and β-actin PCR were run under the same conditions: 1.5μl of 5μM of each primer, 7.5μl of master mix SYBR Green I and 3.5μl of RNase free water were added to 1μl of the 5μg cDNA of a final volume of 15μl in LightCycler capillaries (20μl) (Roche Diagnostics, Mannheim). Thermal cycling was initiated with an incubation step at 50°C for 2 minutes, followed by a first denaturation step at 95°C for 15 minutes, and continued with 45 cycles of 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 15 seconds. Melting curve analysis confirmed the specificity of the amplification products. Serial dilutions of standard preparations of hTLR9 DNA (pCl-neo.TLR9 plasmid) and β-actin cDNA were included to permit standardisation of

data between experiments, and the results were expressed in arbitrary units (AU), based on the ratio of TLR9:β-actin mRNA for each cell line.

CpG ODN activation dose and time course:

Type-B CpG-ODN 10103 was obtained from Coley Pharmaceutical, Canada. In preliminary experiments, 3μg/ml CpG-ODN 10103 was found to be the optimal activating dose for most cell lines, and was used throughout. Cells were either left untreated (culture in growth medium alone) or stimulated with 3μg/ml 10103 CpG-ODN in growth medium for variable times, depending on the experimental read-out.

Effect of CpG-ODN on surface marker expression:

Monoclonal antibody (mAb) staining and flow cytometry were used to investigate the effect of CpG-ODN 10103 on the cellular expression of the adhesion molecule CD54, costimulatory molecules CD80 and CD86, and major histocompatibility complex class II (MHC II). Cells ($5x10^5$ /ml) were stimulated with 3μg/ml of 10103 CpG-ODN for three days, or were left without activation, prior to staining with mAbs (10μl in each case), anti-CD54-PE, anti-CD80-FITC, anti-CD86-PE (Beckman Coulter, UK) and anti-MHC II-FITC (Dako, UK). IgG1-PE and IgG1-FITC mAbs (Beckman Coulter, UK) were used as isotype controls. Analysis was carried out using a FACSCalibur flow cytometer and Cell Quest Pro software (Becton-Dickinson Ltd, UK). Data from 10,000 gated events were analysed, and are expressed as mean channel fluorescence intensity (MFI).

Effect of CpG-ODN on the ability of cells to stimulate in allogeneic MLR:

Stimulation of allogeneic T cell proliferation *in vitro* was estimated in mixed lymphocyte reactions (MLR). Responder peripheral blood mononuclear cells (PBMC) from healthy volunteer donors were obtained by Lymphoprep (Axis-Shield, UK) separation of heparinised venous blood. The experiment was carried out using 2 different healthy donors, with consistent results. Stimulator cells were either treated with CpG-ODN (3μg/ml) or left untreated, prior to being treated with 0.5ml of Mitomycin C (Mit-C; 100 μg/ml) for 1 hour, washed twice with RPMI 1640, and plated in 96 well U-bottomed plates in triplicate wells at 10^5 cells per well in 100 μl culture medium (RPMI 1640 plus 10% FCS and antibiotics). To these cells, 100μl of allogeneic responder cells (normal PBMC) were added at 10^6 cells/ml. The background controls were responder cells

cultured alone, and Mit-C treated stimulator cells alone. The cell cultures were incubated at 37^0C in 5% CO_2 for 5 days. On the fifth day, wells were pulsed with 1 μCi/well [methyl ^{3}H]-thymidine (GE Healthcare, UK) for 16-18 hours. The cells were harvested onto glass fibre filters (1450-421, Wallac, Finland) using a Tomtec cell harvester (Wallac, Finland). After drying, the filters were sealed in sample bags with liquid scintillation Betaplate Scint (Perkin Elmer, UK), and the incorporation of [^{3}H] thymidine into cellular DNA was measured on a 1450 Microbeta plus liquid scintillation counter (Wallac, Finland).

Effect of CpG-ODN on production of IL-6, IL-8, IL-10 and TNF-α:

The secretion of IL-6, IL-8, IL-10 and TNF-α by resting and CpG-ODN-activated cells was analysed using multiplexed bead immunoassays (Upstate Biotechnology, Abingdon, UK). Five x10^5 cells/ml of non-adherent cells, or 1x10^5 cells/ml of adherent cell lines, were split into triplicate wells in 96 well flat-bottomed microtitre plates, and left to grow either without or with 3μg/ml 10103 CpG-ODN for 36 hours, before cell-free supernatants were harvested and frozen in aliquots at -80^{o}C until assayed. Culture supernatants were assayed as previously described [21], using a Luminex100 machine (Luminex Corporation, Austin, TX) and StarStation software (Applied Cytometry Systems, Sheffield, UK). Data are expressed as cytokine concentration (pg/ml).

Effect of CpG-ODN on cellular proliferation:

Cellular proliferation was estimated by incorporation of [methyl-^{3}H] thymidine into replicating cellular DNA. One x10^5 cells/well were left to grow in triplicate wells for three days in flat bottom 96 well plates in the presence or absence of 3μg/ml 10103 CpG-ODN, and then pulsed with [methyl-^{3}H] thymidine (1μCi/well; GE Healthcare, UK) for the next 16-18 hours. The cells were harvested onto glass fibre filters (1450-421, Wallac, Finland) using a Tomtec cell harvester (Wallac, Finland). After drying, the filters were sealed in sample bags with liquid scintillation Betaplate Scint (Perkin Elmer, UK), and the incorporation of [^{3}H] thymidine in cellular DNA was measured on a 1450 Microbeta plus liquid scintillation counter (Wallac, Finland).

Effect of CpG-ODN on NFκB activation:

Nuclear factor kappa B luciferase assay: In order to demonstrate TLR9-mediated nuclear factor kappa B (NFκB) activation, 5x10^5 cells/ml for adherent cells or 5x10^6 cells/ml for

suspension cells were transiently transfected with a luciferase reporter plasmid, (3xNFκB.luc; the gift of Prof. L Ziegler-Heitbrock, University of Leicester, UK), which contained three copies of the NFκB binding sequence from the mouse kappa light-chain enhancer cloned upstream of the TATA box of the pβTATA luci reporter plasmid. Transient transfection was carried out using Effectene Transfection Reagent (Qiagen, UK), according to the manufacturer's instructions, using 0.4μg/well of 3xNFκB.luc construct. Twenty four hours after transfection, cells were washed and split into two groups, each in three wells in 24 well plates (1ml/well in fresh growth medium). One group of cells was left untreated, and the other group was activated with 3μg/ml 10103 CpG-ODN for 18 hours. Control groups of non-transfected cells were treated in the same way. Afterward, cells were harvested, washed with PBS, and the pellet was dissolved in 150μl of 1x reporter lysis buffer (Promega, UK). Twenty microliters of lysate was used for assay with 100μl of luciferase assay system (Promega, UK). The luciferase activity was measured using a Sirius luminometer (Berthold Detection Systems, Germany). Protein concentration was measured using Lowry method for each lysate, and was used to normalize the data.

IκB-α western blot: Five x10^6 cells/ml were either activated for various times (up to 120 minutes) with 10103 CpG-ODN (3μg/ml), or were left unactivated. For each time point, cells were lysed on ice using 0.5ml RIPA lysis buffer (1% NP-40, 0.5% Nadeoxycholate, 50mM Tris HCl, 150mM NaCl, 1mM Na-fluoride, 30mM Na-pyrophosphate, 2mM EDTA, 0.1% SDS, 100μM Na-orthovanadate, 1 mM phenylmethanusulphonylfluoride (PMSF) and protease inhibitors (Sigma) of 10μg/ml Leupeptin, 10μg/ml Aprotinin and 10μg/ml Pepstatin). After lysis, the samples were centrifuged at high speed (14,000 rpm) for 10 minutes at 4ºC, and the supernatants were dried using a bench-top freeze dryer (ModulyoD Freeze Dryer**,** Thermo Fisher Scientific, UK). The lyophilized supernatant was dissolved in 50μl of the lysis buffer. Protein concentration was estimated using a microplate assay (BioRad, Hercules, USA). Twenty micrograms of protein lysate was resuspended in 20 μl Laemmli loading dye with β-ME (BioRad, Hercules, USA) and boiled for 5 minutes at 95ºC, prior to SDS-PAGE to fractionate the proteins. Afterwards the proteins were transferred into a Hybond-C Super nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). The membrane blots were washed in 1x PBS – 0.1% Tween 20 three times for 5 minutes on an orbital shaker, blocked with 5% non-fat dry milk for 1 hour, washed again, and then blotted with 1:500 rabbit polyclonal

anti-phosphor-IκB-α (Ser 32)-R (Santa Cruz Biotechnologies, Santa Cruz, USA) in 1% non-fat dry milk for 24-48 hours. The membranes were then washed and stained with peroxidase labelled polyclonal swine anti-rabbit immunoglobulins/HRP (Dako Cytomation, UK), 1:1000 dilution in 4% non-fat dry milk. Membranes were washed, and the protein bands were visualized by Chemiluminescence ECL Blotting detection kit and exposed to Hyperfilm™ ECL Western (Amersham Biosciences, Little Chalfont, UK). The membranes were stripped and reblotted for total IκB-α protein using rabbit polyclonal IgG anti-IκB-α (C-21; sc-371 Santa Cruz Biotechnologies, Santa Cruz, USA), followed by 1:1000 peroxidase labelled polyclonal swine anti-rabbit immunoglobulins/HRP (Dako Cytomation, UK). To ensure equal protein loading, the same membranes were stained using β-actin/HRP antibody (Santa Cruz Biotechnologies, Santa Cruz, USA) at 1:5,000 dilution, before detection using ECL. Band intensities were quantified using a GS-710 densitometer (Bio-Rad, UK). The relative intensities of phosphorylated and total IκB-α bands were normalised against β–actin.

Statistical Analysis:

Data were analysed using Student's paired t-test using GraphPad prism 4 software version 4.03. Results were considered significant when *p* value was less than 0.05, and highly significant when *p* value was less than 0.01.

Results:

TLR9 expression in human cell lines by semi-quantitative RT-PCR:

We used a semi-quantitative RT-PCR to determine the level of TLR9 mRNA in each cell line, and normalised levels of hTLR9 mRNA against expression of the house keeping gene β-actin. The data are expressed in arbitrary units, as the ratio of TLR9:β-actin mRNA (Table 1). Peripheral blood B-cells from normal healthy donors (included as a positive control for TLR9 expression) expressed TLR9 at a relatively high level. TLR9 mRNA expression levels in a panel of B cell-derived tumour cells was variable, but showed a TLR9:β-actin ratio > 1.0 $x10^{-2}$ AU in all cells except for the myeloma cell lines U266 and Karpas707H. The myeloid lineage cell lines, KG-1 and HL-60 were also negative by this criterion (TLR9:β-actin ratio <1.0 $x10^{-2}$ AU), whilst the monocytic cell line U937 expressed a level of TLR9 mRNA above this threshold. All of a panel of non-haematological cell lines showed low TLR9 expression (TLR9:β-actin < 1.0 $x10^{-2}$ AU).

Untransfected or mock-transfected Hek293 cells had very low TLR9 expression, whilst Hek293 cells which had been stably transfected with TLR9-containing plasmids (Hek293.TLR9 or Hek293.HA/TLR9) expressed TLR9 at levels comparable to the majority of B cell lines tested (TLR9:β-actin > 1.0 $x10^{-2}$ AU; Table 1). Based on these data, we used an arbitrary cut-off level of >1.0 $x10^{-2}$ AU to define cells as TLR9-positive, or TLR9-negative (<1.0 $x10^{-2}$ AU).

CpG-ODN mediated responses of the tumour cell lines:

Having established the levels of TLR9 expression, we investigated the effect of the TLR9 agonist, 10103 CpG-ODN, on the cells, by surface marker expression, cytokine release, cellular proliferation, and NFκB activation.

CpG-ODN mediated surface marker upregulation: Flow cytometry was used to estimate expression of CD54, CD80, CD86 and MHC II by the cells, in the absence or presence of CpG-ODN. As shown in Table 2, CpG-ODN induced upregulation of all four surface markers in normal human B cells. Of nine TLR9-positive tumour B cells tested, six showed upregulation of three or all four cell surface markers, whilst the remaining three showed upregulation of at least one of the cell markers in response to CpG-ODN. In contrast, the TLR9-negative haematological tumour cells, U266, Karpas 707H, KG-1 and HL60, and all of six TLR9-negative non-haematological cell lines, failed to upregulate any of the tested surface markers in response to treatment with CpG-ODN (Table 2). Similarly, mock-transfected Hek293 cells, and Hek293 cells that had been stably transfected with hTLR9 did not upregulate the surface markers in response to treatment with CpG-ODN.

Effect of CpG-ODN on the ability of cells to stimulate allogeneic T cell proliferation in MLR: As CpG-ODN induced the upregulation of the major T cell costimulatory ligand molecules, CD80 and CD86, in several of the TLR9-positive cell lines, we investigated the effect of CpG-ODN on the ability of the cells to stimulate T cell proliferative responses in allogeneic MLR. The highly immunogenic EBV-lymphoblastoid cell line HMy2 was used as a positive control for allogeneic T cell stimulation in the MLR, and induced stronger T cell proliferation than any of the cell lines tested. As illustrated in Figure 1, only the normal B cells (CD19+) and the myeloma cell line RPMI 8226 induced an enhanced stimulation of allogeneic T cells following treatment with CpG-ODN. Both

of these cell lines showed upregulation of CD80 and CD86 in response to CpG-ODN. However, TLR9-positive B-CLL cells (which also upregulated CD80 and CD86), and Ramos cells (which upregulated CD86 but not CD80), did not show a significant increase in induction of T cell proliferation in allogeneic MLR following treatment with CpG-ODN. The TLR9-negative cell lines U266 and Karpas 707H, and all of five non-haematological cell lines, also failed to induce enhanced T cell proliferation following treatment with CpG-ODN.

Effect of CpG-ODN on the induction of cytokines: To study the role of TLR9 in cytokine production, we investigated the effect of CpG-ODN on the production of TNF-α, IL-6, IL-8 and IL-10 by the tumour cell lines. The results for IL-6, IL-10 and TNF-α are shown in Table 3. Normal human B cells, and five of seven tumour cell lines of haematological origin that constitutively expressed hTLR9 at > 1.0 $x10^{-2}$ AU, responded to treatment with CpG-ODN by significantly increasing secretion of IL-6, IL-10 and TNF-α, whilst the remaining two TLR9-positive haematological tumour cells (Ramos and RPMI) increased expression of at least one of these cytokines. In contrast, the TLR9-negative haematological cell lines, KG-1, U266 and Karpas707H, did not increase cytokine secretion in response to CpG-ODN. None of the non-haematological tumour cell lines, including Hek293 cells that had been stably transfected with hTLR9, produced a significant cytokine response to treatment with CpG-ODN (Table 3). We also tested the effect of CpG-ODN treatment on IL-8 production in five of the non-haematological tumour cell lines (and in normal human B cells as controls), as several groups have reported IL-8 production by epithelial cells in response to CpG-ODN [11-13]. Normal human B cells showed a slight (3-fold) increase in IL-8 secretion in response to CpG-ODN, whilst none of the non-haematological tumour cells tested (HeLa, DU-145, HRT-18, MCF-7, and WM1361A) showed a significant upregulation of IL-8 secretion in response to CpG-ODN (data not presented).

CpG-ODN mediated cellular proliferation: Normal human B cells, and TLR9-positive tumour cells B-CLL, TH044, Ramos, Raji, BJAB and RPMI, all showed a significant increase in cellular proliferation in response to treatment with CpG-ODN (Table 4). The TLR9-negative haematological tumour cells KG-1, Karpas707H and U266, and the non-haematological tumour cell lines, MCF-7, DU-145, HRT-18, WM1361A, Hek293 and HeLa, did not show any changes in the cellular proliferation (Table 4). In contrast to the

data on cell surface marker upregulation and cytokine production, CpG-ODN stimulation of TLR9-transfected Hek293 cells resulted in a significant increase in cellular proliferation of both Hek293.TLR9 and Hek293.HA/TLR9 cell lines, whilst mock transfected Hek293 cells did not show any change in cellular proliferation in response to CpG-ODN (Table 4).

Effect of CpG-ODN on NFκB activation: To investigate the effects of CpG-ODN activation of TLR9 on activation of the NFκB pathway, we used a NFκB-luciferase assay. CpG-ODN treatment was associated with a significant increase in NFκB-luciferase activity in normal human B cells, and in all of three constitutively TLR9-positive tumour B cell lines tested (B-CLL, BJAB and RPMI), but had no effect on luciferase activity in the TLR9-negative cell lines, Hek293 and HeLa (Table 5). Mock transfected (pCl-neo or pDisplay) Hek293 cells did not show any NFκB activation in response to CpG-ODN, whereas NFkB activation was observed in both of the TLR9-transfected (Hek293.TLR9 and Hek293.HA/TLR9) cell lines. Activation of NFκB by CpG-ODN was confirmed in Hek293.HA/TLR9 cells by western blotting for phosphor- and total-IκB-α (Figure 2). Induction of phosphor-IκB-α was also seen following CpG-ODN treatment of TLR9-positive Ramos cells, albeit with different kinetics, but no induction of phophor-IκB–α was seen in Hek 293 cells transfected with the control plasmid, pDisplay (Figure 2). These data confirm that TLR9 expression was both required and responsible for CpG-ODN-mediated activation of NFκB in these cell lines.

Discussion

Knowing the expression and function of TLR9 in human normal and tumour cells is crucial for understanding the role of TLR9 in infections, and the potential of CpG-ODN in immunotherapy. In humans, TLR9 is expressed mainly in B-cells and pDCs [3-7], but has also been reported as being expressed in activated neutrophils [8], natural killer (NK) cells [5,10], eosinophils [9], and T lymphocytes [5], in some non-immune cell types, such as pulmonary epithelial cells, airway smooth muscle cells, keratinocytes, intestinal epithelial cells [11-14], and in solid tumours such as lung and intestinal cancers [11,15]. These data suggest that hTLR9 may be more widely expressed than was initially appreciated, and have implications for the use of TLR9 agonists in immunotherapy.

To address this, we studied the expression of TLR9 in more than 20 cells and cell lines of both haematological and non-haematological origin, using a semi-quantitative RT-PCR method. We then correlated the results with the ability of the cells to respond to stimulation with the TLR9 agonist, CpG-ODN, using a range of functional parameters, including the upregulation of cell surface marker expression, secretion of cytokines, increase in cellular proliferation, and activaton of NFkB. Our data indicated that cells with a level of expression of TLR9 mRNA > 1.0 $x10^{-2}$ AU responded to treatment with CpG-ODN, whilst we did not detect any responses to CpG-ODN in cells that expressed < 1.0 $x10^{-2}$ AU TLR9 mRNA.

TLR9 was expressed above the threshold level in normal human B cells and in the majority of tumour B cells and cell lines tested, with the exception of the myeloma cell lines U266 and Karpas707H. The myeloid leukaemia cell lines KG-1 and HL-60 also showed sub-threshold levels of hTLR9 expression, although the monocytic cell line U937 was TLR9-positive. These results are broadly consistent with previous publications in human cells and cell lines of haematological origin [1, 5, 20, 22, 23]. None of the epithelial or melanoma cell lines tested in our study expressed a threshold level or TLR9. In this respect, our data differ from several previous studies, which reported TLR9 expression in a variety of epithelial cells and cell lines [11-15]. These differences may be due to the different cell lines studied, or to different criteria used in interpreting qPCR data on TLR9 expression by the different cell lines.

Whilst qPCR provides a sensitive method for the detection and quantitation of TLR9 mRNA, it does not provide information on TLR9 protein expression in the cells. We were unable to generate quantitative assays for TLR9 protein expression by flow cytometry, western blotting or immunoprecipitation, using commercially available monoclonal and polyclonal antibodies to human TLR9 (data not shown). This may be due to limitations of antigen recognition or antibody avidity, low levels of protein expression, or a combination of these factors. However, confocal microscopy using the same antibodies and an indirect staining method (data not shown), gave data that were broadly consistent with RT-PCR results, suggesting that antibody specificity was not the problem.

Having established the levels of TLR9 expression in the panel of haematological and non-haematological cell lines, we investigated their responses to the TLR9 agonist CpG-ODN. Based on previous reports, we studied the effects of CpG-ODN 10103 on expression of cell surface markers (CD54, CD80, CD86 and MHC class II), cytokine production (IL-6, IL-8, IL-10 and TNF-α), cellular proliferation, and NFκB activation. Several authors have reported CpG-ODN-induced upregulation of CD54, CD80, CD86 and MHC class II (amongst other markers), as well as enhanced cellular proliferation in human normal and tumour B cells [22, 24-26]. Production of IL-6, IL-10 and TNF-α was reported by CpG-ODN stimulated B cells, whilst CpG-ODN-responsive epithelial cells have been reported to secrete IL-6, IL-8 and / or TNF-α [12, 13, 24, 26-28]. Finally, activation of NFκB is central to TLR9-mediated cellular signalling and control of gene transcription [29], and should therefore represent a universal indicator of TLR9 signalling. By using a combination of these functional parameters, we aimed to identify all the CpG-ODN-responsive cells in our study.

In all of the haematological cell lines tested that constitutively expressed TLR9 (> 1.0 $x10^{-2}$ AU), CpG-ODN induced increased cellular proliferation, upregulation of at least one of the cell surface markers and increased secretion of at least one of the cytokines tested. However, the TLR9-positive cell lines varied in both the range and magnitude of these responses. As several of the cell lines showed upregulation of key molecules involved in antigen presentation to T cells, we studied the effect of CpG-ODN treatment of the cells as antigen-presenting cells (APCs) in allogeneic MLR. We observed relatively weak increases in T cell stimulation by CpG-ODN-treated cells, compared with untreated cells, only with normal B cells and RPMI 8226 cells, amongst the TLR9-positive cell lines. Consistent with this, we have found that CpG-ODN treatment appears to be much less potent than CD40 ligation in enhancing the antigen presenting capacity of normal and tumour B cells (Abdulmajed, unpublished data).

In contrast to the TLR9-positive cells, CpG-ODN had no effect on cellular proliferation, surface marker expression or cytokine production in TLR9-negative (< 1.0 $x10^{-2}$ AU) haematological or non-haematological cell lines. Other pro-inflammatory stimuli, such as interferon-γ, have been shown to upregulate expression of CD54 and MHC class II on both epithelial and melanoma cell lines [30-34], and data on TLR9-mediated responses in

other epithelial cells have described release of IL-6, IL-8 and/or TNF-α in response to CpG-ODN [11-13; 27, 28]. We would therefore have expected to see similar responses to CpG-ODN had the cell lines been TLR9-positive. To address this, Hek293 cells were stably transfected with two hTLR9 expressing plasmids. Both Hek293 cell lines transfected with hTLR9 expressed TLR9 (> 1.0 $x10^{-2}$ AU), but did not upregulate any of the cell surface markers or cytokines tested in response to CpG-ODN. However, these cells showed a significant increase in cellular proliferation, and activation of NFκB, in response to CpG-ODN. These results indicate that CpG-ODN mediated activation of NFκB is TLR9 dependent, and enhances cellular proliferation [35], but that cell surface marker upregulation or cytokine production may be independent of NFκB. This is consistent with the finding that the NFκB inhibitor, curcumin, blocked the effects of CpG-ODN on cellular proliferation in TLR9-positive BJAB cells, but had little effect on CpG-ODN-mediated cytokine production or cell surface marker upregulation (Assaf, unpublished data). The results also confirm that TLR9 can mediate cellular responses in non-haematological cells, if expressed at appropriate levels, but suggest that the nature of the responses varies between cells of different origins, such as marrow-derived or epithelial cells. Thus, the lack of induction of surface marker or cytokine expression in the non-haematological tumour cell lines may reflect cell type-specific differences in response to CpG-ODN. However, the absence of cellular proliferation in the epithelial and melanoma cell lines most likely reflects the lack of TLR9 expression in these cells, as all cell lines tested that expressed the threshold level of TLR9 expression (including the transfected Hek293 cells) showed a significant increase in cellular proliferation in response to CpG-ODN.

In summary, our experiments have shown a correlation between a threshold level of cellular TLR9 mRNA expression in both haematological and non-haematological cells, and the ability of the cells to respond to the TLR9 agonist, CpG-ODN. This response involved the NFκB intracellular signalling pathway, but the downstream effects on cytokine synthesis and cell surface marker expression appeared to be cell type-specific, and varied between different cell lines. Thus the effects of CpG-ODN will depend not only on the expression of TLR9 by cells, but also on the cell type(s) involved. These results may have important implications for the role of TLR9 in protective immunity at different tissue sites, and on the use of CpG-ODN in immunotherapy.

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Figure Legend

Figure 1: Allogeneic MLR using PBMC from a healthy, unrelated donor as responder cells, and **A:** unstimulated and CpG-ODN stimulated B cells and B cell lines or **B:** unstimulated and CpG-ODN stimulated solid tumour cell lines as stimulator cells. Results are expressed as a percentage of the T cell proliferative responses induced by the highly immunogenic cell line HMy2. Unstimulated PBMCs from the healthy donor were used as a background control. Statistically significant differences in the ability of CpG-treated cells compared with their untreated counterparts are indicated by horizontal bars over the data; *$p<0.05$.

Figure 2: CpG-ODN induced IκB-α degradation in **A:** TLR9-negative Hek293.pDisplay; **B:** TLR9-positive Hek293.HA/TLR9 cells; **C:** TLR9-positive / CpG-responsive Ramos cells. Cells were treated with 3μg/ml 10103 CpG-ODN for time points up to 120 minutes. Band intensities of phosphor-IκB-α, total IκB and β-actin are shown. **D:** Comparison of phosphor-IκB-α levels, normalised against β-actin, for Hek293.pDisplay, Hek293.HA/TLR9, and Ramos cells.

Table 1. TLR9 expression by cells and cell lines used in the study.

Cells		Description	Cell lineage	TLR9 mRNA (AU)
	$CD19^+$-1	Normal B-Lymphocytes	B cell	**9.83E-01**
	$CD19^+$-2		B cell	**5.41E-01**
	$CD19^+$-3		B cell	**4.70E-01**
	B-CLL	Ex vivo CLL	B cell	**8.17E-02**
	TH044	Mantle cell lymphoma	B cell	**6.06E-01**
	FC029	Mantle cell lymphoma	B cell	**8.68E-02**
	380	Precursor B-ALL	B cell	**4.71E-02**
	LiLa	Precursor B-ALL	B cell	**4.11E-01**
	TANOUE	Precursor B-ALL	B cell	**3.84E-02**
	ELIJAH	Burkitt Lymphoma	B cell	**9.37E-02**
	Ramos	Burkitt Lymphoma	B cell	**7.66E-02**
	RAJI	Burkitt Lymphoma	B cell	**4.52E-02**
	BJAB	Burkitt Lymphoma	B cell	**3.41E-02**
	RPMI 8226	Multiple Myeloma	B cell	**4.19E-01**
	U266	Multiple Myeloma	B cell	**7.60E-04**
	Karpas 707H	Multiple Myeloma	B cell	**3.50E-05**
	HL-60	Promyelocytic Leukemia	myeloid	**9.16E-03**
	KG-1	Acute myeloid leukaemia	myeloid	**6.35E-03**
	U937	Monocytic leukaemia	monocytic	**7.07E-01**
Non-haematological cells	**HeLa**	Cervical adenocarcinoma	Epithelial	**4.67E-03**
	SiHa	Cervical squamous carcinoma	Epithelial	**3.61E-03**
	Hek293	Human embryonal kidney	Epithelial	**4.74E-05**
	DU-145	prostate carcinoma	Epithelial	**8.50E-04**
	HRT-18	Rectum-anus, adenocarcinoma	Epithelial	**2.00E-03**
	MCF-7	Breast adenocarcinoma	Epithelial	**4.46E-05**
	WM1361A	Melanoma	Melanocyte	**6.18E-04**
	Hek293.pCl-neo	Human embryonal kidney (transfected)	Epithelial	**6.54E-05**
	Hek293. pDisplay		Epithelial	**2.03E-03**
	Hek293.TLR9		Epithelial	**5.83E-02**
	Hek293. HA/TLR9		Epithelial	**6.98E-02**

TLR9 levels are expressed in arbitrary units (AU), as a ratio of TLR9:β-actin mRNA

Table 2. CpG-ODN induced surface marker upregulation by cells in the presence or absence of 10103 CpG-ODN.

Cells	TLR9 exp'n	CpG-ODN (μg/ml)	Surface marker expression			
			CD54	CD80	CD86	MHCII
$CD19^+$	+	**0**	**28.4**	**13.7**	**23.0**	**8.9**
		3	**965.5*****	**141*****	**903*****	**161*****
B-CLL	+	**0**	**177.8**	**5.2**	**13.0**	**352.8**
		3	**474*****	**15*****	**59*****	**296*****
TH044	+	**0**	**17.0**	1.8	2.0	27.6
		3	**41.4****	3.0	2.7	2.4
FC029	+	**0**	**73.0**	**28.4**	**14.6**	**12.9**
		3	**330.8*****	**42.6***	**48.3****	**65.5****
380	+	**0**	**79.3**	**5.6**	**122.1**	**596**
		3	**564*****	**9.8****	**180****	**707****
Lila	+	**0**	**63.0**	5.9	101.9	**183.2**
		3	**245*****	5.0	98.3	**486*****
RAMOS	+	**0**	**447.7**	13.6	**35.0**	**31.1**
		3	**1475*****	13.0	**79.8*****	**112*****
Raji	+	**0**	735.1	63.2	**146.8**	673.6
		3	743.8	79.9	**286*****	550.3
BJAB	+	**0**	**712.3**	**78.3**	**494.6**	**772.8**
		3	**1467*****	**114****	**1050*****	**1046*****
RPMI	+	**0**	**557.9**	**6.8**	**81.4**	**11.2**
		3	**1109****	**12.6****	**116.3***	**21.3****
U266	-	**0**	635	13.0	18.2	54.2
		3	578.3	18.3	20.0	33.0
Karpas 707H	-	**0**	71.7	3.1	2.6	22.7
		3	79.5	3.5	3.1	27.2
HL60	-	**0**	1072	96.9	561.1	463.1
		3	889.2	64.3	389.9	307.9
KG-1	-	**0**	ND	1.6	33.7	93.1
		3		2.5	33.8	55.3
HeLa	-	**0**	141.1	4.5	6.4	4.1
		3	132.9	5.5	6.3	4.5
Hek293	-	**0**	6.0	6.2	6.3	6.8
		3	5.9	6.3	6.3	6.9
DU-145	-	**0**	302.3	2.4	2.2	2.6
		3	339.8	2.6	2.3	2.8
HRT-18	-	**0**	44.5	2.5	2.3	2.5
		3	2.2	2.3	2.2	2.4
MCF-7	-	**0**	24.2	4.8	6.4	4.2
		3	22.9	4.4	6.0	3.9
WM1361A	-	**0**	17.3	2.8	2.6	3.0
		3	18.8	3.0	2.6	2.9
Hek 293. pCl-neo	-	**0**	6.1	5.5	6.5	5.5
		3	6.3	4.3	5.0	5.8
Hek293. pDisplay	-	**0**	6.3	5.2	6.2	5.0
		3	6.4	5.0	6.0	5.4
Hek293. TLR9	+	**0**	13.6	8.5	11.2	6.6
		3	5.3	6.8	6.2	4.7
Hek293. HA/TLR9	+	**0**	4.6	4.3	4.5	4.0
		3	4.7	4.0	4.4	4.0

Data present the mean MFI reading of surface marker expression of at least three independent experiments in the absence (0) or presence (3) of 3μg/ml CpG-ODN 10103. Statistically significant differences between non-activated and CpG-ODN-activated cells are highlighted in bold type. * $P<0.05$, ** $P<0.005$, *** $P<0.001$. Cell lines were defined as TLR9 positive or negative on the basis of a TLR9:β-actin ratio of greater than (+) or less than (-) 1.0 $x10^{-2}$ AU. Normal human peripheral blood B cells (CD19+) were used as a positive control.

Table 3. Effect of CpG-ODN on the secretion of IL-6, IL-10 and TNF-α by cells in the presence or absence of 10103 CpG-ODN.

Cells	TLR9 expression	CpG-ODN (μg/ml)	Cytokine release (pg/ml)		
			IL-6	IL-10	TNF-α
CD19$^+$	+	**0**	**70.7±4.1**	**1.2±0.00**	**15.1±0.75**
		3	**1944±108.4****	**27.5±1.4****	**689.1±42.5****
B-CLL	+	**0**	**19.6±1.0**	**<1**	**<1**
		3	**873.7±63.4****	**676.1±21.25*****	**30.9±6.0***
TH044	+	**0**	**38.7±1.6**	**1.0±0.0**	**1.0±0.0**
		3	**1226±2.25*****	**11.0±0.3*****	**25.0±0.4*****
FC029	+	**0**	**19.0±0.35**	**2.0±0.3**	**4.0±0.4**
		3	**553.0±14.9*****	**173.0±5.3*****	**187.0±8.0****
LiLa	+	**0**	**<1**	**<1**	**<1**
		3	**170.8±5.7****	**21.7±3.0****	**4.0±0.8***
Ramos	+	**0**	<1	**13.0±0.4**	1.4±0.5
		3	<1	**107.8±2.45*****	1.4±0.5
BJAB	+	**0**	**1.0±0.2**	**443.8±11.9**	**4.85±1.7**
		3	**13.4±1.1****	**3240±72.7*****	**39.7±1.3****
RPMI	+	**0**	<1	19.8±1.0	**1.8±0.8**
		3	1.35±0.5	21.1±2.2	**39.0±4.5***
U266	-	**0**	1883.3±63.1	1.0±0.05	2.1±0.8
		3	1883.3±59.9	1.2±0.1	1.4±0.0
Karpas	-	**0**	<1	<1	1.4±0.5
		3	<1	<1	1.4±0.5
KG-1	-	**0**	<1	<1	<1
		3	3.4±1.5	<1	<1
HeLa	-	**0**	126.6±19.4	<1	<1
		3	114.8±12.9	<1	<1
DU-145	-	**0**	259.0±17.1	<1	1.9+0.0
		3	232.3+10.6	<1	1.4±0.5
HRT-18	-	**0**	<1	<1	<1
		3	<1	<1	<1
MCF-7	-	**0**	9.1±0.8	5.0±0.0	1.4±0.5
		3	5.8±0.8	5.0±0.0	1.4±0.0
WM1361A	-	**0**	52.0±3.8	<1	1.4±0.0
		3	33.1±3.2	<1	1.4±0.0
Hek 293. pCl-neo	-	**0**	3.8±0.0	<1	<1
		3	3.8±0.0	<1	<1
Hek293. pDisplay	-	**0**	4.2±0.4	<1	<1
		3	3.3±0.5	<1	<1
Hek293. TLR9	+	**0**	2.3±0.9	<1	<1
		3	2.4±0.0	<1	<1
Hek293. HA/TLR9	+	**0**	5.9±0.8	<1	<1
		3	5.5±0.4	<1	<1

Cytokine release in cell supernatants was assayed using Beadlyte® Human Multi-cytokine Beadmaster™ Kit (Milipore, UK). Data represent the mean concentration

(pg/ml) ± S.E.M of triplicates. Statistically significant differences between non-activated and CpG-ODN-activated cells are highlighted in bold type. * $P<0.05$, ** $P<0.005$, *** $P<0.001$. Cell lines were defined as TLR9 positive or negative on the basis of a TLR9:β-actin ratio of greater than (+) or less than (-) 1.0 $x10^{-2}$ AU. Normal human peripheral blood B cells (CD19+) were used as a positive control.

Table 4. CpG-ODN induced cellular proliferation of cells in the presence or absence of 10103 CpG-ODN.

Cells	TLR9 expression	CpG-ODN (μg/ml)	Cellular proliferation (cpm)
Medium	**Control**	**0** **3**	16±3 15±5
CD19$^+$	+	**0** **3**	**990.3±109.6** **20100±1418****
B-CLL	+	**0** **3**	**87.5±43.6** **1726±424.9*****
TH044	+	**0** **3**	**48.7±5.8** **103.0±1.7****
RAMOS	+	**0** **3**	**5515 ± 177.8** **8751±618.1****
RAJI	+	**0** **3**	**76784 ±3703** **101424±2258***
BJAB	+	**0** **3**	**90270±4934** **111900±3474 ****
RPMI	+	**0** **3**	**33106 ±1829** **61686±1881*****
U266	-	**0** **3**	35983 ±489.6 27079±897.6
Karpas	-	**0** **3**	8686 ± 274.0 8449±610.5
KG-1	-	**0** **3**	3597± 270.1 3652±211.7
HeLa	-	**0** **3**	65292±1597 62450±1469
Hek293	-	**0** **3**	112800 ±1244 93696 ±4263
DU-145	-	**0** **3**	3985±327.4 4614±603.3
HRT-18	-	**0** **3**	5081±658.1 5116±1276
MCF-7	-	**0** **3**	14863±1362 14692±2697
WM1361A	-	**0** **3**	20531±3660 16714±2358
Hek 293. pCl-neo	-	**0** **3**	212502±6534 224821±7842
Hek293. pDisplay	-	**0** **3**	2301±40.5 1885±96.7
Hek293. TLR9	+	**0** **3**	**144261±11076** **208265±1066***
Hek293. HA/TLR9	+	**0** **3**	**190330±5954** **243419±7555***

Cells were left unstimulated or activated with 10103 CpG-ODN for 3 days, and cellular proliferation was measured by [methyl-^{3}H] thymidine incorporation. Data present the mean of triplicate cultures (cpm) ± SD. Statistically significant differences between non-activated and CpG-ODN-activated cells are highlighted in bold type. * $P<0.05$, ** $P<0.005$, *** $P<0.001$. Cell lines were defined as TLR9 positive or negative on the basis of a TLR9:β-actin ratio of greater than (+) or less than (-) 1.0 $x10^{-2}$ AU. Normal human peripheral blood B cells (CD19+) were used as a positive control.

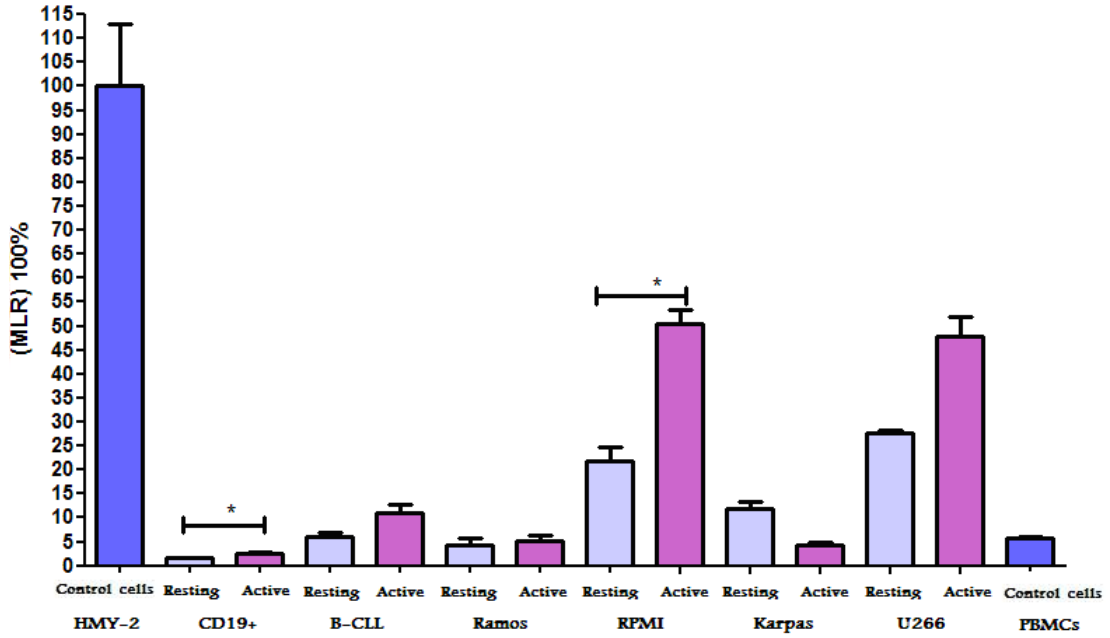
Table 5. NFκB activation in cells in the presence or absence of 10103 CpG-ODN.

Cells	TLR9 expression	CpG-ODN (μg/ml)	NFκB activation (RLU)
CD19$^+$	+	**0**	**143.3±7.1**
		3	**480.7±2.0*** **
B-CLL	+	**0**	**122.7±20.0**
		3	**789.7±45.5****
BJAB	+	**0**	**197.7±9.9**
		3	**703.0±39.2****
RPMI	+	**0**	**5086±188.2**
		3	**104869±7369****
BJAB neg. ctrl	**Control**	**0**	46.0±10.5
		3	49.7±12.9
HeLa	-	**0**	7766±102.0
		3	8009±79.6
Hek293	-	**0**	34007±133.8
		3	33205±410.7
Hek 293. pCl-neo	-	**0**	36735±2073
		3	29813±1391
Hek293. pDisplay	-	**0**	16336±132.5
		3	11130±345.4
Hek293. TLR9	+	**0**	**26037±351.6**
		3	**39860±790.1****
Hek293. HA/TLR9	+	**0**	**38208±1056**
		3	**73961±2766****
Hek293.HA/TLR9 neg.ctrl	**Control**	**0**	187.5±2.887
		3	176.5±39.84

Data represent the mean of luciferase activity (RLU) ± S.E.M of triplicates. Statistically significant differences between non-activated and CpG-ODN-activated cells are highlighted in bold type. * $P<0.05$, ** $P<0.005$, *** $P<0.001$. Cell lines were defined as TLR9 positive or negative on the basis of a TLR9:β-actin ratio of greater than (+) or less than (-) 1.0 x10^{-2} AU. Normal human peripheral blood B cells (CD19+) were used as a positive control. For each cell line tested, a control group of cells was left untransfected, and treated with CpG-ODN. Only baseline luciferase activity was detected, and no effect from CpG-ODN was seen (data only shown for BJAB and Hek293.HA/TLR9).

Figure 1

A.


(MLR) 100%
115
110
105
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
*
*
Control cells
Resting
Active
Resting
Active
Resting
Active
Resting
Active
Resting
Active
Resting
Active
Control cells
HMY-2
CD19+
B-CLL
Ramos
RPMI
Karpas
U266
PBMCs

B.

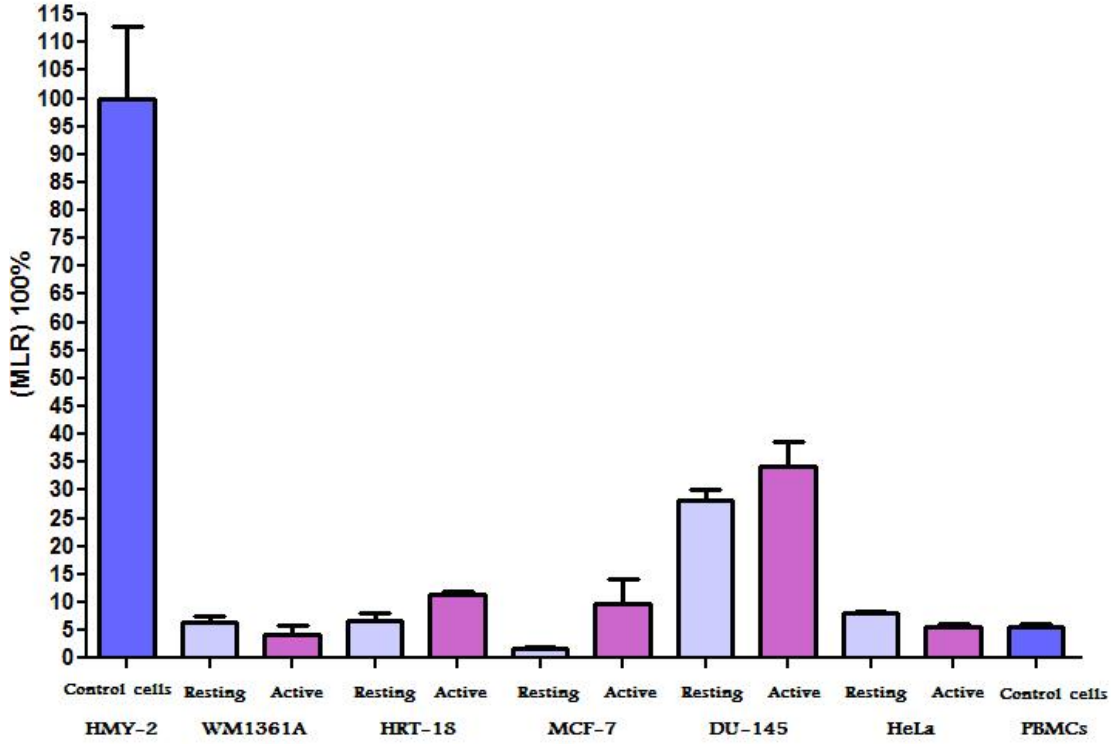
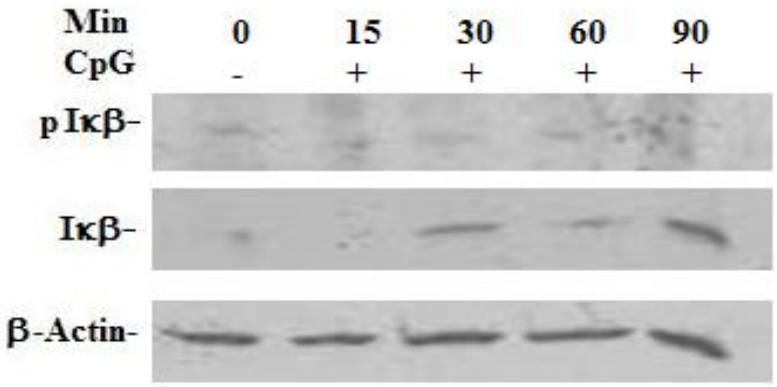
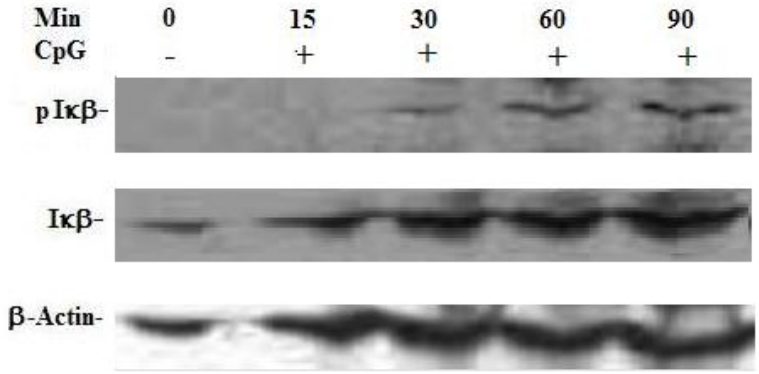

(MLR) 100%
115
110
105
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Control cells
Resting
Active
Resting
Active
Resting
Active
Resting
Active
Resting
Active
Control cells
HMY-2
WM1361A
HRT-18
MCF-7
DU-145
HeLa
PBMCs

Figure 2.

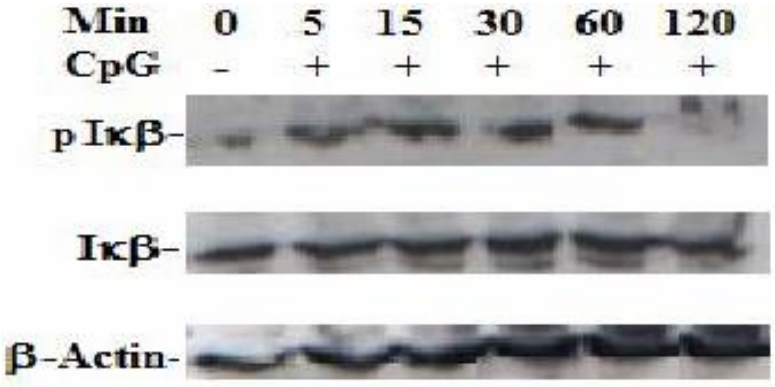
A.


Min
0
15
30
60
90
CpG
-
+
+
+
+
pIκβ-
Iκβ-
β-Actin-

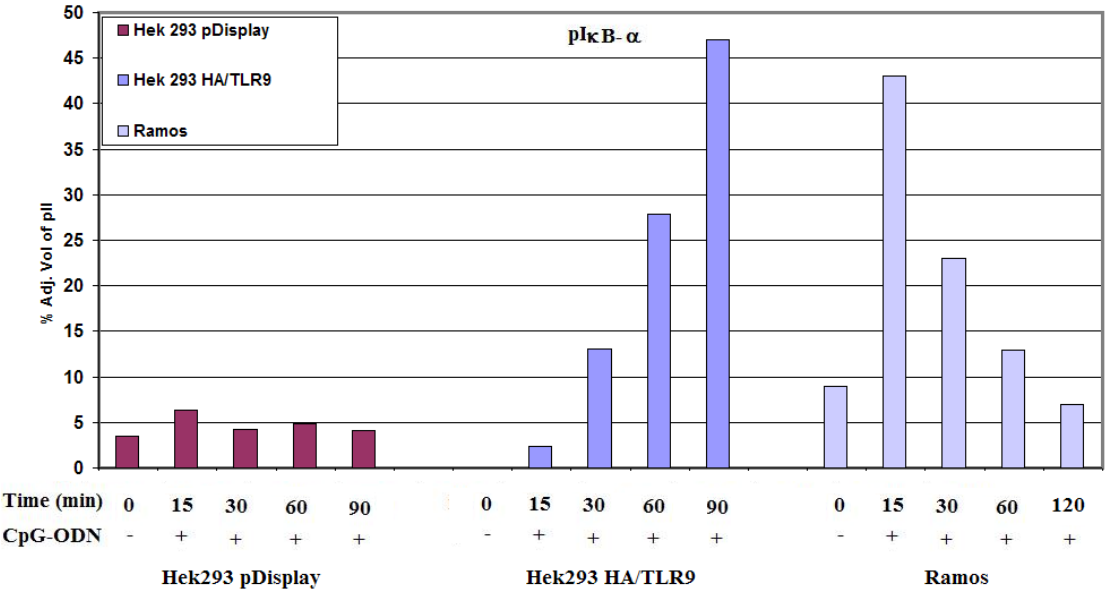
B.


Min
0
15
30
60
90
CpG
-
+
+
+
+
pIκβ-
Iκβ-
β-Actin-

C.


Min
0
5
15
30
60
120
CpG
-
+
+
+
+
+
pIκβ-
Iκβ-
β-Actin-

D.


Hek 293 pDisplay
Hek 293 HA/TLR9
Ramos
pIκB-α
% Adj. Vol of pII
50
45
40
35
30
25
20
15
10
5
0
Time (min) 0 15 30 60 90 0 15 30 60 90 0 15 30 60 120
CpG-ODN - + + + + - + + + + - + + + +
Hek293 pDisplay
Hek293 HA/TLR9
Ramos