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Suppression of B-cell activation and IgE, IgA, IgG1 and IgG4 production by mammalian telomeric oligonucleotides

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Abstract

Background: The fine balance of immunoglobulins (Ig) E, IgG1, IgG4 and IgA in healthy production is maintained by the interaction of B cells with adaptive and innate immune response. The regulation of toll-like receptors (TLRs)-driven innate and adaptive immune effector B-cell response and the role of mammalian telomeric TTAGGG repeat elements represent an important research area.
Methods: Human PBMC and purified naive and memory B cells were stimulated with specific ligands for TLR2, TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 in the presence or absence of telomeric oligonucleotides. B-cell proliferation, differentiation and antibody production were determined.
Results: TLR9 ligand directly activates naive and memory B cells, whereas TLR7 can stimulate them in the presence of plasmacytoid dendritic cells. Human B cells proliferate and turn into antibody-secreting cells in response to TLR3, TLR7 and TLR9, but not to TLR2, TLR4, TLR5 and TLR8 ligands. Stimulation of B cells with intracellular TLR3, TLR7 and TLR9 induced an activation cascade leading to memory B-cell generation and particularly IgG1, but also IgA, IgG4 and very low levels of IgE production. Mammalian telomeric oligodeoxynucleotide (ODN) significantly inhibited all features of TLR ligand-induced events in B cells including B-cell proliferation, IgE, IgG1, IgG4, IgA production, class switch recombination, plasma cell differentiation induced by TLR3, TLR7 and TLR9 ligands.
Conclusion: B cells require specific TLR stimulation, T-cell and plasmacytoid dendritic cell help for distinct activation and Ig production profiles. Host-derived telomeric ODN suppress B-cell activation and antibody production demonstrating a natural mechanism for the control of overexuberant B-cell activation, antibody production and generation of memory.

The fine balance of immunoglobulins (Ig) E, IgG1, IgG4 and IgA is deviated to exuberant production of allergen-specific IgE and weak production of IgG4 in allergic individuals (1). The interaction of the environmental factors including allergens and microbial components with innate and adaptive immune system results in the class switch recombination of immunoglobulins (2). Cells of the innate immunity equipped with toll-like receptors (TLRs) have an immense capacity to recognize a diverse spectrum of microorganisms including bacteria, viruses, fungi and parasites (1–4). While TLR10 ligand is yet to be described in humans, the specific ligands of TLR1 to TLR9 drive innate and adaptive immune effector cells towards inflammation and microbicidal activity (3, 4). TLR1, 2, 4, 5 and 6 are expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 have been shown to

Abbreviations
APRIL, A proliferation-inducing ligand; BAFF-R, B-cell activating factor of the TNF family (BAFF) receptor; BCMA, B-cell maturation antigen; ODN, oligodeoxynucleotide; TACI, transmembrane activator and cyclophilin ligand interactor; AICDA, activation-induced cytidine deaminase.

be expressed in the endosomes of cells (3–6). The intracellular localization of the latter TLRs allows bacteria and virus to be degraded in the endosomes/lysosomes after engulfment and phagocytosis in the innate immune cells during infection. This process is induced by the association of double-stranded RNA to TLR3, single-stranded RNA to TLR7 and TLR8 and unmethylated CpG motifs containing DNA to TLR9 (4, 7–12). TLR distribution intensity in the immune system cells and ligands for TLR has a critical role to understand host–pathogen interactions and the effects on the adaptive immunity. TLR expression shows different aspects in mouse and human cells, especially in B cells. Mouse B cells proliferate and differentiate upon LPS and CpG stimulation (13, 14), whereas human B cells respond to CpG (15) but not to LPS stimulation (16).

Bacterial DNA contains immunostimulatory CpG motifs that stimulate dendritic cells, NK cells and B cells and lead to their proliferation, maturation and secretion of a variety of cytokines, chemokines and immunoglobulins (2, 17, 18). It has been reported that suppressive ODN motifs can selectively block CpG-mediated immune activation. Suppressive motifs are rich in poly 'G' or 'GC' sequences and are present in the DNA of mammals and certain viruses. Suppressive oligodeoxynucleotides (ODNs) expressing arrays of the hexameric TTAGGG repetitive elements patterned after mammalian telomeres (19) have been demonstrated to block CpG-induced cytokine production *in vitro* and inhibit CpG-induced inflammatory reactions such as lethal endotoxic shock in mice (20).

Therefore, we postulated that a natural inhibitory mechanism mediated by mammalian telomere motifs could help to restore B-cell homoeostasis. Stimulation of human PBMC via endosomal-associated TLRs as well as direct activation of naive or memory B cells with CpG ODN and plasmacytoid dendritic cell maturation by TLR7 induces polyclonal B-cell activation, proliferation and IgE, IgA IgG1 and IgG4 production suggesting a mechanism of immunological memory. After class switching in naive B cells, memory B cells and plasma cells that produce immunoglobulin E (IgE^+ cells) develop through a germinal centre IgE^+ intermediate cell without an IgG1 phase. In addition, cellular IgE memory resides in IgE^+ memory B cells, and $IgG1^+$ memory B cells are not an important source of IgE memory (21). Here, we demonstrate that a synthetic analogue of mammalian telomeres containing four repeats of TTAGGG motif suppresses B-cell activation, proliferation and antibody production induced directly or indirectly by TLR3, TLR7 and TLR9 ligands.

Materials and methods

Isolation and purification of cells

PBMCs were obtained from heparinized whole blood or buffy coats from healthy individuals by Ficoll (Biochrom KG, Berlin, Germany) density gradient centrifugation. Cells were washed three times and resuspended in RPMI 1640 medium supplemented as previously described (22). B cells were purified by magnet-activated cell separation after labelling of cells with anti-CD19 microbeads (MACS; Miltenyi Biotec AG, Bergisch Gladbach, Germany). The purity of B cells was >94% as assessed by flow cytometric analysis of cells stained with ECD-labelled anti-CD19 mAb (Immunotech; Beckman Coulter, Marseille, France).

Following B-cell staining with anti-CD19 mAb (Dako AG, Baar, Switzerland) and anti-CD27 mAb (Immunotech, Beckman Coulter), naive and memory B cells were double sorted by flow cytometric sorter (FACS Vantage SE; Beckton Dickenson, Pharmingen, Basel, Switzerland). The purity of naive and memory B cells was >99% as assessed by flow cytometric analysis of cells stained with PE-labelled anti-CD19 mAb (Dako AG, Baar, Switzerland), PC5-labelled anti-CD27 mAb (Immunotech, Beckman Coulter) and FITC-labelled anti-CD123 mAb (Miltenyi Biotec AG). Naive B cells contained <0.1% $CD27^+$ memory B cells and <0.5% plasmacytoid dendritic cells, and memory B cells contained <1% $CD27^-$ B cells and <0.9% plasmacytoid dendritic cells.

Plasmacytoid dendritic cells were isolated from PBMC by positive selection using antibodies against BDCA-4 coupled to microbeads (FcRγ) blocking reagent and the AutoMACS magnetic separation system (Miltenyi Biotec, Bergisch Gladbach).

Cultures of PBMC and B cells

Ligands for TLRs were Pam3-Cys-Ser-$(Lys)_4$ for TLR2 (Calbiochem AG, Laufelfingen, Switzerland), poly(I:C) for TLR3 (Amersham Biosciences, Lausanne, Switzerland), E. Coli-derived lipopolysaccharide for TLR4 (Sigma Chem. Co., Buchs, Switzerland), flagellin for TLR5 (Calbiochem AG), 3M-001 for TLR7 (3M Pharmaceuticals, Minneapolis, MN, USA), 3M-002 for TLR8 (3M Pharmaceuticals) as described previously (23) and CpG2006 for TLR9 (Microsynth GmbH, Balgach, Switzerland). Phosphorothioate-modified ODNs were synthesized at the Microsynth GmbH, Balgach, Switzerland. Sequences of the phosphorothioate ODNs used were as follows: CpG2006 ODN: 5′-TCGTCGTTTGTCGTTTTGTCG TT-3′, control for immunostimulatory ODN (non-CpG): 5′-ATGCACTCTGCAGGCTTCTC-3′, telomeric ODN: 5′-TTAGGGTTAGGGTTAGGGTTAGGG-3′, base scrambled 'control ODN': 5′-ACATAGTCTCTCTCTAGTCAG TCT-3′. All reagents and ODNs used in this study contained <0.1 U/mg endotoxin. There was no cell toxicity of the ODN as investigated by annexin-V and 7-amino actinomycin D staining after 48 h up to 10 μM doses (24). Recombinant IL-2 and IL-4 were from Novartis, Basel, Switzerland. Soluble CD40 ligand (sCD40L) was obtained from the transfected cell line 8-40-1 (sCD40L-CD8-α fusion protein; Institute for Immunology, Basel, Switzerland) (25).

5×10^5 PBMCs or purified naive or memory B cells were stimulated in 500 μl with different TLR ligands in 48-well plates (Costar Corporation, Cambridge, MA, USA). After initial experiments for dose optimization, Pam3-Cys-Ser-$(Lys)_4$ was used at 0.6 μM, poly(I:C) at 25 μg/ml, lipopolysaccharide at 100 ng/ml, flagellin at 1.8 μM, 3M-001 and 3M-002 at 1 μM, CpG2006 and control ODN at 1 μM. IL-2 was used at 4 ng/ml, IL-4 was 25 ng/ml, sCD40L was at 20% v/v

as supernatant (26), IFN-α and IFN-β were 100 ng/ml where indicated. TTAGGG telomeric ODN and control ODN were used in titrated doses and 1 μM in other experiments. Cells were pulsed with 1 μCi/well [^{3}H] thymidine (Dupont and NEN Life Science Products, Basel, Switzerland) for the last 8 h of incubation, and [^{3}H] thymidine incorporation was performed at day 3 for transformed B cells and T cells and at day 5 for primary B cells.

Analysis of B-cell activation and proliferation by FACS, determination of IgG1, IgG4, IgA and IgE by ELISA, Ig-secreting cells by ELISPOTs and immunoglobulin mRNA transcription determination by real-time PCR (refer to Methods in the Supporting Information).

Statistical analysis

Results are shown as mean ± SD. Nonparametric statistical comparison between groups was performed by Mann–Whitney *U*-test. The number of donors indicated in the figure legends includes all donors analysed in the relevant experiment.

Results

TLR3, TLR7 and TLR9 stimulations induce B-cell proliferation, which is suppressed by telomeric ODN

Activation and clonal expansion of B cells is an essential event for immunoglobulin production. To investigate the role of different TLRs on human B-cell activation, proliferation and different isotypes of immunoglobulin production, we used different TLR-stimulated PBMCs, purified B cells and naive and memory B-cell subsets isolated from peripheral blood of healthy humans. First, PBMCs were stimulated with ligands of TLR2, TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 in the presence of IL-2 for 5 days. [^{3}H] thymidine incorporation was determined after 5 days. Ligands for TLR3, TLR7 and TLR9 induced significant cell proliferation. Human telomeric ODN significantly suppressed TLR3-, TLR7- and TLR9-mediated PBMC proliferation in a dose-dependent manner (Fig. 1A) reaching up to 60%, 90% and 80% inhibition at 5 μM doses, respectively (Supporting Information Fig. S1A). For comparison, control ODN did not show significant suppressive activity (Fig. S1B). Telomeric ODN, 1 μM, significantly suppressed PBMC proliferation stimulated by TLR3, TLR7 and TLR9 ligands (Fig. 1B).

The next step was to identify the proliferating cell type within PBMC upon TLR3, TLR7 and TLR9 stimulations. Interestingly, stimulation of TLR3, TLR7 and TLR9 induced $CD19^+$ B cell, but not $CD4^+$, $CD8^+$ T-cell or $CD16^+$ NK cell proliferation (Fig. 2). Mammalian telomeric ODN significantly inhibited B-cell proliferation induced by TLR3, TLR7 and TLR9.

The exact identification of B-cell subsets is instrumental to understand their dynamics under physiological and pathological conditions. Human memory B cells are currently identified according to the expression of CD27, which is absent on naive B cells. It was shown that naive and memory B cells respond differently to the same TLR ligand especially to CpG (16, 27, 28). In addition, it was recently demonstrated that under certain conditions including cell–cell contact and appropriate cytokine and growth factor support, $CD27^-$ naïve B cells increase TLR9 expression, proliferate in response to CpG stimulation without B-cell receptor signalling and differentiate into plasma cells (27). To specifically differentiate the proliferative responses of naive and memory B cells induced by TLR3, TLR7 and TLR9 stimulation, highly purified $CD19^+CD27^-$ naive and $CD19^+CD27^+$ memory B cells were stimulated. Upon TLR9 stimulation, naïve and memory B cells proliferated without the help of type 1 IFNs or plasmacytoid dendritic cells (Fig. 3A, B). TLR3 and TLR7 ligands, however, failed to directly induce the proliferation of highly pure naive and memory B cells. TLR7 stimulated a small fraction of naïve B cells in the presence of plasmacytoid dendritic cells, and memory B cells in the presence of IFN-α, IFN-β or plasmacytoid dendritic cells. Again, mammalian telomeric ODN significantly suppressed TLR7- and TLR9-ligand-induced $CD19^+CD27^-$ naive and $CD19^+CD27^+$ memory B-cell proliferation independent of the combination with IFN-α, IFN-β and the presence of plasmacytoid dendritic cell help.

These data demonstrate direct and indirect activation and clonal expansion of B cells by TLR stimuli. TLR3 and TLR7 ligands cannot directly stimulate naive and memory B cells. In a mixed population of PBMC, when they coexist with the T-cell help or dendritic cells, they become capable of B-cell stimulation. As a strong stimulator for B cells, CpG induces direct activation of memory and a fraction of naive B cells. TLR7 stimulates B cells in the presence of IFN-α, IFN-β secreted by the plasmacytoid dendritic cells.

Regulation of class switch recombination and IgE, IgG1, IgG4, IgA production by TLR3, TLR7, TLR9 ligands and telomeric ODN

B cells are continuously exposed to substances that stimulate the innate immune response from commensal flora bacteria in respiratory and gastrointestinal mucosas and they produce immunoglobulins against pathogens. After demonstration of the difference in TLR3-, TLR7- and TLR9-ligand-induced direct or indirect B-cell proliferation, we examined the effects of these ligands on AICDA mRNA expression and immunoglobulin synthesis by B cells and PBMC as well as the role of IL-2, IL-4 and sCD40L stimulation.

PBMCs were stimulated with ligands for TLR3, TLR7 and TLR9 in the presence and absence of telomeric ODN and control ODNs. IL-2, IL-4 and sCD40L were used to mimic T-cell help and promote B-cell clonal expansion. IgE, IgG1, IgG4 and IgA were measured on day 5 cell lysates (as mRNA) and day 12 culture supernatants (Fig. 4A, B, C). IgE antibody synthesis and Iε-Cε RNA transcripts expression were induced by TLRs in combination with IL-4 and sCD40L, but not induced with IL-2 and sCD40L in healthy individuals (data not shown).

As observed in B-cell proliferation, the profiles of immunoglobulin production showed differences in PBMC and purified B cells (Fig. 4A, B and Fig. S2). In PBMC, stimulation via TLR3, TLR7 and TLR9 even in the absence of IL-2 induced IgG1, IgG4 and IgA in detectable quantities. IL-2

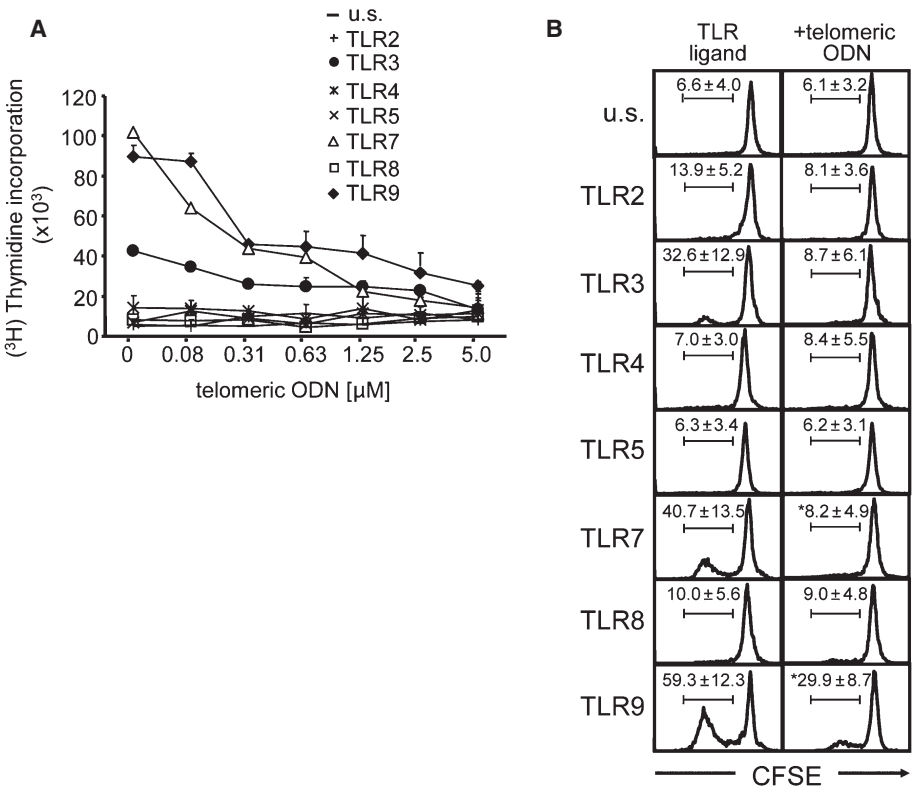

Figure 1 Suppression of TLR-induced human peripheral blood mononuclear cell (PBMC) proliferation. (A) Suppression of TLR-induced PBMC proliferation at different concentration of telomeric oligodeoxynucleotide (ODN). Human PBMCs 10^6/ml were stimulated with ligands for TLR2 (0.6 μM, Pam3-Cys-Ser-(Lys)4), TLR3 (25 μg/ml, poly I:C), TLR4 (100 ng/ml, lipopolysaccharide), TLR5 (1.8 μM, flagellin), TLR7 (1 μM, 3M-001), TLR8 (1 μM, 3M-002) and TLR9 (1 μM, CpG2006) in the presence of 4 ng/ml IL-2. Telomeric ODN was titrated 1:2 starting at 5 μM. To determine proliferative responses on day 5, cells were pulsed with 1 μCi/well [3H] Thymidine, and [3H] Thymidine incorporation was determined after 8 h. (B) To further analyse the effects of TLRs and telomeric ODN on PBMC proliferation, the decrease in CFSE intensity of the cells was measured by flow cytometry and was used as a measure of the extent of cell division. Human PBMCs from healthy donors were labelled with CFSE and stimulated with TLR2, TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 ligands in the presence of IL-2 with and without 1 μM of telomeric ODN. TLR-induced cell proliferation stained by CFSE was determined on day 5 with flow cytometry. Compared to the unstimulated control group, TLR3, TLR7 and TLR9 ligands induced significant cell proliferation. Even at higher doses used in preliminary experiments, TLR2, TLR4 TLR5 and TLR8 ligands did not induce detectable cell proliferation in human PBMC. Mean ± SD of percentage of proliferating cells in five different experiments is shown in each histogram. Telomeric ODN significantly suppressed cell proliferation in all conditions ($P < 0.01$).

significantly increased the synthesis of IgG1, IgG4 and IgA stimulated with TLR3, TLR7 and TLR9 ligands. Stimulation of cells with sCD40L had no significant effect on the production of immunoglobulins in the absence of IL-2, whereas IL-2 and sCD40L combination boosted IgG1 levels, and they had no additional contribution to the induction of IgG4 and IgA. Increased expression of Iγ-Cγ1, V_HDJ_H-Cγ4 and Iα1/2-Cα1/2 RNA transcripts for IgG1, IgG4 and IgA in IL-2 and sCD40L-stimulated PBMC with TLR3, TLR7 and TLR9 verified our results (Fig. 4C). Consistent with the proliferation studies, telomeric ODN significantly suppressed IgE, IgG1, IgG4 and IgA secretion and their mRNA expression independent of the type of TLR ligand in the presence of IL-4, IL-2 and sCD40L experimental conditions. Upon exposure to the infections or immunizations, activation-induced cytidine deaminase (AICDA) regulates class switch recombination and somatic mutation in B cells (29). The expression of AICDA mRNA was measured after 5 days (Fig. 4D). Telomeric ODN suppressed the expression of AICDA mRNA even in PBMC cultured with IL-2 + sCD40L and in combination with TLR3, TLR7 and TLR9 ligands. Control ODN did not show any stimulatory or inhibitory effect on the effect of TLR ligand and telomeric ODN.

We then investigated the effect of TLR3, TLR7 and TLR9 stimulation on antibody-forming cell frequency and whether it is also inhibited by telomeric ODN. Human PBMCs were stimulated with TLR3, TLR7 and TLR9 ligands in the presence of telomeric ODN, IL-2 and sCD40L. Secreted immunoglobulins were captured on ELISPOT plates between day 10 and day 11 of cultures. Stimulation via TLR3, TLR7 and TLR9 significantly increased IgG1, IgG4 and IgA producing cell frequency (Fig. 5A). Particularly, a relatively high frequency of cells produced IgA after TLR3, TLR7 and TLR9 ligand stimulation. It has to be noted here that secreted

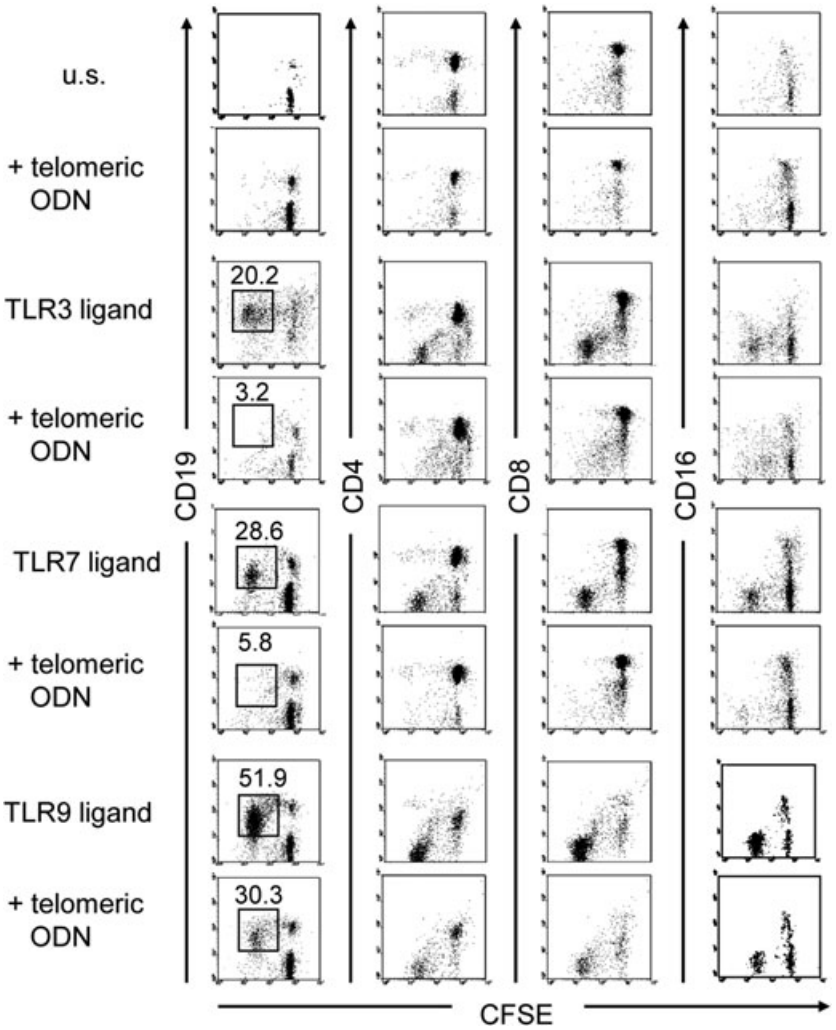

Figure 2 Telomeric ODN inhibits B-cell proliferation induced by TLR3, TLR7 and TLR9. Human PBMCs from healthy donors were labelled with CFSE and stimulated with ligands for TLR3, TLR7 and TLR9 in the presence of IL-2 with and without telomeric ODN at the concentrations indicated in the Fig. 1. Cells were counterstained with CD19, CD4, CD8 and CD16. The numbers in the boxes represent the percentage of the proliferating CD19-stained cells. One representative of four experiments is shown. Telomeric ODN significantly suppressed $CD19^+$ cell proliferation in all conditions ($P < 0.01$). In these experiments, IL-2 consistently induced the expansion of B cells with these three stimuli in PBMCs. IL-2 also induced the proliferation of a small fraction of CD4, CD8 and CD16 positive cells. This fraction was more visible in $CD4^+$ cells, however, was always less than 4.5% of the cells and was not suppressed by telomeric ODN.

immunoglobulins determined in the supernatants by ELISA represent a cumulative value after 12 days. However, ELISPOTs represent the number of Igs-secreting cells in a certain time fragment within the course of cultures. Telomeric ODN significantly suppressed the number of antibody-forming cells producing IgG1, IgG4 and IgA in all experiments (Fig. 5B) supporting the data obtained by ELISA.

Suppression of TLR3, TLR7 and TLR9 ligand induced B-cell differentiation by telomeric ODN

As B cells undergo differentiation towards plasma cells to produce immunoglobulins, we next aimed to investigate whether B-cell differentiation is affected by TLR ligands and whether telomeric ODNs influence B-cell differentiation. Human PBMCs were double stained with mAbs for CD19, CD20, CD27, CD38, CD80, CD86, B-cell activating factor of the TNF family (BAFF) receptor, transmembrane activator and cyclophilin ligand interactor (TACI) and B-cell maturation antigen (BCMA), which are expressed on B cells depending on the stage of maturation and activation of the B cell. Stimulation of PBMC via TLR3, TLR7 and TLR9 in the presence of IL-2 resulted in a significant increase in the numbers of $CD19^+$ B cells. These proliferating B cells expressed high levels of CD27, CD38 and CD86, demonstrating that a fraction of proliferating B cells stayed as memory B cells and

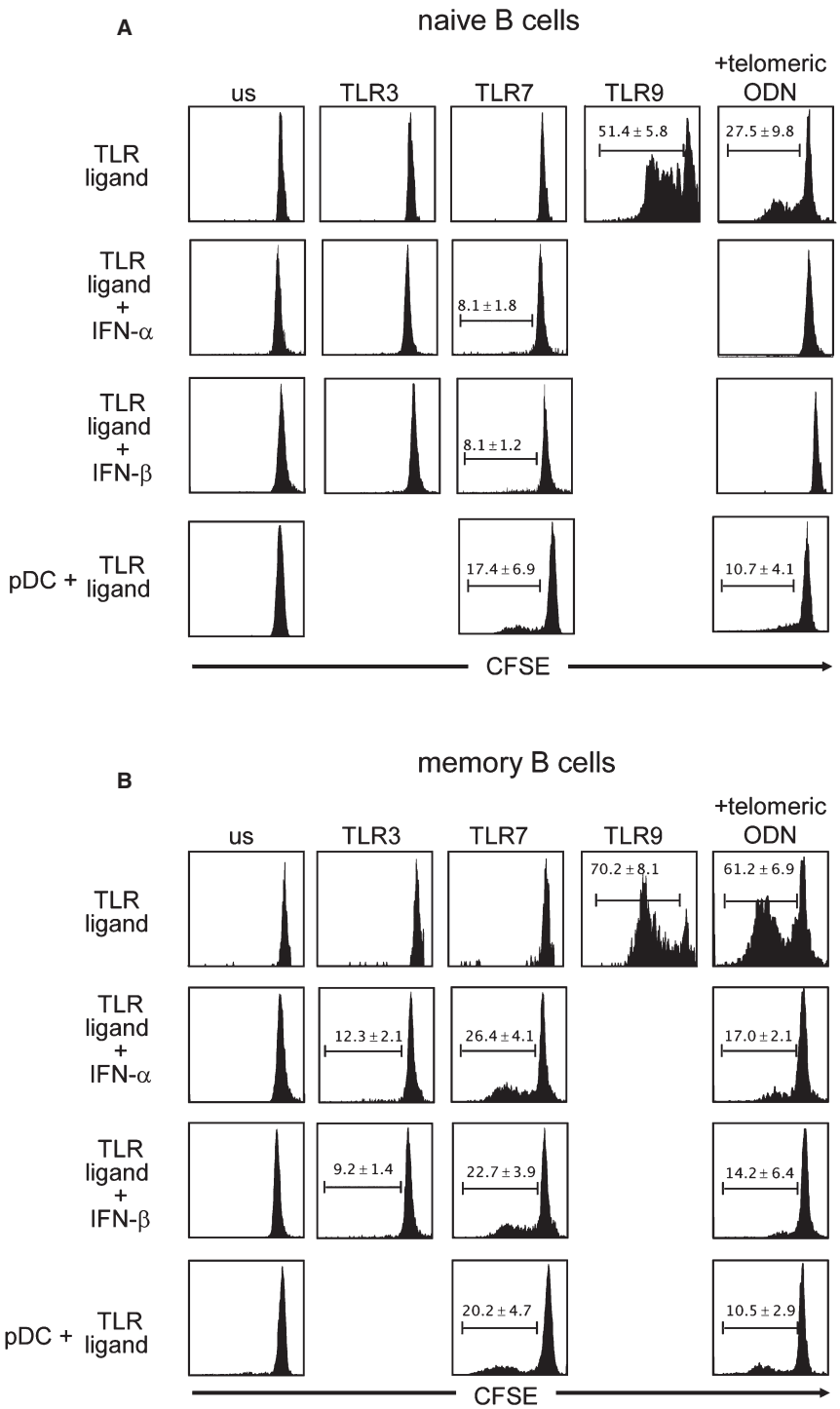

Figure 3 Distinct stimulation of naive and memory B-cell proliferation by TLR3, TLR7 and TLR9 ligands and suppression by telomeric ODN. Purified $CD19^+CD27^-$ naive and $CD19^+CD27^+$ memory B cells were labelled with CFSE to investigate their proliferation by TLR3, TLR7 and TLR9 stimulation with and without interferon-alpha (100 ng/ml), interferon-beta (100 ng/ml). Human plasmacytoid dendritic cells 4×10^4 were added to naïve and memory cells. (A) TLR9 ligand induce the proliferation of naive B cells. The addition of plasmacytoid dendritic cells induces the proliferation of naïve B cells by TLR7 ligand. (B) High proliferation rate of memory B cells with CpG (TLR9 ligand) and moderate proliferation by TLR7 ligand with IFN-α, IFN-β and the addition of plasmacytoid dendritic cells. The proliferation rates of naïve and memory B cells were suppressed by telomeric ODN. One representative of three experiments is shown.

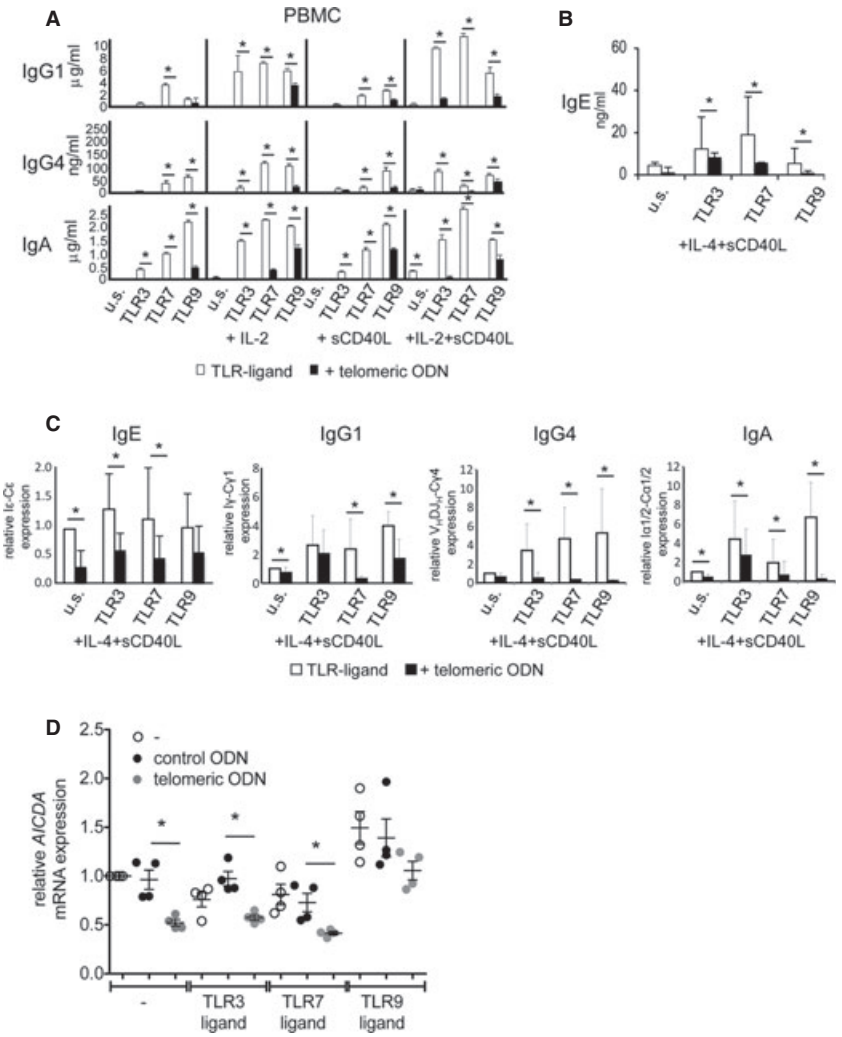

Figure 4 Different requirements in IgE, IgG1, IgG4 and IgA production by PBMC with TLR3, TLR7 and TLR9 stimulation and their suppression by telomeric ODN. (A) IgG1, IgG4 and IgA immunoglobulin synthesis by PBMC 0.5×10^6/ml in 500 μl in 48-well plates was stimulated with TLR3, TLR7 and TLR9 ligands in the absence or presence of IL-2 and sCD40L or both. TLR7 and TLR9 ligands and telomeric ODN were used at 1 μM, and TLR3 ligand was used at 25 ug/ml. IgG1, IgG4 and IgA were measured in day 12 supernatants. (B) PBMCs 0.5×10^6/ml in 500 μl in 48-well plates were stimulated with TLR3, TLR7 and TLR9 ligands in the presence of IL-4 and sCD40L. IgE was measured in day 12 supernatants by ELISA. (C) Relative Iε-Cε, Iγ-Cγ1, V_HDJ_H-Cγ4, Iα1/2-Cα1/2 RNA transcripts for IgE, IgG1, IgG4 and IgA quantified with real-time PCR. The measurements after TLR ligand stimulations were expressed as fold increase relative to the measurement from unstimulated cells. Results are representative of four experiments. (D) Suppression of AICDA mRNA expression by telomeric ODN. PBMCs cultured with sCD40L + IL-2 were stimulated TLR3, TLR7 or TLR9 ligands alone or in combination with telomeric or control ODN. mRNA expression of AICDA was measured after 5 days and its expression is calculated relative to sCD40L+IL-2. Horizontal bar shows mean (±SEM). Results are representative of four independent experiments. $*P < 0.05$.

their APC function was increased and some of them matured to antibody-forming cells (Fig. S3A). The formation of memory B cells ($CD19^+CD27^+$) was significantly inhibited by telomeric ODN. This was in parallel to suppression of $CD20^+$, $CD86^+$ and CD38 high$^+$ B cells. The expression rate of BAFFR, TACI and BCMA showed different patterns depending on TLR ligand types (Fig. S3B). TLR3 stimulation induced moderate increase in BAFFR, TACI and BCMA expression; TLR7 stimulation induced TACI and BCMA expression, and finally, the expression rate of these B-cell surface markers was not stimulated by TLR9 ligand. $CD27^+$ and $CD86^+$ B cells development, and $TACI^+$ and $BCMA^+$ B cells development were suppressed by telomeric ODN. In contrast, BAFFR-expressing B cells were not suppressed, whereas BAFFR-negative B cells showed a significant decrease by telomeric ODN.

Finally to evaluate the plasma cell differentiation in B cells, we determined surface CD138 expression in parallel to

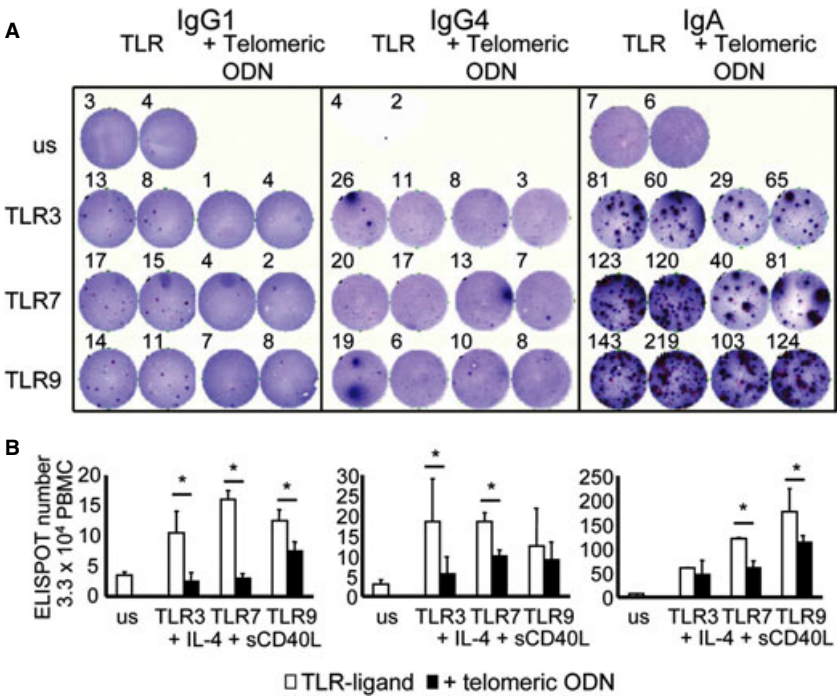

Figure 5 The number of TLR3-, TLR7- and TLR9-ligand-induced IgG1, IgG4 and IgA producing cells decreases by telomeric ODN. (A) Human PBMCs were stimulated with TLR3, TLR7 and TLR9 ligands with and without telomeric ODN. 3×10^4 PBMCs from each condition on day 10 were incubated for 24 h. The ELISPOTs of duplicate wells for each treatment condition on ELISPOT plates are shown. The numbers of spots in each well is indicated on upper left corner. (B) Results representative of four independent experiments are shown in the bar graphs. The *open* bar represent TLR stimulation and the *filled* bars represent the addition of telomeric ODN for IgG1, IgG4 and IgA spots number. $*P < 0.05$.

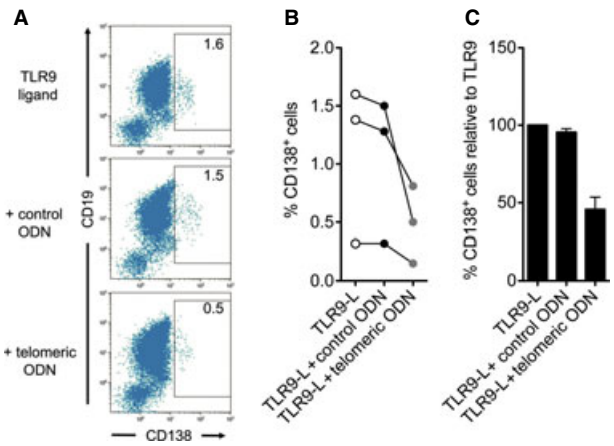

Figure 6 Suppression of plasma cell differentiation by telomeric ODN. PBMCs cultured with sCD40L+IL-4 were stimulated TLR3, TLR7 or TLR9 alone or in combination with telomeric or control ODN. (A) Surface CD138 expression was measured by flow cytometry after 10 days culture. $CD3^-CD16^-CD14^-7\text{-}AAD^-$ cells were gated. Stimulation with TLR3 or TLR7 ligands did not induce detectable CD138 expression. One representative example is shown as dot plots. (B) Connected dots show percentage of $CD138^+$ from three independent experiments. (C) Bar graphs show mean (±SEM) percentages of $CD138^+$ cells relative to TLR9 ligand stimulated cells (set to 100%). Results are representative of three independent experiments, $*P < 0.05$.

$CD19^+$ expression. Surface CD138 expression, which is a highly and specific marker of plasma cells, was determined in 10 days of PBMC cultures with sCD40L + IL-4 and stimulated by TLR3, TLR7 or TLR9 alone or in combination with telomeric or control ODN. Stimulation with TLR3 or TLR7 ligands did not induce detectable CD138 expression, but TLR9 ligand induced its expression at the level of 1.6% of $CD138^+CD19^+CD3^-CD16^-CD14^-7\text{-}AAD^-$ cells (Fig. 6A).

Control ODN did not change the ratio of $CD138^+CD19^+$ cells induced by TLR9 ligand. However, telomeric ODN significantly suppressed the $CD138^+CD19^+$ plasma cell differentiation from B cells induced by TLR9 ligand (Fig 6B, C).

Discussion

The present study demonstrates that TLR3, TLR7 and TLR9 have distinct stimulatory pathways for B-cell activation and immunoglobulin production. We demonstrate here a synthetic analogue of mammalian telomeres suppresses direct and indirect effects of TLR3, TLR7 and TLR9 ligands as strong inducers of B-cell proliferation, differentiation and immunoglobulin production.

Here, we report that in addition to direct stimulation of B cells via TLR9, stimulation of PBMC via TLR3 and TLR7 indirectly leads to human B-cell proliferation and immunoglobulin production. Although TLR3, TLR7 and TLR9 are expressed at the same cellular location, in the endosomes, their expression rates differ depending on the type of the cells. In addition to TLR9, human peripheral blood B cells express high levels of TLR1, TLR6 and TLR10, intermediate levels of TLR7 and low levels of TLR2 and TLR4, but not TLR3 (28). In the present study, we demonstrate that stimulation via TLR9 directly activates $CD27^+$ memory B cells and a fraction of $CD27^-$ naive B cells. As shown by Huggins et al. (27), CpG stimulate not only $CD27^+$ memory B cells, but $CD27^-$ naïve B cells under special *in vitro* conditions. The present study demonstrates that CpG stimulates highly purified naïve and memory B cells and this stimulation is potentialized in the presence of IL-2. Both TLR3 and TLR7 stimuli significantly induced B-cell proliferation only when PBMCs were stimulated, suggesting that there is an indirect effect on B cells mediated by other cells or mediators that are present within the PBMC population. The role of other factors was demonstrated in the present study by co-cultures of highly purified cells. The role of IL-2 is demonstrated as one of the essential factors for B-cell proliferation and antibody production by TLR3 and TLR7 stimuli. It was recently shown that IFN-α released by plasmacytoid dendritic cells upon stimulation with TLR7 was responsible for B-cell polyclonal expansion and differentiation into Ig-producing plasma cells (30). In the present study, the proliferation of purified human B cells, particularly memory B cells, in the presence of IFN-α, IFN-β and plasmacytoid dendritic cells induced by TLR7 shows that this stimulatory effect was mediated via plasmacytoid dendritic cells. The activation of plasmacytoid dendritic cells by TLR7 results in the secretion of IFN-α and IFN-β that induce B-cell activation, proliferation and IgE, IgG1, IgG4 and IgA production.

Whereas previous studies reported that TLR9 stimulation by CpG inhibited CD40/IL-4-driven IgE synthesis in human B cells and IgG1 and IgE synthesis in murine B cells (31, 32). A recent study by Ozcan et al. (33) demonstrates that murine naïve B cells were stimulated by TLR9 ligand with the cooperation of CD40, TACI and IL-4 produce IgG1 and IgE. PBMC stimulation via TLR3, TLR7 and TLR9 even in the absence of IL-2, IL-4 and sCD40L induced IgG1, IgG4 and IgA, in detectable quantities and IgE with the combination of IL-4 and sCD40L. In contrast to PBMC, purified human $CD19^+$ B cells produce IgG1, IgG4 and IgA upon TLR9 stimulation but not TLR3 and TLR7 (Fig. S2). The production of IgG1, IgG4 and IgA was increased by TLR3 and TLR7 in the presence of IL-2 and this response was boosted when combined with sCD40L, but sCD40L alone did not induce additive effect on purified B cells. The production of IgE by purified B cells was not induced by TLR3, TLR7 and TLR9 in the presence of IL-4 and sCD40L contrary to the findings in PBMC. The production amount of IgE by intracellular TLRs is not robust when compared to IgG1, IgG4 and IgA in PBMC and, moreover, was not detectable in human purified B-cell cultures. This finding suggests that intracellular TLR ligands may have a critical role in changing the balance against IgE and more in the direction of IgG1, IgG4 and particularly IgA in allergic diseases (26).

In the present study, we investigated step by step all aspects of B-cell activation, proliferation, Ig class switch recombination, AICDA expression and plasma cell differentiation and demonstrated that mammalian telomeric ODN significantly suppressed all of the features. Suppression of B-cell activation and Ig production by suppressor ODN represents an important way for the treatment of diseases related to B-cell over activation. It can also be used for allergic diseases. Rituximab, a chimeric mAB to CD20, that efficiently eliminates circulating and overactive B cells that have CD20 on their surfaces and is therefore used to treat diseases characterized by high number of active B cells. A pilot study was already conducted in extrinsic atopic dermatitis characterized by highly elevated concentrations of IgE, and the early results of treatment with rituximab suggest that it may be a promising treatment option for patients with severe atopic dermatitis (34, 35).

Previous studies have demonstrated that suppressor ODN possessing four repeats of TTAGGG motifs inhibits CpG-induced inflammation in mice (20, 36, 37). Human telomeres consist of tandem repetitive arrays of the hexameric sequence TTAGGG (19). The enzyme telomerase is a cellular ribonucleoprotein responsible for adding telomeric repeats onto the 3′ ends of chromosomes and thus compensates for cell division-associated telomere loss (37). Elongation of telomeres with the telomerase results in an extended lifespan of a cell. Therefore, it was suggested that the length of the telomere rather than telomerase itself is responsible for limiting cell proliferation. Suppressive activity of TTAGGG motifs has been demonstrated to correlate with the ability of the sequence to form G-tetrads (18). Individual mammalian cells contain phosphodiester TTAGGG motifs and some of them including some synthetic analogues are in single-stranded form. Therefore, it can be expected that high cellular concentrations of TTAGGG elements with suppressive capacity can be released to microenvironment following host cell death or following cell division. It is possible that self-DNA released by injured cells could down-regulate TLR-mediated inflammation response.

Another way to control TLR responses is the TLR tolerance phenomenon, which is explained by the unresponsiveness of the immune cells to the same or different TLR ligands upon the occurrence of repeated or chronic stimulation through TLRs (38). The tolerant state in macrophages and B cells was associated with reduced NF-κB, MAPK activation and c-Jun phosphorylation (39). However, B cells may receive general signals through TLRs and specific signals through BCR. The prestimulation of B cells with TLR7 may result in reduced B-cell proliferation and IgM secretion upon subsequent TLR7 restimulation, but simultaneous BCR signalling may prevent or reverse TLR7- or TLR9-mediated TLR tolerance in human B cells. This distinct response of TLRs and BCR to the TLR7 and TLR9 ligands may be a potential way to study the effects of telomeric ODNs on B-cell responses induced by TLR ligands. The TLR9 ligand, which is nucleic acid sequence, may bind to TLR9 localized in the endosomes. Telomeric ODNs that are also nucleic acid sequence too may have a potential to regulate B-cell response similar to the tolerance phenomenon that takes place by repeated or chronic stimulation through TLRs.

In both PBMC and B-cell cultures, mammalian telomeric ODN suppressed TLR3-, TLR7- and TLR9-ligand-induced B-cell proliferation without regarding direct or indirect stimulation. Whereas the generation of both TACI and BCMA positive and negative B cells were suppressed, the BAFFR-expressing B cells were resistant to suppression by telomeric ODN. Moreover, telomeric ODN significantly suppressed the proliferation of the antibody-forming cells. These findings were fully supported by demonstration of significant decrease in secreted Igs to the supernatants of PBMC and purified B cells by telomeric ODN. It remains to be elucidated whether the different levels of B-cell memory and serum antibodies to different pathogens depend on the amount of cell death and released telomeric ODN to the microenvironment during exposure and generation of the immune response.

It has been suggested that environmental stimuli such as CpG maintain continuous polyclonal memory B-cell activation, proliferation and differentiation (40). In this way, a constant level of plasma cells and serum antibodies could theoretically be maintained throughout a human lifespan. Our study showed that in addition to CpG, TLR3 and TLR7 ligands have a capacity to induce B-cell activation in PBMC, which was significantly enhanced by IL-2. These findings demonstrate that direct or indirect stimulation of human B cells through endosomal-associated TLRs plays a pivotal role in polyclonal B-cell activation and immunoglobulin production to maintain long-term serological memory in humans. Suppression of this response by host-derived telomeric ODN suggests a natural mechanism to overcome hyperactivation of B cells and hyperproduction of immunoglobulins under chronic inflammatory conditions. The distinct effects of intracellular TLR3, TLR7 and TLR9 and telomeric ODN on B-cell activation, proliferation, AICDA expression, Ig class switch recombination, IgE, IgG1, IgG4, IgA production and plasma cell differentiation suggest a crucial role for the immunomodulation of B cell-mediated diseases as well as allergic inflammation.

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Author contributions

C.S. designed and performed experiments, collected and analysed data, and wrote the paper; W.v.V. performed experiments of AICDA expression and CD138 expression on plasma cells; M.A. performed ELISPOT experiments, collected and analysed data, and developed Ig ELISAs; O.U.S. performed experiments, ELISA and RT-PCR analyses; J.Z. assisted experiments, performed data analysis and collected data; O.K. provided scientific input by data discussions and evaluation of the data, and suggestions for scientific direction; S.S.A. provided TLR7 and 8 ligands and designed experiments with them, interpreted and discussed data; I.G. provided TLR9 ligand and telomeric ODN, and assisted in data analysis and wrote the paper; C.A. was coordinator of the study, provided financial support as principal investigator for this study, put essential scientific input into discussions and giving directions and wrote the paper.

Conflict of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Percentage suppression of TLR-induced PBMC proliferation at different concentration of telomeric ODN.

Figure S2. Different requirements in IgG1, IgG4 and IgA production by B cells with TLR3, 7 and 9 stimulation and their suppression by telomeric ODN.

Figure S3. TLR3-, TLR7- and TLR9-ligand induced B-cell differentiation and suppression by telomeric ODN.

Data S1. Methods.

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