

Original article

Cytokine and Ig-production by CG-containing sequences with phosphorodiester backbone and dumbbell-shape

Background: Immunostimulatory oligodeoxynucleotides (CpG-ODN) usually contain phosphorothioate (PS) backbones for nucleotide protection, which may result in some nonspecific side-effects like prolongation of coagulation time.

Objective: The aim of the present study was to investigate the immunomodulatory potential of DNA molecules without PS backbones. Thus, we designed phosphorodiester (PO) molecules with a dumbbell-like covalently-closed structure (dSLIM-30L1).

Methods: We analyzed their effects on peripheral blood mononuclear cells (PBMC) from spontaneous high and low immunoglobulin (Ig)E producer (allergic and nonallergic donors) in comparison with linear CpG-ODN (lin-30L1) with PS backbones, using enzyme-linked immunosorbent assay and flow cytometry.

Results: We observed a decrease of spontaneous IgE levels in PBMC from high IgE producer of approximately 27% with both dSLIM-30L1 and lin-30L1. In addition, both molecules enhanced the production of IgA, IgM and IgG1/IgG2, but with a slightly different pattern. Both molecules stimulated the secretion of the T_H1 -like cytokines interleukin (IL)-2, interferon- γ and IL-12p40 and the pro-inflammatory cytokine IL-6. The immunomodulatory potential of dSLIM-30L1 and lin-30L1 was also effective in PBMC from nonallergic donors, as was confirmed for IL-2, IL-12p40, IgG1/IgG2 and IgM.

Conclusion: Our data show an inhibition of IgE production but also enhancement of the inflammatory cytokine response in PBMC from allergic and non-allergic donors by covalently-closed PO-based dSLIM-30L1 with a pattern similar to that of linear PS-based lin-30L1, while avoiding PS-modifications and thus PS-mediated side-effects. Whether such molecules are useful for the treatment of allergic diseases will need further clarification by appropriate *in vivo* studies.

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Atopic dermatitis (AD) belongs to the atopic diseases, such as allergic rhinitis or allergic asthma, and is a frequently occurring chronic skin disease primarily affecting younger age groups (1). The extrinsic AD is characterized by a deviated immune response with hyper reactivity to various, normally tolerated antigens of the environment. Clinically AD is characterized by subacute and chronic eczematous skin lesions. Especially severely affected patients exhibit elevations of total serum immunoglobulin (Ig)E and the presence of several specific IgE levels towards common allergens. The affected skin sites are infiltrated mostly by $CD4^+$ T cells and eosinophils,

but also harbor an increased number of mast cells (2, 3). The pathophysiology of AD includes several immunological deviations: an unknown genetic predisposition leads to an imbalanced T_H2 -type dominated immune response with consecutive interleukin (IL)-4, IL-5, IL-6 and IL-10 production resulting in eosinophil activation, but also IgE production. Subsequently, skin infiltrating macrophages and eosinophils produce IL-12, promoting interferon (IFN)- γ production and initiating the chronic phase of AD (4, 5).

Thus, molecules with T_H1 -promoting properties may be useful for the prevention of allergic diseases, including AD, if the primary T_H2 -type response is inhibited. One group of such immunostimulatory compounds are oligodeoxynucleotide with nonmethylated cytosine-guanine motifs (CpG-ODN), which resemble microbial DNA and serve as 'danger signals'. These CpG-ODNs not only increase the production of the pro-inflammatory cytokine

Abbreviations: AD, atopic dermatitis; CG, cytosine-guanine dinucleotide; dSLIM, double stem-loop immunomodulator; IL, interleukin; IFN- γ , interferon- γ ; Ig, immunoglobulin; ODN, oligodeoxynucleotide; PBMC, peripheral blood mononuclear cells; PO, phosphorodiester; PS, phosphorothioate; T_H , T helper cell.

IL-6, but also promote a T_H1-response with induction of IL-12 and IFN- γ secretion, and natural killer and dendritic cell activation (6–8). Therefore, they have been successfully used as T_H1-promoting adjuvants (9–11). To circumvent the degradation of linear single-stranded ODN with phosphorodiester (PO) backbones usually ODN with phosphorothioate (PS) modifications were used, enhancing their stability *in vitro* and *in vivo* (12–16). In addition to the increased stability, such PS-based CpG-ODN have been shown to be more potent in stimulating B cell proliferation than PO-based CpG-ODN (6, 17).

Immunization with allergens in mice with plasmids containing cytosine-guanine dinucleotide (CG) motifs is more effective in inhibiting IgE formation and inflammation than with plasmids lacking CG motifs (18). Furthermore, a previous study indicated that PS-ISS inhibits IgE production and stimulates IFN- γ and IL-12 production in human peripheral blood mononuclear cells (PBMC) from atopic patients (19, 20). However, PS-modifications may result in several unwanted side-effects: first, ODN with PS backbone lead to prolongation of the blood clotting time via inhibition of intrinsic tenase complex (21). Secondly, PS-ODN can bind nonspecifically to various proteins, i.e. transcription factors, and thus may affect cell signaling (22). Thirdly, PS-based ODN result in acute toxicities in rhesus monkeys via complement activation (23, 24). Fourthly, specific PS-ODN may lead to severe side-effects in mice, like reduced immune responses, lymphoid follicle destruction and organ enlargement (25). A possibility to avoid PS-modification is to protect the open ends of linear PO-ODN by generating a short covalently-closed dumbbell-like structure (26). These dumbbells show an increased stability toward cellular nuclease degradation without PS-modification resembling natural DNA (27, 28).

In the present study, we designed a PO-based molecule with a covalently-closed structure (dSLIM-30L1) and studied its immunomodulatory potential on PBMC from patients with AD and healthy donors in comparison with PS-based linear CpG-ODN (lin-30L1).

Material and methods

Patients

Heparinized blood samples were obtained from polysensitized AD patients from the Department of Dermatology and Allergy of the Charité Berlin (Germany). All of them were sensitized towards pollen, house dust mites and cat dander proven by positive skin prick test and specific serum IgE levels ranging from 0.35 to 100 KU/ml. The blood drawings from AD patients for *in vitro* investigations were approved by the local ethics committee. We determined the total serum IgE levels in all patients by using the Pharmacia CAP system (Uppsala, Sweden) according to the manufacturer's instructions. The basal IgE levels in PMBC from AD patients ($n = 18$; age: range 21–45 years; mean 31 years) after 10 days of culture ranged from 0 to 45.5 ng/ml (mean 5.7 ng/ml)

Table 1. Characterization of the allergic patients

Patient number	Basal IgE production* (ng/ml)	Age (years)	Sex
AD 1	45.5	27	f
AD 2	15.8	38	f
AD 3	13.9	38	f
AD 4	13.6	37	f
AD 5	3.6	32	m
AD 6	2.6	36	m
AD 7	2.5	22	f
AD 8	1.5	29	f
AD 9	0.9	45	f
AD 10	0.9	36	m
AD 11	0.8	22	f
AD 12	0.7	21	m
AD 13	0.3	32	m
AD 14	0.2	26	m
AD 15	0.0	22	f
AD 16	0.0	36	m
AD 17	0.0	24	f
AD 18	0.0	31	f

*Levels in the supernatants from PBMC (10^6 /ml) cultured for 10 days (mean of AD patients: 5.7 ng/ml).

(Table 1). As control heparinized blood samples were obtained from healthy, age-matched donors, who were nonallergic by history.

Immunomodulatory molecules

For generation of the double-stem-loop immunomodulating oligonucleotide (dSLIM-30L1) homodimers of 5' phosphorylated single stranded hairpin oligonucleotides (5'-CCTAGGGGTACCACCTTCATTGGAAAACGTTCTTCGGGGCGTTCTTAGGTGGTAACC-3') were produced by ligation with T4-DNA ligase (MBI Fermentas, Vilnius, Lithuania) resulting in a covalently-closed molecule (29). Nonligated oligonucleotides were degraded with T7-DNA-polymerase (MBI Fermentas) and the product purified by anion-exchange chromatography on Fractogel EMD-DMAE resin (Merck, Darmstadt, Germany) followed by ethanol precipitation. The DNA was resuspended in phosphate buffered saline (PBS). The content of endotoxins was determined by an end-point LAL-Test (Biowhittaker, Walkersville, MD, USA) to be below 10 EU/mg DNA. The dSLIM-30L1 is covalently-closed by single stranded loops of 30 nucleotides on both sides of the molecule each of which contains three nonmethylated CG motifs (Fig. 1). The linear PS-based CpG-ODN has the sequence 5'-TCATTGGAAAACGTTCTTCGGGGCGTTCTT-3' (PS-backbone is underlined) and is termed lin-30L1. Control molecules without CG motifs (dSLIM-no30L1, lin-no30L1) were designed with TG dinucleotides instead of CG dinucleotides, exchanging one pyrimidine (C) with another (T). Other usual conversions (CG $\rightarrow$ GG or CG $\rightarrow$ GC) would have created new CG motifs.

Cell culture

Cell preparation was performed as described previously (30, 31). Briefly, PBMC were separated from heparinized whole blood by Ficoll density gradient centrifugation (Biochrom AG, Berlin, Germany) and washed repeatedly in PBS. Cell cultures were performed in flat-bottomed 24- and 96-well plates in RPMI 1640 medium supplemented with 10% FCS, glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μ g/ml).

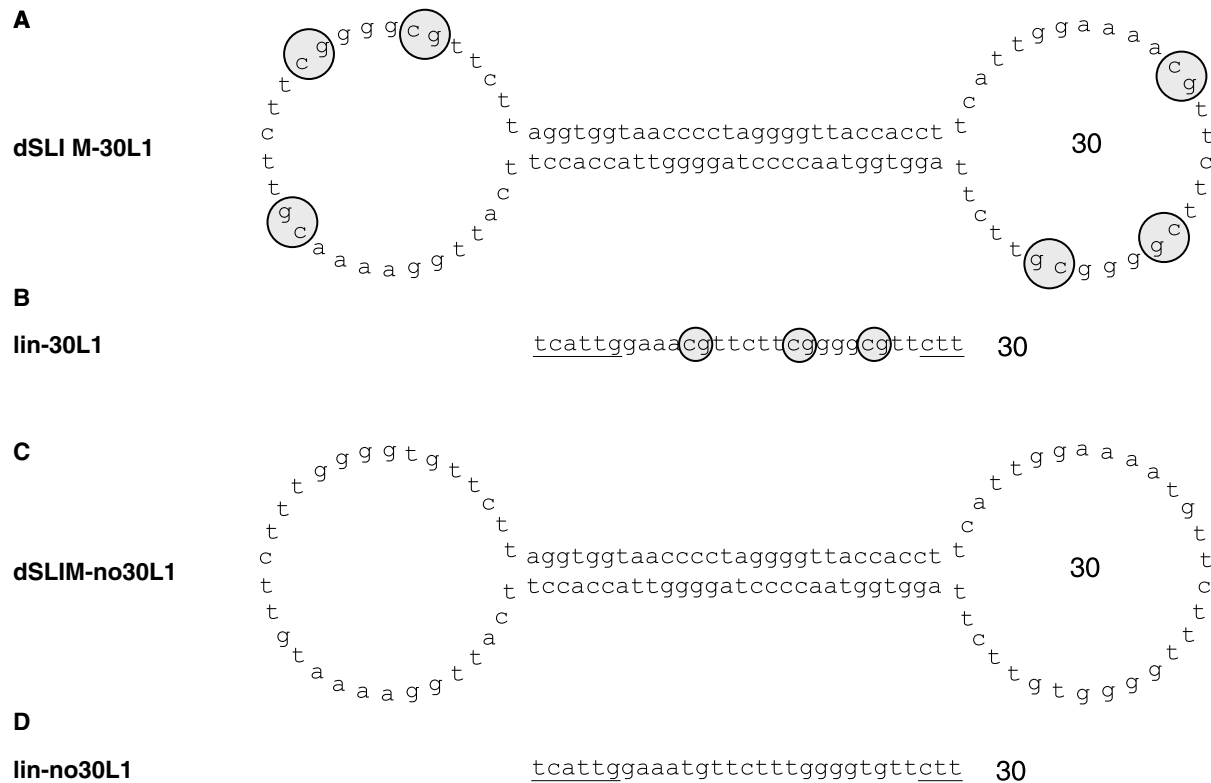


Figure 1. Sequence and structure of the double stem-loop immunomodulator (dSLIM). dSLIM-30L1 (A) and lin-30L1 (B) and control molecules dSLIM-no30L1 (C) and lin-no30L1 (D). dSLIM-30L1 has a double stem-loop phosphodiester (PO)-structure with 28 bp in the stem and 30 nucleotides in each loop. Each loop also contains three cytosine-guanine dinucleotide (CG) motifs (circles). The control dSLIM-no30L1 lacks the CG motifs instead of TG nucleotides. Lin-30L1 is a linear molecule with a PO-backbone, three CG motifs (circles) and partially PS-backbone (underlined). The control lin-no30L1 lacks the CG motifs instead of TG nucleotides.

ELISA

Immunoglobulin and cytokine enzyme-linked immunosorbent assay (ELISA) was performed with supernatants of 10^6 cells/ml which were cultured for 10 or 2 days, respectively (30, 31). Immuno plates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with the respective primary antibody (anti-human IgE-Fc mAb HP 6061, 2 µg/ml; IgA 0.48 µg/ml; IgM 0.36 µg/ml diluted in 0.1 M bicarbonate buffer). Then the wells were blocked for 1 h with 2% BSA-Tris buffer. After washing, supernatants and internal standards were incubated in duplicates for 3 h at room temperature. Thereafter, the second antibody (biotinylated anti-human IgE detection mAb HP 6029, 10 µg/ml, IgA 0.12 µg/ml; IgM 0.12 µg/ml) was added and incubated for another 2 h. The wells were incubated after washing for 1 h with alkaline phosphatase conjugated streptavidin. Following the final reaction with phosphatase substrate (Sigma, Dreieich, Germany), plates were read in a micro plate ELISA reader at 405 nm, and the amount of the respective Ig was calculated according to the standard curve. Cytokine levels were determined in supernatants of 1×10^6 PBMC/ml cultured for 48 h according to the manufacturer's instruction (IFN-γ and IL-2: R&D Systems, Minneapolis, MN; IL-6: Becton Dickinson, Heidelberg, Germany; IL-12p40: Biosource, Ratingen, Germany). The sensitivity ranges of the ELISAs were: IgE 0.2–50 ng/ml, IgA 0.5–30 mg/dl, IgM 0.5–

30 mg/dl, IL-2 15–1000 pg/ml, IFN-γ 31–2000 pg/ml, IL-12p40 3.5–500 pg/ml, IL-6 31–2000 pg/ml.

Flow cytometry

Flow cytometric analysis was performed with PBMC which had been cultured for 4 days in a concentration of 1×10^6 cells/ml. After washing with PBS, 1×10^6 cells were resuspended in 100 µl staining buffer (2% BSA in PBS; pH 7.4; 0.1% sodium azide), triple-stained with 20 µl anti-CD19-PerCP (Becton Dickinson), 10 µl anti-CD138-PE (Becton Dickinson) and with 10 µl of anti-CD23-FITC antibody (Biosource), and incubated on ice for 30 min. The cells were then washed with PBS. The isotypes IgG1-FITC, IgG1-PerCP and IgG1-PE (Becton Dickinson) were used as negative controls. The measurements were carried out using a FACSCalibur flow cytometer (Becton Dickinson) and evaluated using CellQuest software.

Statistics

Statistical analysis was performed using the paired *t*-test (two sided). Comparison of specific stimulation *vs* nonstimulated control is shown while using the relative values. A *P*-value lower than 0.05 is considered as statistically significant.

Results

Dumbbell-shaped PO-based dSLIM

Common linear CpG-ODNs are protected via PS-modifications. As these PS are known to account for side-effects like prolongation of coagulation, unspecific binding of proteins and acute toxicities in primates (21–24), we developed a covalently-closed structure with PO-backbone and a dumbbell-like shape (Fig. 1). This double stem-loop immunomodulatory molecule (dSLIM-30L1) has a stem of 28 base pairs and two loops with each 30 nucleotides including three CG motifs. dSLIM-30L1 is compared with a linear PS-containing CpG-ODN with the same nucleotide sequence, lin-30L1, to estimate its immunomodulatory potential in AD (Fig. 1). To provide the same number of CG motifs, we used 1 μ M dSLIM-30L1 and 2 μ M lin-30L1, when indicated.

dSLIM-30L1 and lin-30L1 decrease spontaneous IgE production in PBMCs from AD patients

Phosphorothioate-based CpG-ODN are known to decrease the synthesis of IgE from allergic donors (19, 20). We incubated PBMC from AD patients with dSLIM-30L1 and lin-30L1 *in vitro* and determined the spontaneous IgE production using an ELISA. Both dSLIM-30L1 and lin-30L1 decreased the spontaneous IgE secretion comparably (mean decrease: 27.8 and 27.3%; *P*-values: 0.033 and 0.097, respectively) (Fig. 2A). We then investigated the effect of dSLIM on CD40/IL-4-dependent IgE production. The data show that incubation of PBMCs with dSLIM significantly inhibited IgE production after CD40/IL-4 stimulation as well (mean decrease 62.6%; *P*-value: 0.023) (Fig. 2B).

Stimulation with dSLIM-30L1 and lin-30L1 enhances secretion of IgM, IgA and IgG

We examined next whether the synthesis of IgM and IgA, which are known to be stimulated by PS-ODNs, were also affected in by dSLIM-30L1 and lin-30L1 treatment of PBMC from AD patients. The data show that both dSLIM-30L1 and lin-30L1 increased IgA (12.3- and 2.3-fold; *P*-values: 0.007 and 0.134, respectively) and IgM production (7.7- and 21.5-fold; *P*-values: 0.005 and 0.009, respectively) (Fig. 3A and C). The stimulation of IgM by dSLIM-30L1 and lin-30L1 was confirmed in PBMC from healthy donors (Fig. 3B). However, lin-30L1 exhibited a stronger stimulatory activity on IgM production, whereas dSLIM-30L1 has a higher ability to stimulate IgA production and this difference was significant (IgA: 0.032; IgM: 0.037). To investigate the effect of dSLIM-30L1 on Ig production in more detail, we analyzed IgG1 and IgG2 levels after stimulation with the respective immunomodulator. Intriguingly, both dSLIM-30L1 and lin-30L1 moderately enhanced IgG2 and IgG1 and production in PBMC from AD patients (*P*-values for IgG1: dSLIM-30L1,

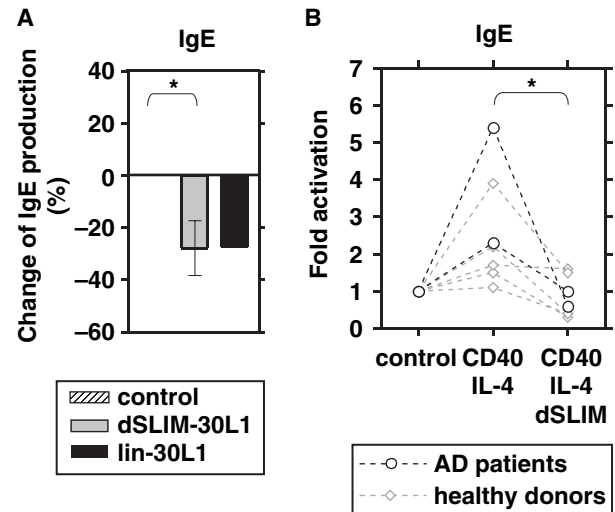


Figure 2. Double stem-loop immunomodulator (dSLIM) inhibits basal and CD40/IL-4-stimulated IgE production from allergic donors. (A) Peripheral blood mononuclear cells from atopic dermatitis patients were incubated for 10 days with the respective immunomodulator (no treatment, $n = 8$; dSLIM-30L1, $n = 8$; lin-30L1, $n = 6$). The respective IgE level compared with no treatment is shown in percent (mean $\pm$ SE). Concentrations of molecules were as follows: dSLIM-30L1 1 μ M and lin-30L1 2 μ M. (B) Treatment of PBMC from healthy donors (circles) and AD patients (squares) with CD40/IL-4 with and without the respective dSLIM ($n = 7$). Each circle/square represents one sample and different treatments of this sample are connected via lines. Concentrations of dSLIM molecules were 1 μ M. Statistical significances are denoted as follows: * < 0.05 .

$P = 0.130$; lin-30L1, $P = 0.207$; for IgG2: dSLIM-30L1, $P = 0.009$; lin-30L1, $P = 0.019$), with dSLIM having moderate trend towards a higher IgG2/IgG1 ratio (Fig. 3D). Because of the low basal level of IgG1 and IgG2, we confirmed the results with total IgG production in PBMC from healthy donors. As expected, both molecules increased total IgG production, indicating their broad immunomodulatory potential (data not shown).

B-cell differentiation by dSLIM-30L1 and lin-30L1

As production of various Igs was enhanced by dSLIM-30L1, we analyzed the effect of dSLIM-30L1 on the phenotype of B-cells in the analyzed population. After stimulation of PBMC with dSLIM-30L1 or lin-30L1 we determined the numbers of CD19⁺, CD23⁺ and CD138⁺ cells via flow cytometric analysis. We detected a significant higher percentage of CD19⁺ B-cells after stimulation with dSLIM-30L1 (1.6-fold; *P*-value: < 0.001), which was slightly higher compared to lin-30L1 (1.2-fold; *P*-value: 0.127) (Table 2). This higher percentage of CD19⁺ B-cells may be a result of B-cell proliferation or other effects on cell composition. Regarding

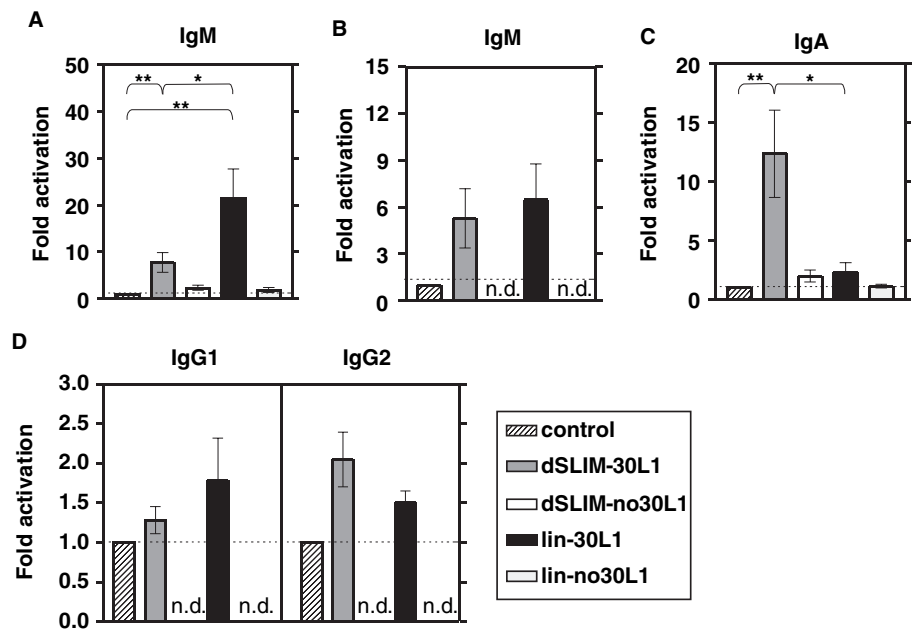


Figure 3. Double stem-loop immunomodulator (dSLIM) enhances IgM, IgA and IgG secretion. (A) IgM, (C) IgA. Peripheral blood mononuclear cells (PBMC) from atopic dermatitis (AD) patients were incubated for 10 days with the respective immunomodulator (no treatment, $n = 18$; dSLIM-30L1, $n = 18$; dSLIM-no30L1, $n = 8$; lin-30L1, $n = 10$; lin-no30L1, $n = 10$). (D) IgG1 (left) and IgG2 (right). The fold increase of respective Ig level compared with no treatment is shown (mean $\pm$ SE). Absolute values (pg/ml) for AD patients were as follows: no treatment, IgM: 19.8 ± 5.7 , IgA: 24.0 ± 6.5 ; dSLIM-30L1, IgM: 90.6 ± 16.5 , IgA: 130.9 ± 31.9 ; lin-30L1, IgM: 169.5 ± 38.8 , IgA: 37.1 ± 2.9 . (B) IgM. PBMC from healthy donors ($n = 3$). Concentrations of molecules were used as follows: dSLIM-30L1 or dSLIM-no30L1 1 μ M and lin-30L1 or lin-no30L1 2 μ M. n.d., not determined. Statistical significances are denoted as follows: * < 0.05 ; ** < 0.01 .

Table 2. Effect of double stem-loop immunomodulator (dSLIM) on the percentage of CD19⁺ B-cells from allergic patients

Treatment	CD19 ⁺ B-cells* (mean)
Control	1.0 $\pm$ 0.0
dSLIM-30L1	1.6 $\pm$ 0.1
lin-30L1	1.4 $\pm$ 0.1

Flow cytometry of peripheral blood mononuclear cells treated for 4 days with the respective immuno-modulator; concentrations of molecules: 1 μ M dSLIM-30L1 and 2 μ M lin-30L1.

*The fold increase of CD19⁺ cell numbers compared with no treatment control is shown (mean $\pm$ SE); $n = 10$.

CD23⁺ (low affinity IgE receptor) or CD138⁺ (plasma cells) expression, the total amount of positive cells was low (CD23⁺: mean 0.03%, range 0.01–0.06%; CD138⁺: mean 0.28%, range 0.02–0.69%) and we did not find any significant changes in positive cell numbers or density of marker molecules on the cell surface when comparing dSLIM-30L1 and lin-30L1-treated to untreated PBMC (data not shown).

dSLIM-30L1 and lin-30L1 induce cytokine production

The potential of directing T_H1-responses through increased production of the cytokines IL-2, IFN- γ and IL-12 is another known property of CpG-ODN (32).

Therefore, we stimulated PBMC from AD donors with dSLIM-30L1 and lin-30L1 and analyzed the secretion of IL-2, IFN- γ , and IL-12 subsequently. Both, dSLIM-30L1 and lin-30L1 stimulated the secretion of these cytokines (Fig. 4A, C and D). The induction of IL-2 and IFN- γ production by both, dSLIM-30L1 and lin-30L1, was significant and comparable (IL-2: 2.0- vs 2.9-fold, P -values: 0.002 vs 0.003; IFN- γ : 3.7- vs 4.4-fold, P -values: 0.003 vs 0.002). However, dSLIM-30L1 led to a lower secretion of IL-12p40 than lin-30L1 but both changes were significant (1.8- vs 3.6-fold, P -values: 0.017 vs 0.035). The induction of IL-2 and IL-12p40 was confirmed with PBMC from healthy donors, in which both dSLIM-30L1 and lin-30L1 stimulated the IL-2 and IL-12p40 production (Fig. 4B & data not shown). Interestingly, also IL-6 production was stimulated differently by both molecules: dSLIM-30L1 increased IL-6 secretion 4.1-fold and lin-30L1 had a significantly greater effect on IL-6 production (14.8-fold) (P -values: 0.005 and 0.051, respectively) (Fig. 4E).

Discussion

In the present study, we developed a covalently closed dumbbell-like immunomodulatory structure with PO-

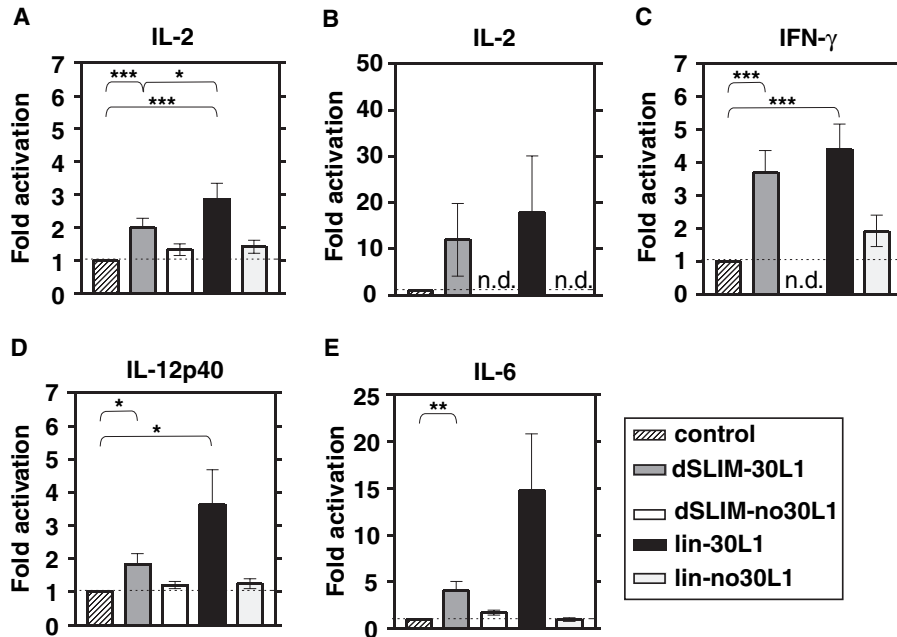


Figure 4. Induction of IL-2, IFN- γ , IL-12p40 and IL-6 secretion by dSLIM. (A) IL-2, (C) IFN- γ , (D) IL-12p40, (E) IL-6. Peripheral blood mononuclear cells (PBMC) from AD patients were incubated for 2 days with the respective immunomodulator (for IL-2 and IL-12p40: no treatment, $n = 18$; dSLIM-30L1, $n = 18$, dSLIM-no30L1, $n = 8$; lin-30L1, $n = 10$; lin-no30L1, $n = 10$; for IFN- γ : no dSLIM-no30L1 were studied and all others were $n = 10$; for IL-6: no treatment, $n = 17$; dSLIM-30L1, $n = 17$, dSLIM-no30L1, $n = 8$; lin-30L1, $n = 9$; lin-no30L1, $n = 9$). The fold increase of cytokine level compared with no treatment is shown (mean $\pm$ SE). Absolute values (pg/ml) for AD patients were as follows: no treatment, IL-2: 4.5 ± 1.5 , IFN- γ : 52.5 ± 8.6 , IL-12p40: 5.6 ± 1.2 , IL-6: 34.2 ± 7.9 ; dSLIM-30L1, IL-2: 15.8 ± 3.3 , IFN- γ : 172.7 ± 26.0 , IL-12p40: 9.9 ± 1.4 , IL-6: 139.5 ± 31.4 ; lin-30L1, IL-2: 27.7 ± 6.8 , IFN- γ : 229.6 ± 44.8 , IL-12p40: 26.2 ± 6.8 , IL-6: 514.4 ± 190.7 . Concentrations of molecules were used as follows: dSLIM-30L1 or dSLIM-no30L1 1 μ M and lin-30L1 or lin-no30L1 2 μ M. (B) IL-2 after treatment of PBMC from healthy donors ($n = 3$). n.d., not determined. Statistical significances are denoted as follows: * < 0.05 ; ** < 0.01 ; *** < 0.005 .

backbone, termed double stem-loop immunomodulatory molecule (dSLIM) and analyzed its immunomodulating effect in PBMC from allergic but also from nonallergic donors.

We show in our *in vitro* model system that these dSLIM molecules, dSLIM-30L1, inhibit IgE production but stimulate the production of IgA, IgM and IgG (with a moderate trend towards IgG2). They furthermore induce the production of T_H1 cytokines like IL-2, IFN- γ and IL-12p40 but also the pro-inflammatory cytokine IL-6. As expected, the effects of linear lin-30L1 were comparable with dSLIM-30L1. In fact, lin-30L1 was sometimes more effective (for IgM, IL-12p40, IL-6, IgG/IgG1) which may be explained by the double amount of lin-30L1 vs dSLIM-30L1 (2 μ M vs 1 μ M) which was used to provide an equal amount of CG motifs. However, experiments with equal molarities of dSLIM-30L1 and lin-30L1 revealed that the differences in strength of cytokine and Ig stimulation may also likely be due to either variations between different individuals (33) and/or to different effects of dSLIM-30L1 and lin-30L1. In addition, samples from both AD patients and healthy donors showed stimulation of IL-2, IL-12p40 and IgM production,

indicating a comparable effect of these molecules in cells from allergic and nonallergic donors.

CpG-ODN are potent immunostimulatory molecules driving B-cell proliferation, antigen presentation and T_H-responses (32, 34). To protect the open ends of the linear oligonucleotides, PS-backbones are commonly used. Despite their immunological effects, these structures exert nonspecific side-effects like prolongation of coagulation time (21). In addition, PS-ODN can act as competitors for promoter regions by binding transcription factors and changing the transcription of genes (22). Furthermore, it was shown that PS-based and PO-based CpG-ODN exhibit differences in activating macrophages (35). On the contrary, ODN with PO-backbones lack the long-term stability of PS-based ODN because of degradation by exonucleases. One may speculate that the side-effects of PS-based ODN may be due to their increased half-life and not related to the PS-backbones. Surprisingly, experiments using a PO-based dSLIM with the same dumbbell-shaped backbone but with a different number of CG motifs in their loops varied in their immunomodulatory potential, which makes this explanation unlikely (M. Schmidt, unpublished data). In addition, we observed

a similar serum and storage stability of dSLIM and lin-30L1 (M. Schmidt, unpublished data). Thus, our dSLIM structure may lack PS-mediated side-effects and avoids nuclease-mediated degradation because of its covalently closed form. This is further supported by our recent data which showed a significantly lower potency of PO-based CpG-ODN – exhibiting the same sequence as lin-30L1 – compared with dSLIM-30L1 or lin-30L1 (M. Schmidt, unpublished data).

In general, covalently-closed PO-based dSLIM had an at least comparable immunomodulatory potential to PS-based CpG-ODN, which was previously described to be 200-fold greater than that of linear PO-based CpG-ODN (6, 17, 35).

Interestingly, the observed reduction of IgE levels was not only independent of backbone structure but also of the CG motif content and sequence: different dSLIM molecules reduced IgE levels after CD40/IL-4 stimulation (data not shown).

For the analysis of our *in vitro* experiments we included samples from AD patients with different IgE levels. Nevertheless, the general immunomodulatory potential of dSLIM-30L1 and lin-30L1 in PBMC from patients with IgE levels < 1 ng/ml ($n = 10$) was comparable with patients with IgE production of > 1 ng/ml ($n = 8$) (data not shown). These results indicate that immunomodulation by dSLIM in AD patients may be independent of their clinical status (i.e. IgE level). This is in line with the observation that PBMCs from normal donors were stimulated by dSLIM comparable with PBMCs from AD patients. The IgE production depends on a complex immune response and requires at least the delivery of two signals, namely IL-4 and CD40L-CD40 interaction. However, several other molecules are involved in IgE regulation. It has been shown that IL-6 promotes IL-4-dependent activation of IgE production (36), although in

this study IL-6 production was increased while a spontaneous IgE production was reduced (Fig. 2). It can be speculated that the repressing effect of dSLIM-30L1 and lin-30L1 on IgE production was stronger than the stimulating effect of IL-6 itself. These observations are in line with a previous study detecting a PS-based CpG-ODN stimulation of IL-6 production in PBMC from allergic donors, but also an inhibition of IL-4-dependent IgE synthesis (20). In that study the decreased IgE-production was related to a down-modulation of the IL-4R by CpG-ODN and may explain also our finding of reduced IgE production, although IL-4 production itself is rather enhanced by CpG-ODN. Interestingly, despite the equal reduction of IgE by dSLIM-30L1 and lin-30L1 the dSLIM-30L1 molecule exhibits a lower induction of the pro-inflammatory cytokine IL-6 which is an intriguing trend but not significant ($P = 0.071$).

The immunological response in AD patients is characterized by an initial T_H2 - and a chronic T_H1 -dominated phase (4). Therefore, one can speculate that immunomodulators (like dSLIM) containing CG motifs may be useful for the prevention of the initial T_H2 -dominated phase. Whether these molecules are of any use in chronic phases, which are dominated by IFN- γ -producing T cells, will need to be clarified. However, possibly other immunomodulators with modified sequences and/or structures can direct an immune response, which may also be useful for the treatment in the chronic T_H1 -driven phase of AD.

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