Gene Ther Mol Biol Vol 8, 319-326, 2004

Phosphorothioated CpG Oligonucleotide induced hemopoietic changes in mice

Research Article

Priya Aggarwal[1], Ruma Ray[2] and Pradeep Seth[1*]

Departments of Microbiology[1] and Pathology[2], All India Institute of Medical Sciences, New Delhi-110029, India

***Correspondence**: Pradeep Seth MD FAMS FNASc, Professor and Head, Dept of Microbiology, All India Institute of Medical Sciences, New Delhi-110029; Phone: 91-11-26588714; Fax: 91-11-26588641; Email pseth@aiims.aiims.ac.in, sethpradeep@hotmail.com

Key words: CpG motifs; 1826-ODN; Splenomegaly; Hemopoiesis

Abbreviations: cytotoxic T lymphocyte, (CTL); extramedullary hemopoiesis, (EMH); human immunodeficiency virus, (HIV); oligodeoxynucleotides, (ODNs); pathogen-associated microbial patterns, (PAMPs); reactive follicular hyperplasia, (RFH); Toll like receptors, (TLRs)

Received: 17 May 2004; Accepted: 25 May 2004; electronically published: May 2004

Summary

Bacterial DNA and the synthetic CpG-oligodeoxynucleotides (ODNs) derived thereof have attracted attention because they activate cells of the adaptive immune system (lymphocytes) and the innate immune system (macrophages). They induce a Th1 biased immune response upon activation of the immune cells. In this paper we addressed whether unmethylated phosphorothioated CpG ODN (for example 1826 CpG-ODNs) affected hemopoiesis. We observed an overall Th1 dominant response upon *in-vitro* stimulation of naïve splenocytes with 1826-ODN. Immunizing mice with immunostimulatory CpG motifs led to transient splenomegaly, with a maximum increase of spleen weight at 4 weeks post immunization. Thereafter the splenomegaly regressed. The induction of splenomegaly by CpG-ODNs was dose-dependent with the maximum spleen weights recorded at the 250 μg immunizing dosage of 1826-ODN. In addition, the splenomegaly was also associated with dose dependent extramedullary hemopoiesis and reactive follicular hyperplasia in the spleens and lymph nodes, which could be of therapeutic relevance particularly in patients with life threatening chronic and persistent infectious diseases like visceral leishmaniasis and HIV infection.

I. Introduction

CpG oligodeoxynucleotides (ODNs) are a novel pharmacotherapeutic class with profound immunomodulatory properties. CpG ODN shows Th1 biased immune responses and promise as vaccine adjuvant and in the treatment of asthma, allergy, infection, and cancer. Several groups have studied the effect of CpG ODNs on the various arms of the immune system: B cells, T cells, NK cells, and dendritic cells (Krieg et al, 1995; Ballas et al, 1996; Davis et al, 1998). They have also studied its effect on the release of various cytokines important from an immunological standpoint. Overall CpG induces a Th1 like pattern of cytokine production that is dominated by IL-12 and IFN-γ with little secretion of Th2 cytokines. Recent work demonstrates the powerful adjuvant effect of CpG-ODNs, which can be used to trigger protective and curative Th1 responses in vivo (Chu et al, 1997; Lipford et al, 1997a, b; Zimmermann et al, 1998). When combined with specific antigen *in-vivo*, CpG ODNs can serve as a strong stimulus for T-cell activation, as well as for proliferation of antigen specific cytotoxic T lymphocyte (CTL) effectors.

It is known that bacterial stimuli (Lipopolysaccharide or Complete Freunds Adjuvant containing heat-killed mycobacteria) can trigger increased splenic hemopoiesis (McNeill et al, 1970; Apte et al, 1976; Staber et al, 1980), possibly via macrophage-derived hemopoietic growth factors that stimulate the generation and mobilization of the blood cells necessary to combat bacterial infections (Morrison et al, 1995). Here, we show that 1826-CpG-ODNs displayed the capacity to potentiate hemopoiesis. In addition, we observed that Phosphorothioated-ODNs with CpG motifs cause splenomegaly in Balb/c mice. We conclude that CpG ODN likely exerts systemic effects on spleens and lymph nodes.

II. Materials and methods

A. CpG Motifs (1826-ODN)

An unmethylated, phosphorothioated CpG motif, 1826-ODN, (5'-TCCATGAC*G*TTCCTGA*CG*TT-3') was synthesized commercially (Biosynthesis, USA). This ODN has 2 CpG motifs separated by 7 bases in between them. The ODN preparation had < 0.1 EU of endotoxin per milligram of ODN as assessed by a Limulus Amebocyte Lysate assay - E-TOXATE (Sigma, USA).

B. Animals

6-8 weeks old, inbred female Balb/c mice were purchased from National Central for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India.

C. *In vitro* stimulatory effect of 1826-ODN on naïve murine spleen cells

Normal mice were euthanised with an overdose of pentobarbital and spleens were removed aseptically. The spleen cells were collected, enumerated and resuspended in RPMI medium with 10% FCS to the required concentration. One million naïve spleen cells from unimmunized Balb/c mice, were plated in each well of a six-well tissue culture plate and incubated with different doses on 1826-ODN in duplicate wells (2,10,50 and 250µg/well). The control wells did not contain any ODN. The culture supernatants were collected at 24,36,48 and 72 hours for quantification of secreted IL-2, IFN-γ, IL-4 and IL-10 by murine cytokine ELISA kits (R&D Systems) according to the manufacturer's instructions.

D. Immunization of mice

Five mice per group were injected with different doses of 1826-ODN (2,10,50 and 250µg/mouse) intradermally. The mice were boosted with the same dose two weeks later. The control mice received normal saline intradermally. Mice were sacrificed at 4, 6, 8 and 24 weeks post-immunization respectively and spleen and lymphnodes were collected for histopathology. For determination of splenomegaly, fat and contiguous tissue around the spleens was trimmed off and the spleens were weighed.

E. Histopathology

After removal, the spleens and lymphnodes were fixed in 10% neutral-buffered formalin and subsequently fine sections (5-µ thick) were taken for histopathology. The tissue sections were then processed in Histokinette machine (Leica TP1020) for microscopic evaluation. This processing included fixation in 70% ethanol for 1 hour followed by 80% and absolute ethanol for 1 hour each. Then they were treated with acetone and xylene for 1 hour each, for the clearing of tissues. Finally, they were impregnated with melted paraffin wax (60°-62°C) for 1 hour. The tissue sections were mounted on slides, and stained with hematoxylin and eosin.

III. Results

A. *In vitro* stimulatory effect of 1826-ODN on naïve murine spleen cells

Nonspecific stimulatory effect of the 1826-ODN was evaluated quantitatively on naïve spleen cells, by evaluating release of Th1 and Th2 cytokines in the culture supernatants **(Figure 1)**. Murine IL-2 was detectable only with 2µg of 1826-ODN. The IL2 level showed a steady increase with the increasing incubation time and was 265 pg/ml at 72 hours. On the other hand, only 20 pg/ml of IL-2 was detected at 72 hours with 10 µg dose of the ODN. Similarly, higher amounts of IFN-γ levels were also detected with 2-µg dose.

Th2 cytokine, IL-10, was secreted in relatively higher amounts at all doses in comparison to the other cytokines. The maximum secretion was seen with 2 µg dose with the values of 115, 490, 405 and 510 pg/ml at 24, 36, 48 and 72 hours time points respectively. The IL-10 cytokine levels were comparatively low with 10 µg dose of ODN. With the increasing dose of ODN to 50 and 250 µg, the IL-10 cytokine secretion levels further decreased. The IL-10 cytokine levels at 250-µg dose were barely detectable. On the other hand, IL-4 cytokine secretion was not detected in the culture supernatants at all doses at all time points. Control wells, incubated without ODN did not show any secretion of either IL-10 or IL-4 cytokines.

B. Mouse splenomegaly assay

Splenomegaly was observed to be highly dose dependent **(Figure 2)**. There was a significant increase in the spleen weights with the increasing dose of 1826-ODN at all time points. Maximum spleen weights were recorded at 4 weeks time point. Thereafter, the spleen size and weight decreased significantly over time during next 5 months. Massive splenomegaly was observed with the 250-µg dose of 1826-ODN at 4 weeks time point with an average spleen weight of 0.65338 +/- 0.075049 grams,

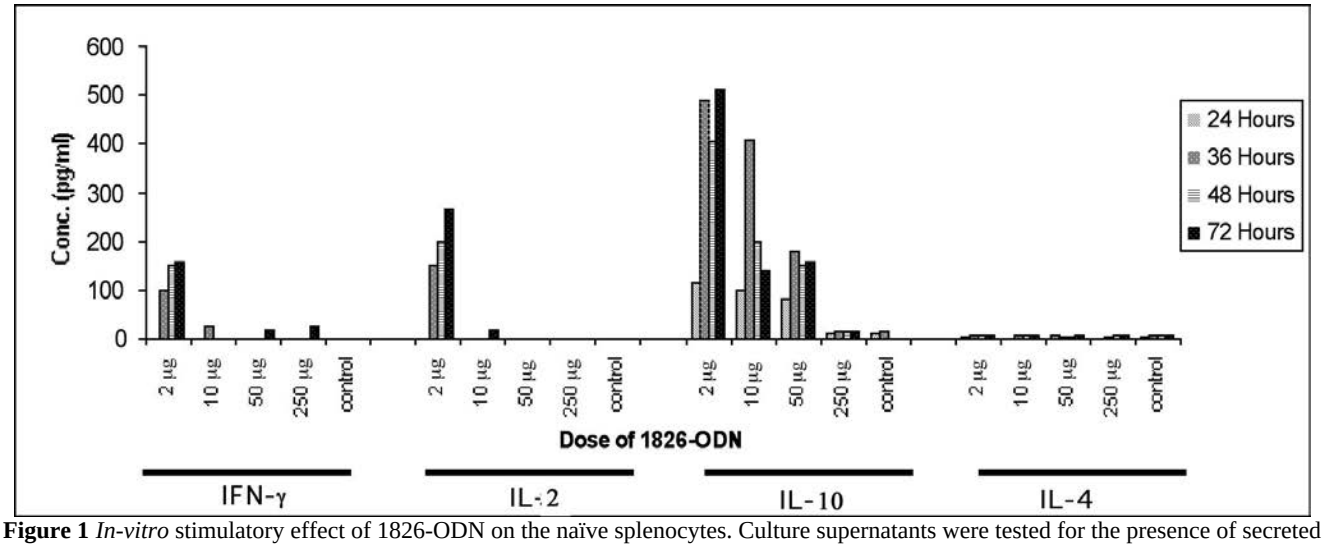

Figure 1 *In-vitro* stimulatory effect of 1826-ODN on the naïve splenocytes. Culture supernatants were tested for the presence of secreted murine Th1 (IFN-γ and IL-2) and Th2 cytokines (IL-10 and IL-4).

which was 9.6 times more than the average spleen weight of mice injected with normal saline. At 6 months time point also, the average spleen weight for 250-µg dosage was 1.5 folds greater than the average spleen weight of mice injected with normal saline. On the other hand splenic weights of mice immunized with 2µg, 10µg and 50µg doses of 1826-ODN at 4 weeks time point were 4.8, 3.2 and 3 folds more than the spleen weight of mice injected with normal saline, respectively.

C. Histopathology

Histological changes were studied in the spleens at 6 weeks time point and in both spleens and lymph nodes at 6 months time point **(Table 1a and b)**. Spleens showed increasing degree of extramedullary hemopoiesis (EMH) and reactive follicular hyperplasia (RFH) with prominent germinal centers with the increasing doses of 1826-ODN **(Figure 3a)**. EMH was diagnosed by the presence of immature hemopoietic precursors including megakarycytes (**Figure 3c**). There was a prominent expansion of white pulp of the spleens and formation of germinal centers with all the doses of 1826-ODN as compared to the spleens of mice injected with normal saline, which were histologically normal **(Figure 3e).** Spleens of mice injected with 250-µg-1826-ODN showed severe degree of reactive follicular hyperplasia with EMH **(Figure 3b**). Red pulp showed histiocytes with abundant eosinophilic cytoplasm. There were prominent germinal centers. Numerous megakaryocytes were present in the red pulp. The spleens of mice at 6 months time point also showed EMH but to a lesser degree than that observed at 6 weeks time point. Here also, the degree of reactive hyperplasia increased with the increasing dose of 1826-ODN, with maximum at 250 µg CpG ODN dosage. **Figure 3(c)** shows EMH with megakaryocyte formations in the spleen section of 10-µg dose of ODN. **Figure 3(d)**

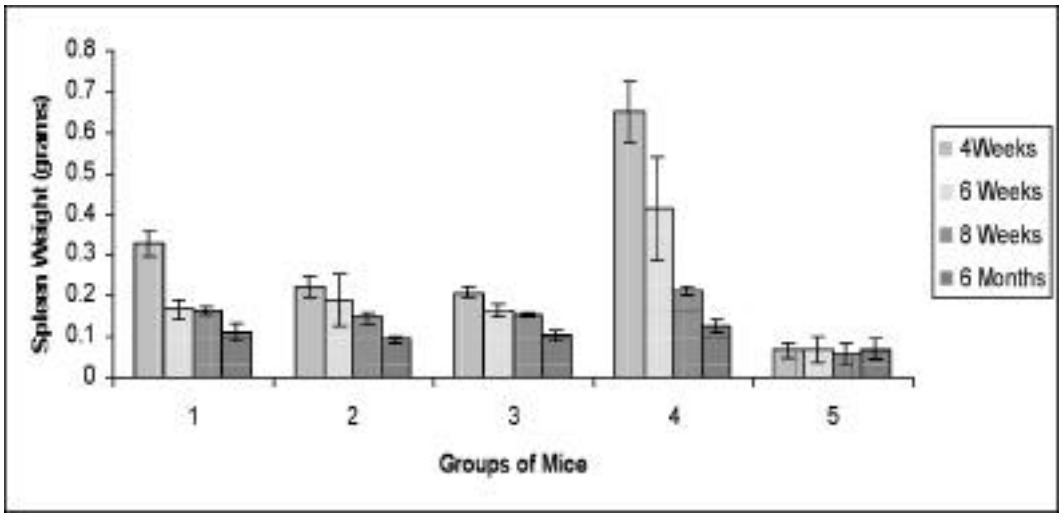

Figure 2 Mouse splenomegaly assay. The mice were immunized with different doses of 1826 ODN (2µg (group 1), 10µg (group 2), 50µg (group 3), 250µg (group 4)) intradermally. The control group (group 5) received normal saline. The spleens were harvested at 4 weeks, 6 weeks 8 weeks and 24 weeks post immunization and weighed. Each group had 5 mice. The average spleen weight is expressed in grams.

Table 1a. Observation chart showing the histological changes in the respective spleen and lymph node sections of mice injected with escalating doses of 1826-ODN **(a)** at 6 weeks time point **(b)** at 6 months time point post immunization.

	2 µg ODN	***10 µg ODN***	***50 µg ODN***	***250 µg ODN***	***Normal Saline***
Spleen	*Reactive follicles *Prominent expansion of white pulp	*Reactive follicles *Prominent white pulp *Hyperplasia	*Expansion of white pulp with reactive follicular hyperplasia * Extramedullary hemopoiesis	*Severe degree of reactive follicular hyperplasia *Red pulp shows histiocytes with abundant eosinophilic cytoplasma *Prominent germinal centers *Formation of Megakaryocytes * Extramedullary hemopoiesis	Histologically normal

Table 1b.

	2 µg ODN	*10 µg ODN*	*50 µg ODN*	*250 µg ODN*	*Normal Saline*
Spleen	Histologically normal	Extramedullary hemopoiesis *Formation of Megakaryocytes	Extramedullary hemopoiesis *Formation of Megakaryocytes *Severe degree of reactive follicular hyperplasia * Formation of germinal centers * Small epitheloid cells granuloma with in center of reactive white pulp.	Extramedullary hemopoiesis *Formation of Megakaryocytes *Severe degree of reactive follicular hyperplasia *Formation of Megakaryocytes in red pulp	Histologically normal
Lymph Node	Histologically normal	Sinus histiocytosis	lymph node not found	* Few reactive secondary follicles with germinal center	Histologically normal

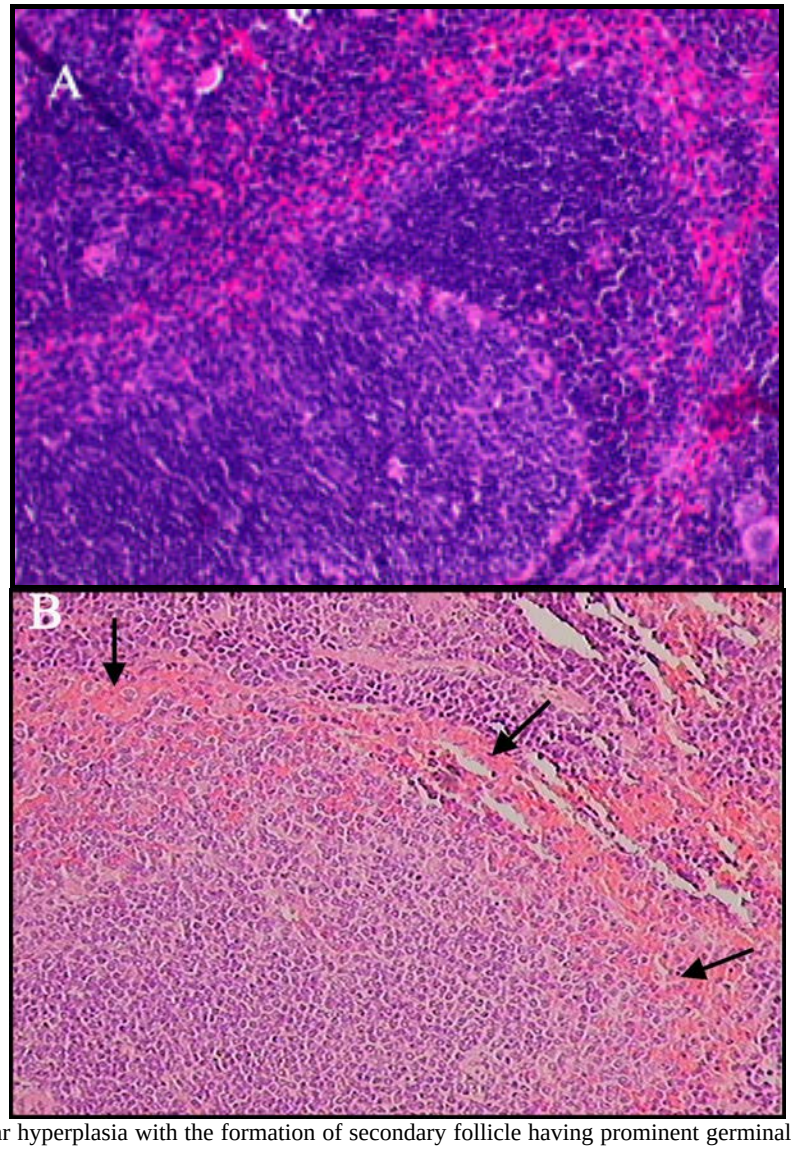

Figure 3 Reactive follicular hyperplasia with the formation of secondary follicle having prominent germinal center in spleen from mice injected with **(a)** 50µg and **(b)** 250 µg of 1826-ODN at 6 weeks time point (40X). The arrows are demarcating an expanding follicle.

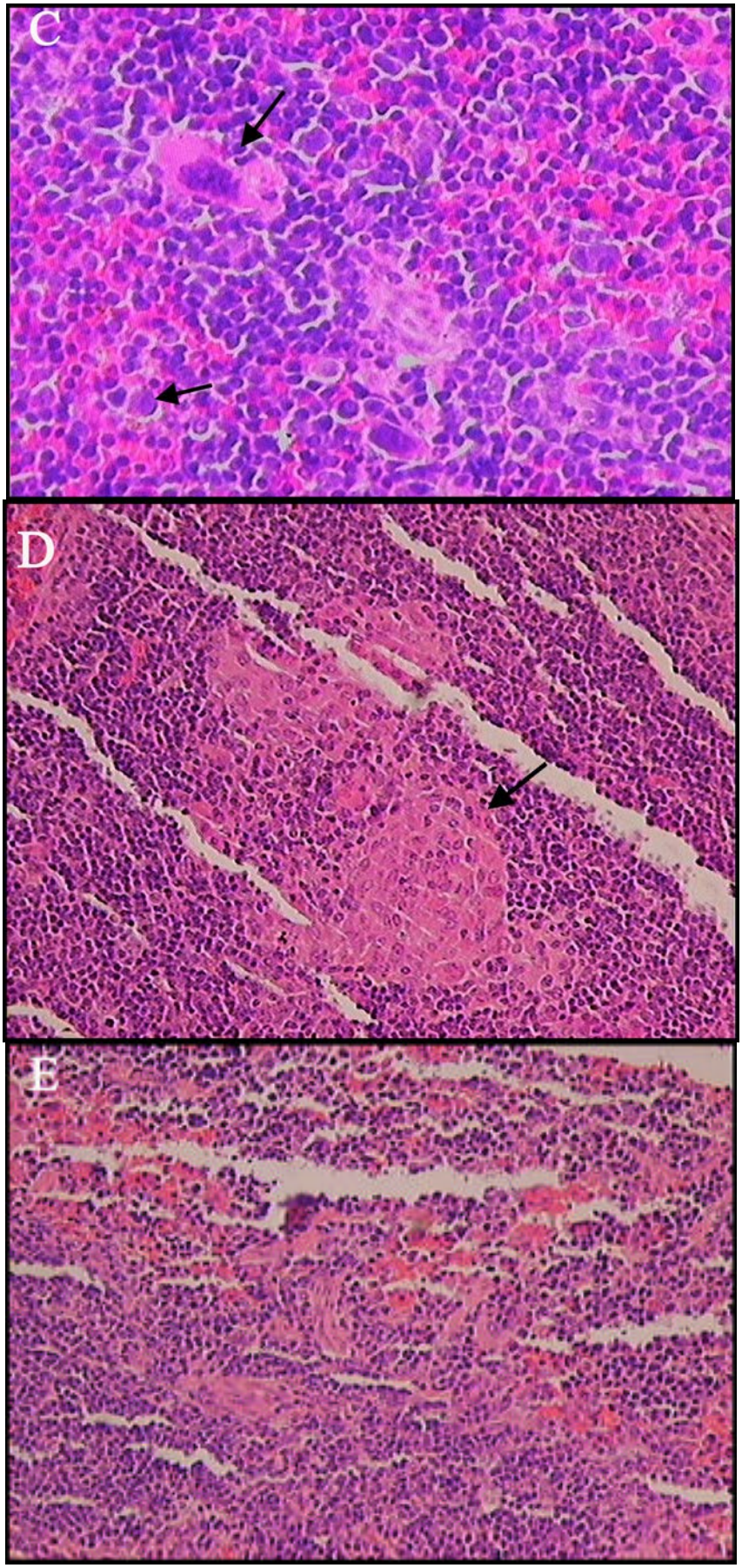

Figure 3(c) Extramedullary hemopoiesis with the formation of megakaryocytes (arrows) in the spleen from mice injected with 10ug of 1826-ODN at 6 months time point (40X). **(d)** Granuloma formation (arrows) with small epitheloid cells in the spleen from mice injected with 50 ug of 1826-ODN at 6 months time point **(e)** Spleen from mice injected with normal saline (40X).

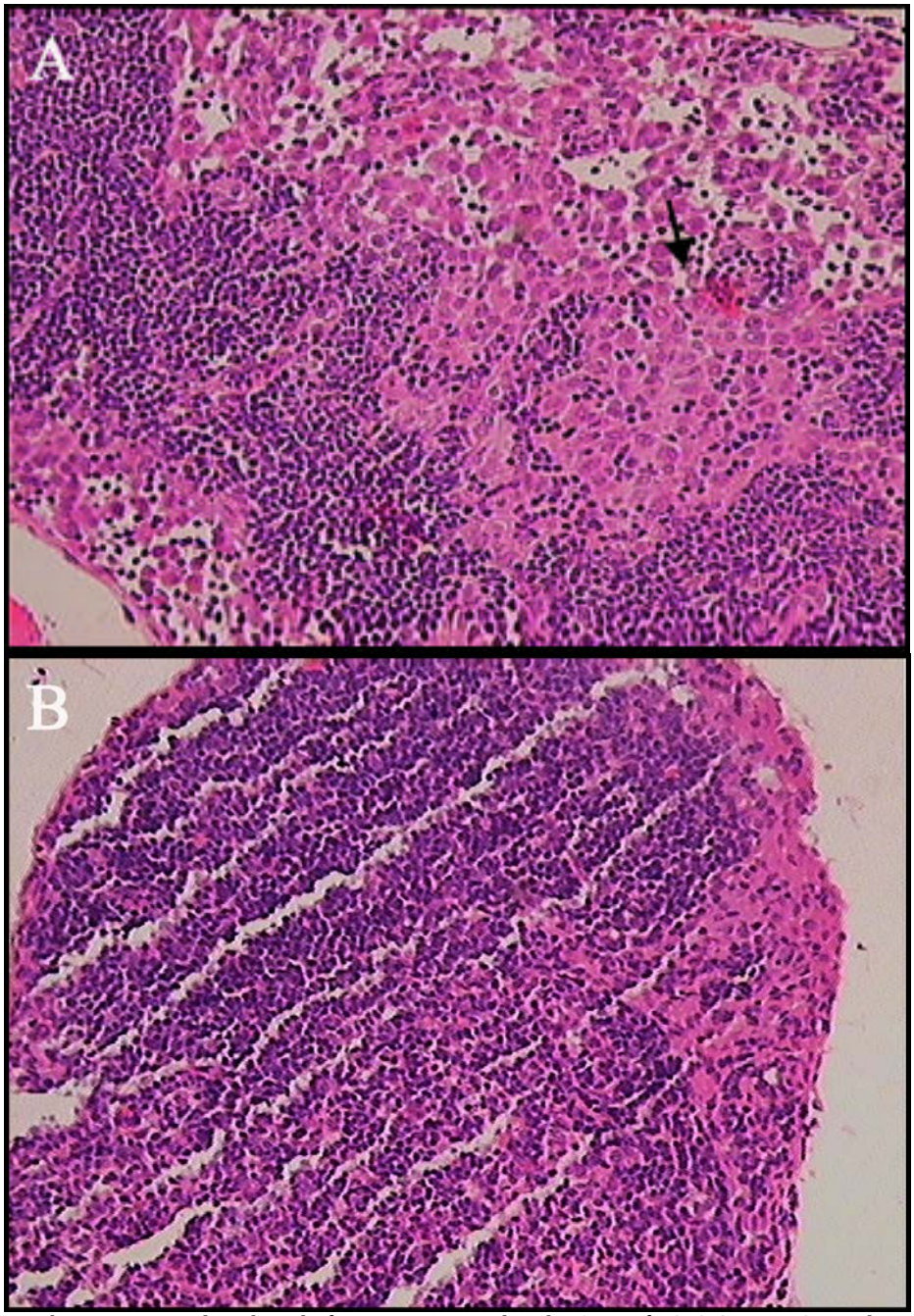

Figure 4(a) Focal sinus histiocytosis in lymph node from mice injected with 10 µg of 1826-ODN at 6 months time point (40X) The arrow is pointing towards a collection of histiocytes. **(b)** the lymph node from mice injected with normal saline (40X).

shows the spleen section of mice injected with 50 µg ODN dose, at 6 months time point, where granuloma can be seen with small epitheloid cells.

IV. Discussion

In this study, we describe and characterize the *in vitro* cytokine response of spleen cells and *in vivo* extramedullary hemopoiesis in spleen and lymph nodes in mice induced by CpG-ODNs. Specific CpG sequences appear to be important for elicitation of Th1-type immunity and enhancement of vaccine efficacy. As our understanding about the mechanisms of action of various CpG-ODN improves, it should be possible to predict effects on immune responses *in vivo* based on the results of *in vitro* assays. At the present time, *in vitro* assays are most useful in initially screening CpG-ODN for immunostimulatory activity and to determine its optimizing dosage to use in *in vivo* models. In our study, CpG-ODN 1826 induced significant Th1 cytokine responses (IFN-γ and IL-2) *in vitro*, on splenocytes from normal mice. The induction of cytokines by the naïve spleen cells can be explained by the presence of Toll like receptors (TLRs) on the cells. These evolutionary conserved receptors, homologues of the Drosophila Toll gene, recognize highly conserved structural motifs only expressed by microbial pathogens, called pathogen-associated microbial patterns (PAMPs). Stimulation of TLRs by PAMPs initiates a signaling cascade that involves a number of proteins, such as MyD88 and IRAK (Medzhitov et al, 1997). TLR9, which is localized

intracellularly, is involved in the recognition of specific unmethylated CpG-ODN sequences. This signaling cascade leads to the activation of the transcription factor NF-kB that induces the secretion of pro-inflammatory cytokines and effector cytokines that direct the adaptive immune response. There may be physiologic or pathologic conditions where TLR-9 would be expressed in nonimmune cells, in which they would be expected to become CpG responsive. Carlow et al, (1998) has described CpG-induced stimulation of L cells, which are of stromal origin, to produce IFN-γ upon transfection with plasmid DNA. Bacterial DNA or a CpG ODN has also been reported to induce human gingival fibroblasts to activate NFκB and secrete IL-6 (Takeshita et al, 1999). The only cells that are directly activated upon exposure to CpG DNA are the TLR-9 expressing cells like B cells and pDC (Bauer et al, 2001; Krug et al, 2001). Klinman et al, (1996) has also shown that a DNA motif consisting of an unmethylated CpG motif rapidly stimulates B cells in a polyclonal and antigen-nonspecific fashion, to produce IL-6 and IL-12, CD4+ T cells to produce IL-6 and IFN-γ, and NK cells to produce IFN-γ *in-vitro*. CpG PTO (phosphorothioated) was most effective in inducing *in-vitro* proliferation of splenocytes. The IL-12 p40 levels peaked at 500nM concentration ODN with cytokine levels of 7500pg/ml after 36 hours of incubation. Similarly, the IL-6 levels peaked to 7000pg/ml at 1000nM concentration of ODN (Zimmermann et al, 2003). Zelenay et al, (2003) have also shown that 1826 ODN induced naïve splenocytes to secrete high levels of IL-6 and IL-12 and modest levels of IFN-γ *in-vitro*.

Splenomegaly phenomenon was transient and highly dose dependent. There was a significant increase in the spleen weights with the increasing dose of CpG motifs reaching maximum at 4 weeks post-immunization and thereafter regressing gradually over next 20 weeks. Massive splenomegaly was observed in the mice injected with 250-μg dose of 1826-ODN at 4 weeks time point with a 9.6 fold increase in the splenic weight as compared to that of mice injected with normal spleen. An antisense ODN against the *rev* gene of the human immunodeficiency virus (HIV) caused a profound degree of B cell proliferation and massive splenomegaly *in-vivo* in mice (Branda et al, 1993). Mice treated with high doses of immune stimulatory phosphorothioated CpG ODN developed massive splenomegaly and increased spleen granulocyte macrophage colony forming units (GM-CFUs) and early erythroid progenitors (burst-forming units-erythroid) (Sparwasser et al, 1999). Treatment of rodents with phosphorothioate oligodeoxynucleotides induces a form of immune stimulation characterized by splenomegaly, lymphoid hyperplasia, hyper-γ-globulinemia and mixed mononuclear cellular infiltrates in numerous tissues. Splenomegaly and B-lymphocyte proliferation increased with the dose or concentration of oligodeoxynucleotides (Monteith et al, 1997). Splenomegaly appeared to occur, at least in part, as a result of stimulation of B-lymphocyte proliferation. Bhagat et al, (2003) have also reported splenomegaly in Balb/c mice to the extent of 153 mg after 48 hours of subcutaneous injection of a single dose of 5mg/kg immunomers.

In the spleen sections of mice at 6 weeks time point, there was increasing degree of extramedullary hemopoiesis and reactive follicular hyperplasia with prominent germinal centers, with the increasing doses of 1826-ODN. Thus, the transient splenomegaly observed in CpG motifs injected mice was dose dependent and associated with extramedullary hemopoiesis. CpG ODN has a profound effect on hematopoietic function. CpG-ODNs activate dendritic cells and macrophages to secrete large amounts of hemopoietically active cytokines, including IL-6, GM-CSF, IL-1, IL-12, and TNF-α (Ballas et al, 1996; Aggarwal and Seth, unpublished data). To date, it is unclear which of these cytokines, singly or synergistically, triggers the extramedullary hemopoiesis described here. It is also conceivable that CpG-ODNs target bone marrow stroma cells to release hemopoietically active cytokines. CpG-ODNs, which are operationally similar to LPS, may trigger extramedullary hemopoiesis via the induction of cytokines mobilizing BM progenitor cells to the spleen (Apte et al, 1976; Tokunaga et al, 1992). Even before the identification of the CpG motif, several investigators using antisense ODN noted the induction of sequence-specific extramedullary hematopoiesis and induction of hematopoietic colony formation (Hatzfeld et al, 1991; McIntyre et al, 1993). More recently, these effects were shown to be CpG specific. Histologically, an increased number of large immature blasts and erythroblasts were detected, reaching maximum at day 6, suggesting hemopoietic activity (Sparwasser et al, 1999).

Our findings in this study demonstrate that phosphorothioate oligonucleotide 1826-ODN exerts stimulatory effects in mouse model. Recent data from our laboratory also suggest that CpG-ODNs potentiate the immune responses induced by HIV-1 Indian Subtype C vaccine constructs in mice (manuscript under preparation) perhaps by augmenting the hemopoiesis. Thus, it may be possible to use CpG-ODN as therapeutic agents in patients with early or limited HIV disease.

Acknowledgments

This study was supported by the research grant from the Department of Biotechnology, Ministry of Science and technology, Govt. of India, under Prime minister's, Jai Vigyan Mission Program.

References

Apte R N, Galanos C, Pluznik DH **(1976)** Lipid A, the active part of bacterial endotoxins in inducing serum colony-stimulating activity and proliferation of splenic granulocyte/macrophage progenitor cells. **J Cell Physiol** 87, 71-78.

Ballas ZK, Rasmussen WL, Krieg AM **(1996)** Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. **J Immunol** 157, 1840–1845.

Bauer S, Kirschning CJ, Hacker H, Redecke V, Hausmann S, Akira S, Wagner H, Lipford GB **(2001)** Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. **Proc Natl Acad Sci USA** 98,

9237–9242.

Bhagat L, Zhu FG, Yu D, Tang J, Wang H, Ekambar R, Zhang KR, and Agrawal S **(2003)** CpG penta- and hexadeoxyribonucleotides as potent immunomodulatory agents. **Biochem Biophys Res Commun** 300, 853-861.

Branda RF, Moore AL, Mathews L, Mc- Cormack JJ, Zon G **(1993)** Immune stimulation by an antisense oligomer complementary to the rev gene of HIV-1. **Biochem Pharmacol** 45, 2037–2043.

Carlow DA, Teh SJ, Teh HS **(1998)** Specific antiviral activity demonstrated by TGTP, a member of a new family of interferon-induced GTPases. **J Immunol** 161, 2348–2355.

Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV **(1997)** CpG oligodeoxynucleotides act as adjuvants that switch on T helper (Th1) immunity. **J Exp Med** 186, 1623-1631.

Davis HL, Weeratna R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM, Weeranta R **(1998)** CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. **J Immunol** 160, 870-876. Erratum in: **J Immunol (1999)** 162, 3103. Weeranta R [corrected to Weeratna R].

Hatzfeld J, Li ML, Brown EL, Sookdeo H, Levesque JP, O'Toole T, Gurney C, Clark SC, Hatzfeld A **(1991)** Release of early human hematopoietic progenitors from quiescence by antisense transforming growth factor β1 or Rb oligonucleotides. **J Exp Med** 174, 925–929.

Klinman D, Yi A K, Beaucage SL, Conover J and Krieg AM **(1996)** CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete Interleukin 6, interleukin 12, and interferon γ. **Proc Nat Acad Sci USA** 93, 2879-2883.

Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM **(1995)** CpG motifs in bacterial DNA trigger direct B-cell activation. **Nature** 374, 546–549.

Krug A, Towarowski A, Britsch S, Rothenfusser S, Hornung V, Bals R, Giese T, Engelmann H, Endres S, Krieg AM, Hartmann G **(2001)** Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. **Eur J Immunol** 31, 3026–3037.

Lipford GB, Bauer M, Blank C, Reiter R, Wagner H, Heeg K (**1997a)** CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. **Eur J Immunol** 27, 2340-2344.

Lipford GB, Sparwasser T, Bauer M, Zimmermann S, Koch ES, Heeg K, Wagner H **(1997b)** Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. **Eur J Immunol** 27, 3420-3426.

McIntyre KW, Lombard-Gillooly K, Perez JR, Kunsch C, Sarmiento UM, Larigan JD, Landreth KT, Narayanan R **(1993)** A sense phosphorothioate oligonucleotide directed to the initiation codon of transcription factor NF-κB p65 causes sequence-specific immune stimulation. **Antisense Res Dev** 3, 309–322.

McNeill TA **(1970)** Antigenic stimulation of bone marrow colony-forming cells. **Immunology** 18, 61-72.

Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. **(1997)** A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. **Nature** 388, 394-397.

Monteith DK, Henry SP, Howard RB, Flournoy S, Levin AA, Bennett CF, Crooke ST **(1997)** Immune stimulation--a class effect of phosphorothioate oligodeoxynucleotides in rodents. **Anticancer Drug Des** 12, 421-432.

Morrison SJ, Uchida N, Weissman IL **(1995)** The biology of hematopoietic stem cells. **Annu Rev Cell Dev Biol** 11, 35-71.

Sparwasser T, ltner LH, Koch ES, Luz A, Lipford GB, and Wagner H **(1999)** Immunostimulatory CpG-Oligodeoxynucleotides Cause Extramedullary Murine Hemopoiesis. **J Immunol** 162, 2368–2374.

Staber FG, Metcalf D **(1980)** Cellular and molecular basis of the increased splenic hemopoiesis in mice treated with bacterial cell wall components. **Proc Natl Acad Sci USA** 77, 4322-4325.

Takeshita A, Imai K, Hanazawa S **(1999)** CpG motifs in Porphyromonas gingivalis DNA stimulate interleukin-6 expression in human gingival fibroblasts. **Infect Immun** 67, 4340–4345.

Tokunaga T, Yano O, Kuramoto E, Kimura Y, Yamamoto T, Kataoka T, Yamamoto S **(1992)** Synthetic oligonucleotides with particular base sequences from the cDNA-encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. **Microbiol Immunol** 36, 55-66.

Zelenay S, Elias F and Flo J **(2003)** Immunostimulatory effects of plasmid DNA and synthetic oligodeoxynucleotides. **Eur J Immunol** 33, 1382-1392.

Zimmermann S, Egeter O, Hausmann S, Lipford GB, Röcken M, Wagner H, Heeg K **(1998)** CpG oligonucleotides trigger curative Th1 responses in lethal murine leishmaniasis**. J Immunol** 160, 3627-3630.

Zimmermann S, Heeg K, and Dalpke A **(2003)** Immunostimulatory DNA as adjuvant: efficacy of phosphodiester CpG oligonucleotides is enhanced by 3' sequence modifications. **Vaccine** 21, 990-995.

Dr. Pradeep Seth