

CpG DNA redirects class-switching towards “Th1-like” Ig isotype production via TLR9 and MyD88

Ling Lin¹, Andrea J. Gerth¹ and Stanford L. Peng^{1,2}

¹ Department of Internal Medicine/Rheumatology, Washington University School of Medicine, St. Louis, USA

² Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, USA

Unmethylated CpG-containing DNA plays a critical role in immunity via the augmentation of Th1 but suppression of Th2 T cell responses. We describe here that CpG motifs also redirect isotype production by murine B cells to “Th1-like” Ig isotypes (IgG2a, IgG2b, and IgG3) while suppressing Th2 isotypes (IgG1 and IgE). Using genetically mutant B cells, we find that the IgG2a, IgG2b and IgG3 isotypes are transcriptionally regulated via the promotion of class-switching, in a manner critically dependent upon TLR9 and MyD88. Thus, CpG DNA redirects Ig isotype production by regulating the specificity of class-switch recombination.

Key words: CpG DNA / B lymphocyte / Th1/Th2 cell / Gene rearrangement / Cellular differentiation

Received	11/11/03
Revised	20/2/04
Accepted	26/2/04

1 Introduction

DNA molecules containing unmethylated CpG motifs comprise one of an ever-growing number of defined pathogen-associated molecular patterns that modulate adaptive immune responses [1, 2]. These motifs play critical roles in the augmentation and/or initiation of autoimmune responses by serving as adjuvants for autoreactive T cells [3], promoting Th1 responses largely via the induction of IL-12 [4–6], and costimulating autoreactive B cells [7]; conversely, they suppress pathogenic Th2 responses, including allergic airway inflammation [8, 9]. Given the constant exposure of most complex organisms to such DNA motifs, CpG sequences thus likely supply a tonic modulatory signal to ongoing immune responses, and serve as useful adjuvants during immunomodulation and vaccination strategies [2].

In this context, a critical feature of CpG-containing DNA remains its potential to redirect Ig isotype production by B cells during ongoing immune responses [2, 10]. In several animal models of vaccination, infection, and allergy, CpG DNA promote antigen-specific antibodies of the “Th1-like” isotypes IgG2a, IgG2b, and IgG3, while suppressing the Th2-like (and IL-4-related, since IL-4 promotes Th2 responses) isotypes IgG1 and IgE, presumably

ably in large part due to the effects of CpG on T cells [2, 4]. However, recent studies indicate that CpG DNA may also directly stimulate B cells, suppressing IgG1 and IgE production [11, 12], though the mechanistic details by which this regulation takes place remain largely unknown.

Cellular recognition of CpG-containing DNA predominantly requires the Toll-like receptor 9 (TLR9) [13–15], which stimulates a myeloid differentiation marker 88 (MyD88)-dependent signaling pathway [16, 17]. We therefore investigated the molecular mechanisms that govern CpG-induced redirection of Ig isotype production in MyD88-deficient and TLR9-deficient B cells, and found that the Th1-like isotypes were indeed regulated transcriptionally at their germline loci. Thus, CpG-containing DNA modulates Ig isotype production by B cells by regulating class-switching to Th1-like Ig via regulation of the germline transcripts.

2 Results

2.1 CpG DNA redirects Ig isotype secretion to non-Th2 IgG isotypes

Since CpG-containing DNA can promote Th1 responses and suppress Th2 responses *in vivo* [4–6, 8, 9], we predicted that they likely redirect B cell differentiation toward a “Th1-like” B cell phenotype in general [18], inducing multiple non-Th2-related isotypes. To test this, we exposed B cells undergoing activation with soluble

[DOI 10.1002/eji.200324736]

Abbreviations: MyD88: Myeloid differentiation marker 88 TLR9: Toll-like receptor 9

anti-CD40 and recombinant IL-4 to CpG oligonucleotides, and assessed the quantities of secreted Ig (Fig. 1, open bars). Strikingly, exposure to CpG oligonucleotides significantly suppressed the production of the IL-4-related isotypes IgE and IgG1, resulting in a 10–20-fold suppression of the former, and a 2–4-fold suppression of the latter ($p < 0.01$ for both isotypes). At the same time, CpG DNA strongly promoted the secretion of IgG2a, IgG2b, and IgG3, increasing the amount secreted from undetectable to 10–20 ng/ml for IgG2a and IgG3 ($p < 0.001$), and stimulating the secretion of IgG2b over 20-fold ($p < 0.001$). These effects were not replicated with GpC-containing oligonucleotides, indicating that the Ig redirection requires the CpG motif.

2.2 Ig redirection by CpG partially requires MyD88 and TLR9

We therefore speculated that CpG redirection required both TLR9 [13–15] and MyD88 [16, 17], and investigated

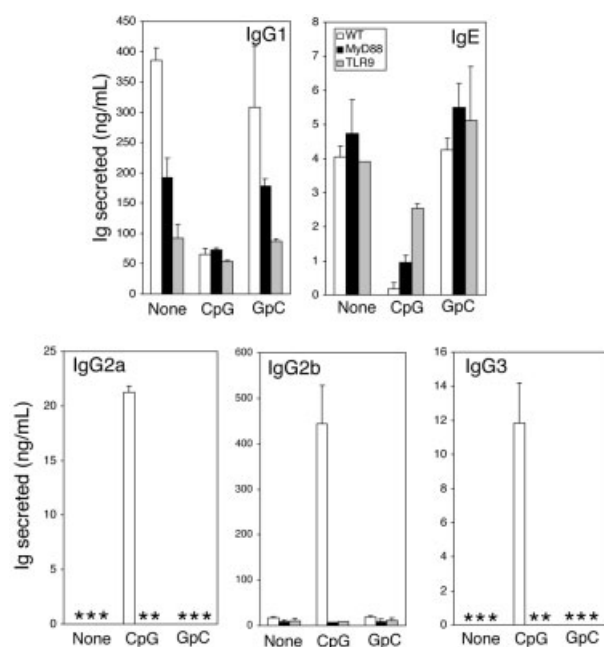


Fig. 1. CpG suppresses IL-4-related isotype production and induces “Th1-like” Ig isotype production. Resting splenic B cells from wild-type (WT; open bars), MyD88-deficient (black bars), or TLR9-deficient (gray bars) C57BL/6 mice were incubated in the presence of anti-CD40 antibody and recombinant IL-4 for 10 days, in the presence or absence of a CpG-containing or control (GpC-containing) oligonucleotide. Secreted Ig were quantified by ELISA of culture supernatants. Shown are data from one experiment consisting of four simultaneously tested animals, representative of three experiments. Error bars indicate standard deviations, and are too small to be visible in some cases. Asterisks (*) indicate undetectable by this assay (<100 pg/ml).

the ability of CpG DNA to redirect Ig production in TLR9-deficient and MyD88-deficient B cells (Fig. 1, black and gray bars, respectively). Both MyD88- and TLR9-deficient B cells were severely deficient in their ability to generate Th1-like IgG isotypes in response to CpG: they were completely impaired in the ability to induce detectable levels of IgG2a and IgG3, and were unable to augment IgG2b production above baseline (anti-CD40+IL-4-induced) quantities ($p < 0.0001$).

Interestingly, CpG DNA was clearly capable of suppressing both IgG1 and IgE production by both MyD88- and TLR9-deficient B cells: although the former appeared to produce IgE in levels slightly higher than wild-type counterparts, perhaps indicating a role for MyD88 in the regulation of Ig production by anti-CD40+IL-4, CpG clearly induced a 5–6-fold suppression ($p < 0.001$), and similarly induced a 2–3-fold suppression in IgG1 production (Fig. 1, upper panels). The effect in TLR9-deficient cells was more modest, with CpG inducing only at most a 2-fold suppression in IgE and IgG1. Nonetheless, taken together, these results indicated that the classical TLR9-MyD88 pathway is critical for Th1-like isotype promotion, but does not as strongly apply to the Th2 (IL4-related) isotypes.

2.3 MyD88- and TLR9-dependent Ig redirection by CpG involves redirection of class-switching

Notably, these defective Ig responses in MyD88- and TLR9-deficient B cells could not be explained simply by CpG-mediated effects upon cell proliferation and/or apoptosis, since MyD88- and TLR9-deficient B cells proliferated comparably to wild-type cells treated with anti-CD40 and IL-4 (not shown). Instead, since the suppression of the IL-4-related isotypes by CpG appears to reflect the inhibition of class-switching to IgG1 and IgE [11], we suspected that the promotion of the Th1-like isotypes might reflect the augmentation and/or initiation of class-switching to IgG2a, IgG2b, and IgG3. Therefore, we examined both germline and post-switch transcripts for these isotypes in wild-type, MyD88- and TLR9-deficient B cells during stimulation in the presence or absence of CpG DNA (Fig. 2).

Strikingly, both MyD88 and TLR9 were absolutely required for the induction of class-switching to each of these isotypes, as evidenced by analyses of both germline and post-switch transcripts: only wild-type B cells exposed to CpG DNA produced significant levels of germline and post-switch transcripts of IgG2a, IgG2b and IgG3, whereas wild-type cells, whether or not exposed to GpC DNA, as well as MyD88- and TLR9-

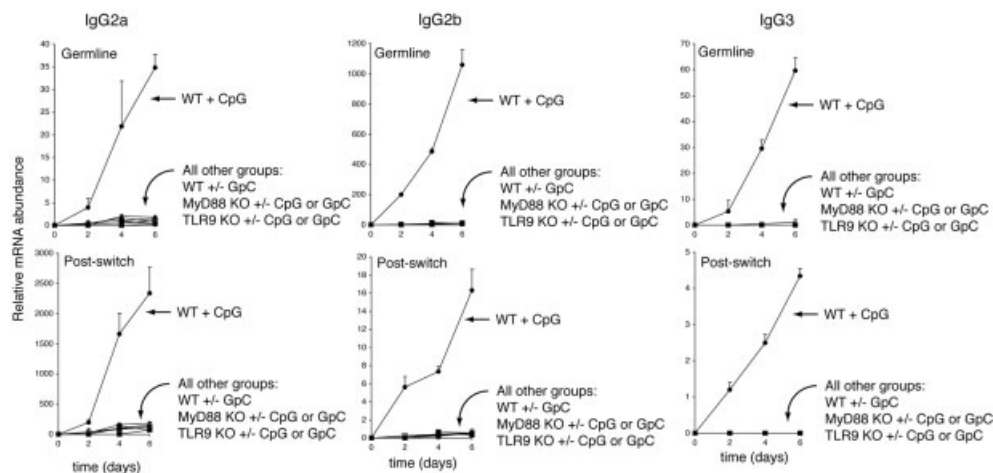


Fig. 2. CpG induces class-switching to “Th1-like” IgG isotypes in a MyD88- and TLR9-dependent fashion. Resting splenic B cells from wild-type (WT), MyD88-deficient, or TLR9-deficient C57BL/6 mice were incubated in the presence of anti-CD40 antibody and recombinant IL-4, in the presence or absence of a CpG- or control GpC-containing oligonucleotide. On the days indicated, cDNA was then assessed for the quantity of germline and post-switch transcripts. Each graph reflects data from one experiment consisting of four simultaneously tested animals, representative of at least three experiments. Error bars indicate standard deviations.

deficient cells, whether or not exposed to CpG or GpC DNA, failed to produce significant levels of any ($p < 0.00001$ for all isotypes, comparing CpG-treated wild-type cells with any other group). Thus, the induction of the Th1-like Ig isotypes by CpG DNA requires TLR9- and MyD88-dependent transcriptional activation of the respective germline Ig loci, leading to subsequent class-switch recombination.

3 Discussion

These findings indicate that CpG DNA induces class-switching to “Th1-like” Ig isotypes by promoting transcription at their germline loci. These events are dependent upon both TLR9 and MyD88, presumably due to a requirement to induce and/or activate transcriptional activators and/or regulators to induce germline transcription. At the same time, CpG suppresses the production of the “Th2-like,” IL-4-related isotypes IgG1 and IgE, but interestingly this effect does not appear to require MyD88 and/or TLR9, indicating that other receptors and/or signal transduction pathways likely contribute to mediating the effects of CpG DNA [17]. As such, the classical pathway of CpG-TLR9-MyD88 primarily promotes the type 1 lymphocyte fate in B cells, at least in terms of the types of Ig isotypes generated.

Although little is known about specific transcriptional regulators of the germline loci of these “Th1-like” isotypes, it is likely that one or more transcription factors, such as T-bet [11, 12, 19], is induced by CpG oligonucle-

otides and is responsible for the initiation and/or augmentation of class-switching to those isotypes in response to CpG stimulation, analogous to the role of T-bet in IgG2a induction in response to IFN- γ [19, 20]. Indeed, T-bet-deficient B cells are unable to generate IgG2a in response to CpG oligonucleotides, although they can produce IgG2b and IgG3 ([12] and our unpublished data). As such, additional transcription factors likely participate, such as members of the NF- κ B family, which play critical roles in signal transduction of TLR pathways [21], and have been implicated in the germline regulation of the $\gamma 2b$ and $\gamma 3$ loci [22, 23]. However, the relatively delayed appearance of $\gamma 2b$ and $\gamma 3$ switch transcripts (typically at least 48 h after CpG exposure), in contrast to the earlier (~6 h) induction of other CpG target genes like T-bet ([11] and our unpublished data) suggests that not NF- κ B factors, but rather one of their target genes, may in fact represent the true, specific regulators of these loci in response to CpG. Continued investigation within this context will likely yield novel targets in the regulation of class-switching, and therefore in the regulation of autoimmune, allergic, and infectious diseases.

4 Materials and methods

4.1 Mice

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). MyD88-deficient mice [24] of the C57BL/6 background were graciously provided by Dr. Emil

Table 1. Real-time PCR primers used in this study

Locus	Primer 1	Primer 2
IgG2a ^b germline	5'-ACTGGTGGACCGAGGAAGG	5'-CCAGTGGATAGACCGATGGG
IgG2a ^b post-switch	5'-TCTGGACCTCTCCGAAACCA	5'-GGGCCAGTGGATAGACCGAT
IgG2b germline	5'-CTCACACACAGAAGAATGGACCA	5'-AGTTGTATCTCCACACCCAGGG
IgG2b post-switch	5'-GAAACCAGGCACCGCAAAT	5'-AGTTGTATCTCCACACCCAGGG
IgG3 germline	5'-GCAAGATCTCTGCAGCAGAAATC	5'-CCAGGGACCAAGGGATAGACA
IgG3 post-switch	5'-TCTGGACCTCTCCGAAACCA	5'-CCAGGGACCAAGGGATAGACA

Unanue (St. Louis, MO, USA). TLR9-deficient [13] mice of the C57BL/6 background were purchased from the European Mouse Mutant Archive (Orleans, France). All animals were bred and raised under specific pathogen-free conditions at the Washington University School of Medicine, and all experiments were performed in compliance with the relevant laws and institutional guidelines, as overseen by the Animal Studies Committee of the Washington University School of Medicine.

4.2 B cell cultures and ELISA

For *in vitro* analyses, B cells were purified by negative selection against CD43, yielding >98% IgM⁺CD69⁺ B cells (Miltenyi Biotec, and data not shown). Cells were stimulated by 2 µg/ml anti-CD40 antibody (BD Pharmingen, San Diego, CA, USA) with 10 ng/ml recombinant murine IL-4 (Pepro-Tech, Inc, Rocky Hill, NJ, USA), as described previously [19]. Phosphorothioate CpG (5'-TCCATGACGTTCTGACGTT) or control GpC (5'-TCCATGAGCTTCTGAGTCT) oligonucleotides were added where indicated at 3 µM, as described previously [11]. Ig secretion was assessed in culture supernatants by ELISA (Southern Biotechnology, Birmingham, AL, USA). Of particular note, IgG2a^b (IgG2c, C57BL/6) was detected using goat anti-mouse-IgG2a (Southern Biotechnology) and biotin-5.7 (anti-mouse-IgG2a^b; BD Pharmingen) as the capture and detection antibodies, respectively, followed by avidin-alkaline-phosphatase (Sigma-Aldrich Co., St. Louis, MO, USA). For purposes of consistency with prior studies [11], we refer herein to this isotype as IgG2a. In addition, for convenience of discussion, we have used the term "Th1-like" to refer collectively to the IgG2a, IgG2b and IgG3 isotypes, since these isotypes have been previously linked to type 1 T cell responses in animal studies [25, 26].

For cellular proliferation analysis, B cells were washed twice with ice-cold PBS, and then resuspended in PBS containing 5 mM carboxyfluorescein diacetate, succinimidyl ester (CFDA SE, CFSE; Molecular Probes, Eugene, OR, USA) at a concentration of 2×10^7 cells/ml. Cells were then incubated for 10 min at room temperature with intermittent agitation, then quenched with an equal volume of heat-inactivated

fetal bovine serum (BioWhittaker, Walkersville, MD, USA), followed by washing thrice with PBS. Cells were then resuspended in complete medium and cultured as above. Cells were analyzed by flow cytometry on a FACSCalibur System (BD Biosciences, San Jose, CA, USA).

4.3 RNA transcript analysis

For RNA analyses, RNA was prepared from cells at the times indicated in the text with the RNeasy[®] Mini Kit (Qiagen, Inc., Valencia, CA, USA) accompanied by DNase treatment, and first-strand cDNA synthesized using oligo(dT) primers and SuperScript[™] II reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA). Samples were then subjected to real-time PCR analysis on an ABI PRISM[®] 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) under standard conditions with specificity reinforced via the dissociation protocol. Gene-specific primers are listed in Table 1, and were validated by comparison with standard PCR primers used to detect germline and post-switch transcripts in wild-type B cells in response to LPS +/- IL-4 or IFN-γ ([20] and our unpublished data). The relative mRNA abundance of each transcript was normalized against tubulin [20], calculated as $2^{(Ct[tubulin] - Ct[gene])}$, where Ct represents the threshold cycle for each transcript.

Acknowledgements: We are grateful to Emil Unanue for MyD88-deficient animals, and Robert Schreiber for the use of the ABI PRISM 7000 Sequence Detection System. This work was supported in part by the Siteman Cancer, Diabetes Research and Training, and the Digestive Diseases Research Core Centers of the Washington University School of Medicine, as well as grants from the NIH (AI01803 and AI057471) and the Lupus Research Institute. S. L. P. is supported in part by an Arthritis Investigator Award from the Arthritis Foundation.

References

- 1 Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A. and Klinman, D. M., CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995. **374**: 546–549.
- 2 Krieg, A. M., CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 2002. **20**: 709–760.
- 3 Segal, B. M., Chang, J. T. and Shevach, E. M., CpG oligonucleotides are potent adjuvants for the activation of autoreactive encephalitogenic T cells in vivo. *J. Immunol.* 2000. **164**: 5683–5688.
- 4 Chu, R. S., Targoni, O. S., Krieg, A. M., Lehmann, P. V. and Harding, C. V., CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J. Exp. Med.* 1997. **186**: 1623–1631.
- 5 Brazzoli Millan, C. L., Weeratna, R., Krieg, A. M., Siegrist, C. A. and Davis, H. L., CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc. Natl. Acad. Sci. USA* 1998. **95**: 15553–15558.
- 6 Krieg, A. M., Love-Homan, L., Yi, A. K. and Harty, J. T., CpG DNA induces sustained IL-12 expression in vivo and resistance to *Listeria monocytogenes* challenge. *J. Immunol.* 1998. **161**: 2428–2434.
- 7 Leadbetter, E. A., Rifkin, I. R., Hohlbaum, A. M., Beaudette, B. C., Shlomchik, M. J. and Marshak-Rothstein, A., Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 2002. **416**: 603–607.
- 8 Kline, J. N., Waldschmidt, T. J., Businga, T. R., Lemish, J. E., Weinstock, J. V., Thorne, P. S. and Krieg, A. M., Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* 1998. **160**: 2555–2559.
- 9 Sur, S., Wild, J. S., Choudhury, B. K., Sur, N., Alam, R. and Klinman, D. M., Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J. Immunol.* 1999. **162**: 6284–6293.
- 10 Wagner, H., Toll meets bacterial CpG-DNA. *Immunity* 2001. **14**: 499–502.
- 11 Liu, N., Ohnishi, N., Ni, L., Akira, S. and Bacon, K. B., CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. *Nat. Immunol.* 2003. **4**: 687–693.
- 12 Peng, S. L., Li, J., Lin, L. and Gerth, A., The role of T-bet in B cells. *Nat. Immunol.* 2003. **4**: 1041.
- 13 Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S., A Toll-like receptor recognizes bacterial DNA. *Nature* 2000. **408**: 740–745.
- 14 Bauer, S., Kirschning, C. J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H. and Lipford, G. B., Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. USA* 2001. **98**: 9237–9242.
- 15 Takeshita, F., Leifer, C. A., Gursel, I., Ishii, K. J., Takeshita, S., Gursel, M. and Klinman, D. M., Cutting edge: Role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. *J. Immunol.* 2001. **167**: 3555–3558.
- 16 Hacker, H., Vabulas, R. M., Takeuchi, O., Hoshino, K., Akira, S. and Wagner, H., Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. *J. Exp. Med.* 2000. **192**: 595–600.
- 17 Heit, A., Maurer, T., Hochrein, H., Bauer, S., Huster, K. M., Busch, D. H. and Wagner, H., Cutting edge: Toll-like receptor 9 expression is not required for CpG DNA-aided cross-presentation of DNA-conjugated antigens but essential for cross-priming of CD8 T cells. *J. Immunol.* 2003. **170**: 2802–2805.
- 18 Shirota, H., Sano, K., Hirasawa, N., Terui, T., Ohuchi, K., Hattori, T. and Tamura, G., B cells capturing antigen conjugated with CpG oligodeoxynucleotides induce Th1 cells by elaborating IL-12. *J. Immunol.* 2002. **169**: 787–794.
- 19 Peng, S. L., Szabo, S. J. and Glimcher, L. H., T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc. Natl. Acad. Sci. USA* 2002. **99**: 5545–5550.
- 20 Gerth, A. J., Lin, L. and Peng, S. L., T-bet regulates T-independent IgG2a class switching. *Int. Immunol.* 2003. **15**: 937–944.
- 21 Zhang, G. and Ghosh, S., Toll-like receptor-mediated NF- κ B activation: a phylogenetically conserved paradigm in innate immunity. *J. Clin. Invest.* 2001. **107**: 13–19.
- 22 Snapper, C. M., Zelazowski, P., Rosas, F. R., Kehry, M. R., Tian, M., Baltimore, D. and Sha, W. C., B cells from p50/NF- κ B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching. *J. Immunol.* 1996. **156**: 183–191.
- 23 Laurencikiene, J., Deveikaite, V. and Severinson, E., HS1,2 enhancer regulation of germline ϵ and γ 2b promoters in murine B lymphocytes: evidence for specific promoter-enhancer interactions. *J. Immunol.* 2001. **167**: 3257–3265.
- 24 Kawai, T., Adachi, O., Ogawa, T., Takeda, K. and Akira, S., Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 1999. **11**: 115–122.
- 25 Balasa, B. and Sarvetnick, N., Is pathogenic humoral autoimmunity a Th1 response? Lessons from (for) myasthenia gravis. *Immunol. Today* 2000. **21**: 19–23.
- 26 Shlomchik, M. J., Craft, J. E. and Mamula, M. J., From T to B and back again: positive feedback in systemic autoimmune disease. *Nat. Rev. Immunol.* 2001. **1**: 147–153.

Correspondence: Stanford L. Peng, Department of Internal Medicine / Rheumatology, 660 South Euclid Avenue, St. Louis, MO 63110, USA
 Fax: +1-314-454-1091
 e-mail: speng@im.wustl.edu