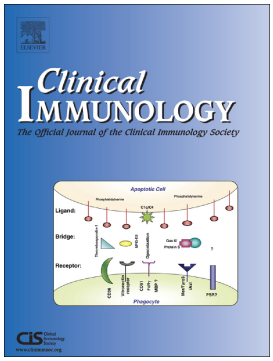

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DOCK8 and STAT3 dependent inhibition of IgE isotype switching by TLR9 ligation in human B cells

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To the Editor,

Hyper IgE Syndromes (HIES) represent a group of disorders whose genetic etiology manifests in an autosomal dominant form (AD-HIES) in the case of heterozygous mutations in signal transducer and activator of transcription (STAT) 3, or in an autosomal recessive form (AR-HIES) in the case of biallelic mutations in dedicator of cytokinesis (DOCK) 8, non-receptor tyrosine-protein kinase (TYK) 2, and phosphoglucomutase (PGM) 3 [1].

DOCK8 is a guanine nucleotide exchange factor for CDC42 that plays an important role in actin cytoskeleton rearrangement and cellular function [2, 3]. DOCK8 interacts with STAT3 and regulates its translocation to the nucleus. [3]. In human B cells, DOCK8 serves as an adaptor that links TLR9 signaling to STAT3 activation through a macromolecular complex that includes MyD88, Pyk2, Src, and Syk [4]. In the absence of DOCK8, TLR9-driven B cell proliferation and IgG production are impaired, but anti-CD40+IL-4-driven IgG and IgE production are intact [4]. Patients with DOCK8 deficiency and those with STAT3 deficiency have elevated levels of serum IgE [5-8], suggesting that DOCK8 and STAT3 inhibit IgE synthesis by human B cells. These observations raise the possibility that the elevated levels of IgE in these two immunodeficiencies could be associated with defective TLR9 signaling via STAT3. We have sought to determine if TLR9 ligation inhibits IgE class switching by normal human B cells *in vitro*, and to examine the roles that DOCK8 and STAT3 might play in this process.

IgE isotype switching requires two signals: crosslinking of CD40 on B cells

by CD40L expressed on activated T cells, and the cytokine IL-4 produced by Th2 cells [9]. We used the human B cell-specific CpG oligodeoxynucleotide (ODN) 2006 (herein called CpG) to determine the effect of TLR9 ligation on anti-CD40+IL-4-mediated IgE secretion by B cells. Addition of increasing concentrations of CpG to anti-CD40+IL-4-stimulated peripheral blood mononuclear cell (PBMCs) from healthy non-allergic donors resulted in significant inhibition of IgE secretion (Fig. 1A), but had no effect on IgG4 secretion (data not shown), indicating that CpG specifically interferes with IgE production. These findings are consistent with a previous report showing that a synthetic CpG ODN inhibits IgE production by anti-CD40+IL-4-stimulated human PBMCs [10]. However, it was not clear if the ODN used in that study was B cell specific, or whether it acted indirectly on B cells through another cell population.

The inhibitory effect of CpG on anti-CD40+IL-4-mediated IgE secretion was also observed when highly purified naïve B cells (>98% $CD19^{+}CD27^{-}IgE^{-}$) were used (Fig. 1B), demonstrating that CpG acts directly on B cells to inhibit IgE production. Addition of CpG did not increase B cells apoptosis or affect their differentiation and activation programs (data not shown). These data demonstrate that TLR9 signaling acts directly on naïve B cells to inhibit IgE isotype class switching.

IgE isotype switching is preceded by the transcription of sterile Cε germline transcripts (*CεGLT*), which is mediated by activation of STAT6 and NF-κB [11]. It is followed by AID-dependent switch recombination, which causes deletional switch recombination and results in the expression of mature *Cε* that

encodes membrane and secreted forms of IgE [11]. Quantitative real time PCR was used to determine the levels of *CεGLT* in naïve control B cells stimulated with anti-CD40+IL-4. Addition of 0.1μM CpG to naïve B cells stimulated with anti-CD40+IL-4 resulted in a highly significant reduction in the level of the *CεGLT* mRNA compared to B cells stimulated with anti-CD40+IL-4 alone (Fig. 1C). Addition of CpG also caused, as expected, a highly significant reduction in the level of the mature *Vh-Cε* transcripts that encode for IgE (Fig. 1D). These data demonstrate that TLR9 signaling interferes with the expression of *CεGLT* and mature *Cε* transcripts by anti-CD40+IL-4-stimulated naïve B cells, leading to decreased IgE production.

To determine if DOCK8 and STAT3 play a role in TLR9-mediated inhibition of IgE production, we examined PBMCs from DOCK8- and STAT3-deficient patients. The mutations in the patients studied are presented in Supplemental Tables E1 and E2. Addition of CpG did not result in significant inhibition of IgE secretion by anti-CD40+IL-4-stimulated cells from DOCK8-deficient patients or STAT3-deficient patients (Fig. 1E, F). These findings indicate that DOCK8 and STAT3 are essential for TLR9-mediated inhibition of IgE production by anti-CD40+IL-4 stimulated cells. Because of the limited amounts of blood available from the patients, we were unable to examine the effect of CpG on their purified B cells. However, considering that the CpG we used is B cell-specific, and given our demonstration that CpG acts directly on normal B cells to inhibit IgE synthesis, we conclude that DOCK8 and STAT3 are essential for TLR9 mediated inhibition of IgE class switching by B cells. Given that STAT3 is

downstream of DOCK8 in TLR9-mediated B cell activation [4], these data indicate that the role of DOCK8 in inhibiting IgE production by B cells depends on its downstream effector STAT3. It has been reported that IL-21 amplifies IgE production by human B cells *in vitro* in a STAT3-dependent manner [12]. Taken together with our data, this suggests that STAT3 might exist in two different complexes generated following TLR9 or IL-21 stimulation, which exert opposite effect on IgE production.

Microbes are a rich source of CpG oligonucleotides, and serum IgE levels are increased in germ free mice [13]. In patients with DOCK8 or STAT3 deficiency, immune activation by repeated antigenic stimulation due to recurrent infections may drive IgE production in the absence of efficient downregulation by microbial CpG-mediated TLR9 activation, contributing to the hyper-IgE gammaglobulinemia seen in these patients.

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Figure legend

Fig. 1. CpG inhibits anti-CD40+IL-4-mediated secretion of IgE by cells from healthy subjects, but not from patients with DOCK8 or STAT3 deficiency. A, B. CpG inhibition of IgE secretion by normal peripheral blood mononuclear cells (A) or naïve B cells (B) stimulated with anti-CD40+IL-4. The results are expressed as percentage IgE in the supernatants of cultures stimulated with anti-CD40+IL-4 alone, set at 100%. **C, D.** Effect of CpG 2006 (0.1µM) on *CεGLT* (C) and *Vh-Cε* (D) mRNA expression by normal B cells stimulated with anti-CD40+IL-4, as determined by qPCR. *CεGLT* mRNA (C) and *Vh-Cε* mRNA (D) were normalized to GADPH mRNA, and the results were expressed as a percentage of these ratios compared to their percentage in B cells stimulated with anti-CD40+IL-4 alone, set at 100%. **E, F.** CpG inhibition of IgE secretion by peripheral blood mononuclear cells from patients with DOCK8 (E) or STAT3 (F) deficiency stimulated with anti-CD40+IL-4. The results are expressed as percentage IgE in the supernatants of cultures stimulated with anti-CD40+IL-4 alone, set at 100%. N=15 in (A), 7 in (B), 9 in (C), 5 in (D), 8 in (E), and 6 in (F). Significance was calculated using the correlation function (A, B, E, F) and student *t* test (C, D) of GraphPad Prism. *, $p<0.05$; ***, $p<0.0001$; ns, not significant.