Gpr97 is essential for the follicular versus marginal zone B-lymphocyte fate decision

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Gpr97 is an orphan adhesion GPCR and is highly conserved among species. Up to now, its physiological function remains largely unknown. Here, we show that Gpr97 deficiency results in an extensive reduction in B220+ lymphocytes in mice. More intensive analyses reveal an expanded marginal zone but a decreased follicular B-cell population in Gpr97−/− spleen, which displays disorganized architecture characterized by diffuse, irregular B-cell areas and the absence of discrete perifollicular marginal and mantle zones. In vivo functional studies reveal that the mutant mice could generate antibody responses to T cell-dependent and independent antigens, albeit enhanced response to the former and weakened response to the latter. By screening for the molecular events involved in the observed phenotypes, we found that lambda 5 expression is downregulated and its upstream inhibitor Aiolos is increased in the spleen of mutant mice, accompanied by significantly enhanced phosphorylation and nuclear translocation of cAMP response element-binding protein. Interestingly, increased constitutive Nf-κB p50/p65 expression and activity were observed in Gpr97−/− spleen, implicating a crucial role of Gpr97 in regulating Nf-κb activity. These findings uncover a novel biological function of Gpr97 in regulating B-cell development, implying Gpr97 as a potential therapeutic target for treatment of immunological disorders.

Cell Death and Disease (2013) 4, e853; doi:10.1038/cddis.2013.346; published online 10 October 2013

Subject Category: Immunity

B cells develop from hematopoietic stem cells in the BM. Early development and commitment to the B-cell lineage depend on diverse transcription factors, including early B-cell factor, PU.1, E2A and PAX5.1 The earliest committed B-cell progenitors in the active cell cycle begin DJ rearrangement, followed by V(D)J rearrangement at the H-chain loci at the pro-B stage. After a productive V(D)J recombination, the cytoplasmic μ heavy chains (cμHCs) are expressed and then paired with surrogate L chains (VpreB and Jμ proteins) to form the pre-B-cell receptor (BCR) at the pro-B-cell stage. Expression of the pre-BCR on the cell surface allows the B cells to encounter cognate antigen and undergo further maturation. Marginal zone (MZ) B cells, located at the outer rim of follicles, represent a smaller fraction of splenic B cells.5 The MZ and FO B-cell subsets differ significantly in phenotype, function and anatomical location. How these subpopulations are selected remains incompletely understood. Growing evidence has supported an important role for transcription factors in regulating MZ and FO B-cell fate. This is largely based on the observations that genetically mutant mice deficient in a series of transcription factors exhibited imbalanced development of MZ and FO B cells.6–11

The adhesion GPCRs, which have 33 members in humans, contain large extracellular N-terminal domain containing a range of protein domains found in cell adhesion proteins, and C-terminal domain homologous to secretin-like GPCR.12–14 N- and C-terminal domains can be autocatalytically cleaved at the membrane-proximal GPCR proteolytic site domain, which is a characteristic feature of this receptor family.15 The majority of adhesion GPCRs are still orphans, for which neither ligand nor function is known. There is increasing

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Keywords: Gpr97; knockout mice; B lymphopoiesis; follicular B cells; lambda 5 gene

Abbreviations: GPCR, G protein-coupled receptor; FO, follicular; MZ, marginal zone; BM, bone marrow; BMD, bone mineral density; PB, peripheral blood; GC, germinal center; PNA, peanut agglutinin; CFA, complete Freund’s adjuvant; IFA, incomplete Freund’s adjuvant

Received 26.4.13; revised 23.7.13; accepted 29.7.13; Edited by Y Shi

Citation: Cell Death and Disease (2013) 4, e853; doi:10.1038/cddis.2013.346 © 2013 Macmillan Publishers Limited All rights reserved 2041-4889/13

www.nature.com/cddis
evidence for the roles of adhesion GPCRs in the central nervous system, immune system and tumorigenesis. For example, CELSR1-3\textsuperscript{16,17} and latrophilin\textsuperscript{18} coordinate neuronal development and neurotransmitter release, respectively. CD97\textsuperscript{19,20} and EMR1-3\textsuperscript{21–23} are involved in coordinating both the innate and the acquired immune responses. GPR124 promotes tumor angiogenesis.\textsuperscript{24}

Although there is no consensus yet about the physiological functions of adhesion GPCRs and their molecular mechanisms of signaling, the existing data suggest that this receptor class mediates essential cell–cell and cell–matrix interactions.\textsuperscript{12,25}

GPR97 is an orphan adhesion GPCR with homology to the better characterized HE6 (human epididymis-specific protein 6) and GPR56 (human brain-specific protein). Like HE6 and GPR56, GPR97 possesses both an exceptionally long extracellular region, characteristic of cell adhesion proteins and an intracellular region reminiscent of other GPCRs.\textsuperscript{26,27} GPR97 was found by searching human genome databases.\textsuperscript{26} Previous work revealed that Gpr97 mRNA is highly expressed in immune cells.\textsuperscript{28} Other study has shown that GPR97 is coupled to G\textsubscript{o}, which means that the inactivation of GPR97 would lead to an increase in cAMP levels in target cells.\textsuperscript{29} To investigate the biological function of Gpr97 in vivo, we have generated Gpr97\textsuperscript{−/−} mice. The phenotypic analyses have demonstrated an indispensable role of Gpr97 in maintaining B-lymphocyte population, especially in regulating constitutive CREB and NF-κB activities.

Results

Expression profile of Gpr97 in mice. To determine the expression pattern of Gpr97 in normal adult mice, we performed semi-quantitative and real-time reverse transcription (RT)–PCR analyses on various mouse tissues. As shown in Supplementary Figure S1, the highest expression level of Gpr97 mRNA was found in BM, and relatively low but detectable expression levels were also observed in heart, kidney and spleen tissues, implicating the tissue compartments where Gpr97 could execute its physiological functions in mice.

Gpr97\textsuperscript{−/−} mice are born alive and appear grossly normal. To explore the physiological function of Gpr97 in vivo, we generated a mouse model with global-targeted deletion of Gpr97 (Figure 1a). The homologous recombination in ES cells was confirmed (Figure 1b), and the genotypes of mice were verified by PCR analysis of genomic DNA (Figure 1c). The absence of Gpr97 expression was confirmed by analyzing the Gpr97 mRNA by RT-PCR (Figure 1d) and protein expression by western blot analysis (Figure 1e). As expected, Gpr97 mRNA and protein were disrupted in the BM of Gpr97\textsuperscript{−/−} mice. However, the mutant mice were viable and reached the adult stage without any gross developmental abnormalities, suggesting that Gpr97 is not indispensable for normal development.

Reduction of B-cell population in Gpr97\textsuperscript{−/−} mice. Considering the features of Gpr97 expression profile in mice, we performed several phenotypic screening tests for detection of some parameters potentially affected due to Gpr97 deficiency. It was found that no significant differences in body weight, ratios of thymus or spleen weight to body weight, as well as bone mineral density between sex- and age-matched WT and Gpr97\textsuperscript{−/−} mice (Supplementary Figure S2a-d). Hematological examination revealed that the numbers of white blood cells, red blood cells and platelets remained unaffected in PB of Gpr97\textsuperscript{−/−} mice (Supplementary Figure S2e). The differential counting of BM cells was performed by flow cytometry using lineage-specific cell-surface markers. The results showed that the percentages of CD3\textsuperscript{+}, Ter119\textsuperscript{−}, CD41\textsuperscript{+} and Gr-1\textsuperscript{−} cells were comparable between WT and Gpr97\textsuperscript{−/−} mice but B220\textsuperscript{−} cell population was decreased in Gpr97-deficient BM cells (Supplementary Figure S2f). More intensive analysis revealed that the reduction of B220\textsuperscript{−} cells was observed not only in BM but also in spleen and PB of the mice lacking Gpr97 as compared with WT littermates (Figure 2a). The percentages of T cells and granulocytes were not significantly different between WT and Gpr97\textsuperscript{−/−} mice as judged by CD3 or Gr-1 staining, respectively (Figure 2b). These results suggested that Gpr97 is essential in maintaining B220\textsuperscript{−} cell population. To address whether the reduction of B lymphocytes was due to impaired cell proliferation or increased apoptosis, single-cell suspensions of splenocytes were cultured with or without LPS (20 μg/ml) stimulation. MTT assay revealed that...
Gpr97−/− splenocytes proliferated as rapidly as WT cells upon LPS treatment (data not shown). However, annexin V/PI staining and FACS analyses showed that B220+ cells deficient for Gpr97 had increased apoptosis when compared with WT cells cultured for 24 h in the presence or absence of LPS (Supplementary Figure S3). We also analyzed Bcl-2, Bax and caspase-3 mRNA levels, as well as caspase-3 activity in splenic B220+ cells, and found that Bcl-2 expression level is decreased and caspase-3 activity is increased in Gpr97−/− splenic B lymphocytes cultured for 24 h with or without LPS (Supplementary Figure S4). Taken together, these observations demonstrate that the increased apoptosis of B220+ cells in Gpr97−/− spleen was attributed to enhanced caspase-3 activity.

Abnormal B-cell subpopulations in BM and spleen of Gpr97−/− mice. To further characterize the reduction of B-cell population in Gpr97-deficient mice, we performed an extensive survey of surface marker expression on B-lineage cells in BM. It was found that mature B (B220+ IgM+ IgD−, M)-cell populations were reduced in Gpr97−/− mice as compared with WT controls. The percentages and absolute cell numbers of mature B cells were reduced by 32.7% and 36.2%, respectively. Although pro-B (CD43−B220int), pre-B/immature B (CD43−B220int) and immature B (B220+ IgMlowIgD−, IM) cell populations in Gpr97 mutant BM were comparable to those found in WT littermates (Figures 3a and b). These findings suggest that Gpr97 is not involved in early B-lineage cell commitment but is required for development of mature B cells in secondary lymphoid tissues.

By analyzing the splenic B-cell compartment, we observed that the percentages of mature B (B220+ CD21intIgMint) and T1 B (B220+ CD21lowIgMhigh) cells in Gpr97 mutant spleens were lower than those in WT controls (Figure 3c, top; 3d, top left). Accordingly, the absolute numbers of mature B and T1 B cells were significantly reduced due to a decrease in total splenic B cells in mutant mice as compared with WT controls (Figure 3d, top right). Meanwhile, the T2 B (B220+ CD21highIgMhigh) cells of mutant mice were found significantly increased with regard to percentages, but no significant changes were observed in the absolute cell numbers (Figure 3c, bottom). By means of flow cytometry, mature B cells in spleen were separated into MZ B and FO B cells in terms of the surface expression of CD21 and CD23 (Figure 3c, bottom). Our results revealed that MZ B cells (B220+ CD21highCD23low−) were markedly increased by 3.8-fold in percentage and 2.5-fold in absolute numbers.
after Gpr97 deletion, whereas FO B cells (B220<sup>+</sup> CD21<sup>int</sup>CD23<sup>high</sup>) were markedly decreased by 9-fold in percentage and 10-fold in absolute numbers (Figure 3d, bottom).

Gpr97<sup>−/−</sup> mice display abnormal splenic architecture. Microscopic evaluation of the spleen of Gpr97<sup>−/−</sup> mice showed a disruption of normal architecture characterized by diffuse, irregular follicular (B-cell) areas with an absence of a discrete perifollicular MZ (Figures 4a and b). These morphological abnormalities were substantiated after immunochemical detection of B cells (Figures 4c and d). To corroborate the proportional differences in the mature B-cell populations between Gpr97<sup>−/−</sup> and WT controls, histological analysis was performed. Sections were stained with anti-IgM for B cells, and MOMA-1 for metallophilic macrophages to delineate the border between the MZ and the follicular area (Figures 4e and f). Consistent with the increased numbers of MZ B cells observed in flow cytometry, the MZ area of Gpr97<sup>−/−</sup> mouse spleen was larger than that of WT controls.

The abnormal architecture of Gpr97<sup>−/−</sup> spleen suggested that immune responses depending on cellular interactions in the follicle might not be fully functional. To test this hypothesis, the germinal centers (GCs) were examined in KO and WT mice after secondary immunization. GCs are typical structures formed in secondary lymphoid organs during the course of a TD immune response, and are the place where affinity maturation, class switching and memory cell formation take place. Microscopic examination of the spleen confirmed the presence of GCs in both KO and WT mice (Figures 4g and h). The results revealed that PNA-binding GC B cells clearly developed in the spleen of Gpr97<sup>-KO</sup> mice, which indicated an intact GC formation despite Gpr97 deletion.

Impaired immunoglobulin production, humoral primary and secondary immune responses in Gpr97<sup>−/−</sup> mice. To determine whether Gpr97 deficiency had an effect on Ig production, Ig titers were assessed in 3-month-old WT and Gpr97<sup>−/−</sup> mice by ELISA. As shown in Figure 5a, levels of IgG1, IgG2b and IgA were significantly decreased, whereas IgG2a level was significantly increased in KO mice as compared with WT controls. IgM and IgG3 levels were similar between WT and KO mice. Next, we analyzed the ability of Gpr97-KO mice to mount antibody responses to TI and TD antigens. At day 14 after immunization with TI antigen...
DNP-Ficoll, production of both IgM and IgG3 anti-DNP antibodies was reduced in Gpr97-KO mice as compared with WT controls (Figures 5b and c). The amount of DNP-specific IgM antibodies after TD immunization was lower in Gpr97-KO mice than that in controls. Level of DNP-specific IgG1 instead was higher in mutant mice after TD immunization (Figures 5d and e). These data indicate that Gpr97-deficient animals are able to make both TD primary and secondary immune responses, consistent with the data of the GC formation in Gpr97-KO mice, as well as TI responses, although at reduced levels compared with WT animals.

**Downregulation of lambda 5 expression in Gpr97-deficient mice.** The observation that abnormal B-cell development in KO mice prompted us to evaluate the expression pattern of a multitude of different transcriptional factors by real-time PCR, which have been linked to distinct stages of the life of B lymphocytes, such as differentiation in the BM, migration to the peripheral organs and antigen-induced activation. The mRNA expression levels of several genes were found unaltered as compared with those in WT control except for lambda 5 in both BM (Figure 6a) and spleen (Figure 6b). The lambda 5 transcript is reduced by 27.8% and 84.1% in BM and spleen of Gpr97−/− mice, respectively. To confirm this observation, we carried out flow cytometric analysis to examine the percentage of lambda 5-positive cells in BM. The results showed that the percentage of lambda 5-positive cells was reduced by 18.6% in BM of Gpr97−/− mice as compared with WT controls, and the difference was statistically significant.
A crucial role of Gpr97 in regulating CREB signaling pathway. Given that Gpr97 is an orphan GPCR, we presumed that Gpr97 would be more likely to regulate lambda 5 indirectly. Through a literature search, we found that Aiolos, a member of the Ikaros family of transcription factors, is required for the efficient silencing of lambda 5.\(^9\)

To determine whether the decreased level of lambda 5 is due to an increased Aiolos level in Gpr97\(^{-/-}\) mice, we analyzed the Aiolos level in the spleen by real-time PCR. As expected, an increased Aiolos expression level was observed in the spleen of KO mice as compared with WT controls (Figure 7a). Then, we analyzed the promoter of Aiolos by TFSEARCH software (Computational Biology Research Center, Tsukuba, Japan) for the purpose of finding potential transcription factor binding sites. As predicted, a CREB-binding site was identified in Aiolos promoter region. The transcription factor CREB is one of the components of many signaling cascades, and is regulated by the GPCR-cAMP–PKA pathway. Thus, we examined the levels of pCREB and total CREB in the spleen of Gpr97\(^{-/-}\) mice by western blot analysis. As shown in Figure 7b, the level of pCREB in total cell lysate from the spleen of KO mice was found increased while CREB level remains comparable to that of WT control. Most strikingly, both CREB and pCREB levels in the nuclear extract were found dramatically increased as compared with WT controls, suggesting that Gpr97 could negatively regulate CREB signaling. Thus, we believe that the ablation of Gpr97 leads to increased phosphorylation of CREB, and the increased level of pCREB may enhance the expression level of Aiolos, which down-regulates the expression of lambda 5.

Gpr97 negatively regulates NF-\(\kappa\)B signaling. BM-derived B cells make an important cell fate choice to develop into either FO B cells or MZ B cells in the spleen, which depends on signaling through BCR, Notch2, the receptor for B cell-activating factor and the canonical nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) pathway, as well as signals involved in the migration and anatomical retention of MZ B cells.\(^40\) Hence, it is possible that Gpr97 deletion might affect BAFF, NF-\(\kappa\)B or Notch2 expression in spleen. This possibility was elucidated by the following three experiments. First, we examined the serum BAFF level by ELISA, and found there is no difference between the two groups (Figure 7c). Second, western blot analysis showed that constitutive p105, p65 and p50 protein levels were increased to some extent in the total cell lysates of Gpr97\(^{-/-}\) splenocytes when compared with that of WT control. Accordingly, higher levels of p65 and p50 in the nuclear extracts of Gpr97\(^{-/-}\) splenocytes were observed with comparable levels of p65, p50, as well as p105 proteins in cytoplasmic fraction and responses to LPS stimulation between two groups (Figure 7d). These results indicate that Gpr97 deficiency leads to increased constitutive expression and activation of NF-\(\kappa\)B components examined, suggesting
that Gpr97 may negatively regulate Nf-κb signaling. Third, the level of Notch2 in the spleen had no significant difference between WT and mutant mice, as demonstrated by western blot analysis (Figure 7e).

**Discussion**

A previous study has established a Gpr97-deficient mouse model, which displays no any significant quantitative or qualitative defects in B- and T-cell development.\(^{41}\) Such discrepancy may attribute to the different mouse genetic background to that in this study. The mice used in the previous study have a homogenous 129Sv genetic background. However, the mutant mice used in this study are maintained in a mixed 129Sv/C57BL/6 background. Many studies have already addressed that genetic background is of great importance to immune research.\(^{42,43}\) Through intensive phenotypic analysis, we have demonstrated that Gpr97 has a crucial role in maintaining normal splenic architecture, regulating B-lymphocyte development, specifically follicular B-cell development. To understand the molecular events underlying the impaired B-cell development, we have tested the expression levels of multiple transcription factors which have been demonstrated to have a role in B-cell development. For example, E2A and EBF control the immunoglobulin-gene rearrangement.\(^{44}\) Blimp1 is induced upon terminal differentiation to plasma cells\(^{45}\) and NF-κB is described as a nuclear transactivator of the immunoglobulin-light chain enhancer and a cytokine-inducible transcription factor governing the expression of an important set of genes involved in inflammation and cell survival.\(^{37}\) Lambda 5, a component of pre-BCR, is expressed in B-cell development before conventional L chains. Deficiency of lambda 5 leads to a block in B-cell development in BM at the pre-B-cell stage, resulting in a marked decrease in the number of mature B cells in the periphery. The proportion was increased but not the absolute number of MZ B cells, whereas FO B cells were decreased. Lambda 5 KO mice are able to mount not only primary but also secondary TD and TI responses, albeit at reduced levels,\(^{46,47}\) similar to the phenotypes observed in Gpr97\(^{-/-}\) mice.
To understand the relationship between Gpr97 and lambda 5, we discovered that the expression level of Aiolos, which is required for silencing lambda 5, was significantly increased in the spleen of Gpr97<sup>-/-</sup> mice. Interestingly, the promoter of Aiolos has a CREB-binding site predicted by TFSEARCH software. CREB, a cellular transcription factor, is activated by phosphorylation at Ser-133.<sup>48</sup> The kinase responsible for this activating phosphorylation was identified as cAMP-dependent protein kinase, PKA. PKA activity is regulated by molecules that can alter cAMP levels, and hence by GPCRs, which regulate adenylate cyclase activity. Based on the previous findings, we examined the phosphorylation level of CREB in the Gpr97<sup>-/-</sup> mice and found an increased phosphorylation level of CREB in both nuclear extracts and whole-cell lysates from the spleen of Gpr97<sup>-/-</sup> mice. This observation is in accordance with the previous finding that Gpr97 is coupled to G<sub>o</sub>, which means that the inactivation of Gpr97 would lead to an increase in cAMP levels in target cells. According to these results, it is reasonable that Gpr97 modulates the expression of lambda 5 via Aiolos. We have noted that the follicular type I B-cell population was preserved in Aiolos-deficient mice, in which the strength of BCR signaling was increased, but the number of splenic MZ B cells was markedly decreased, as well as MZ precursor B cells. When the increased BCR signal strength in Aiolos-deficient mice was abrogated by crossing these mice with Xid mice, no less of MZ or MZ precursor B cells was observed, which indicates that increased BCR signaling hampers the differentiation of immature B cells into MZ B cells and Aiolos was not directly required for MZ B-cell development.<sup>59</sup> Taken together, these observations suggest that an increased Aiolos level may partially contribute to the enhancement of MZ B-cell development in Gpr97<sup>-/-</sup> mice.

There are several mechanisms that may explain the cell fate determination to develop into either FO B cells or MZ B cells in the spleen. First, BCR signaling strength is reported to have a critical role in mature B-cell fate decision. FO B-cell development is dependent on strong BCR signaling, whereas signals driving MZ B-cell development are weak.<sup>40</sup> Second, the Notch signaling pathway is essential for mature B-cell fate determination. The mice lacking Notch2<sup>50</sup> Rbpj<sup>51</sup> or Dil<sup>1</sup><sup>52</sup> have defects in MZ B-cell development. Third, BAFF and NF-κB are also required for peripheral B-cell development. In IL-7 transgenic mice which have a dramatic increase in the FO B-cell compartment,<sup>53</sup> MZ B-cell number was reduced probably due to lower overall levels of BAFF. MZ B-cell development is defective in mice that lack p50.<sup>54</sup> The absence of Rel and p65 also affects MZ B-cell development partially, which indicates that p50-Rel and p65-Rel complexes might be required for MZ B-cell development. Accordingly, we examined the levels of BAFF, NF-κB and Notch2 in the spleens of WT and KO mice, and found that nuclear p50 and p65 were markedly increased in Gpr97-null mice, whereas serum BAFF and Notch2 proteins had no differences between WT and KO mice. These findings suggest that the enhanced NF-κB signaling strength may contribute to an increase in MZ B cells in Gpr97<sup>-/-</sup> mice, whose phenotype is opposite to that of p50<sup>-/-</sup> or p65<sup>-/-</sup> mice. The question is how Gpr97 affects the levels of nuclear p50 and p65 in splenocytes. A possible inference is that PKA activation is able to simulate PKC and p38 MAPK, leading to IKK-dependent NF-κB activation.<sup>55</sup>

In summary, our study uncovers a novel biological function of Gpr97 in regulating B-cell development, especially CREB and NF-κB signaling pathways, implying Gpr97 as a potential therapeutic target for treatment of immunological disorders.

Materials and Methods

Mice. The mutant mice were generated by using a homologous recombination method and maintained on a mixed 129Sv/C57BL6 background and housed under specific pathogen-free conditions at a constant room temperature of 22–24°C with a 12-h light/dark cycle. Animal protocols and experiments were approved by the Animal Use and Care Committee of Shanghai Research Center for Model Organisms ( Permit Number: 20110007). Age-matched littermates (12-week old) with different genotypes were used for phenotypic analyses. More detailed description on Materials and Methods can be found in the Supplementary Information on line.

Immunization and determination of serum Ig levels. Tilters of Ig in serum were tested using ELISA. In brief, 96-well plates were coated with goat anti-mouse Ig capture antibody overnight at 4°C, followed by incubation with diluted serum samples and developed with HRP-conjugated goat anti-mouse isotype-specific antibodies and substrate ABTS (Southern Biotechnology, Birmingham, AL, USA). Reactions were read at 405 nm at 10 min after substrate addition using a microplate reader (BioTek, Winooski, VT, USA). To elicit Th antigen responses, gender-matched 12-week-old WT and KO mice were intraperitoneally injected with 50 μg DNP-conjugated Ficoll (Biosearch Technologies, Petaluma, CA, USA). Sera were collected at the time of immunization and 14 days later, and anti-DNP IgM and IgG3 antibody titers were measured by ELISA. For TD antigen responses, 12-week-old WT and KO mice were intraperitoneally injected with 100 μg DNP-keyhole limpet hemocyanin (KLH, Biosearch Technologies) with CFA. After 2 weeks, mice were injected again with 10 μg DNP-KLH with IFA. Sera were collected on the day of the primary immunization, at the time of the secondary immunization, and another week later. Antibodies to DNP were measured by ELISA using plates coated with DNP-BSA (Biosearch Technologies).

Cell culture. Suspensions of splenocytes (after isotonic erythrocyte lysis) were cultured in complete RPMI 1640 medium containing 10% FBS, 1% HEPES, 1% L-glutamine, 1% pen/strep and 0.1% 2-mercaptoethanol with or without LPS (20 μg/ml) for 24 or 48 h. Monitoring of apoptosis or necrosis was performed by staining with annexin V and PI (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instructions. Cells negative for PI but positive for annexin V were defined as apoptotic cells. Late apoptotic and necrotic cells were identified by strong PI staining.

Histological analysis. Spleens were fixed for 24 h in 10% neutral buffered formalin, embedded in paraffin, sectioned (5 μm) and then stained with hematoxylin and eosin. For immunohistochemistry, splenic sections were incubated with the antibodies against B220 (BD Pharmingen, Franklin Lakes, NJ, USA) in blocking buffer overnight at 4°C. After washing, the sections were incubated with biotin-labeled rat antibody (KPL), and labeled developed using an ABC kit (Vector Laboratories, Burlingame, CA, USA). For immunofluorescence, spleens were frozen in Tissue-Tek OCT compound (Sakura, Torrance, CA, USA) and sectioned at a thickness of 8 μm. Sections were then fixed for 15 min in 4% paraformaldehyde (PFA) and stored at −20°C. For labeling, slides were washed, incubated for 10 min in PBS containing 0.25% Triton X-100 and blocked for 30 min in 1% BSA in PBS. Then sections were labeled overnight at 4°C with primary antibody in blocking buffer. After extensive washing in PBS, slides were mounted in Antifade Mounting Medium (Beyotime, Shanghai, China). Primary antibodies used: FITC-MOMA-1 (AbD Serotec, Oxford, UK) and Texas Red-IgM (Vector Laboratories). GC formation in spleen was examined by histological analysis. Sections were labeled with PNA (Vector Laboratories), and color was developed using an ABC kit (Vector Laboratories).

Analyses of mRNA expression. Total RNA was prepared from different mouse tissues using Trizol Reagent (Invitrogen) according to manufacturer’s instructions. mRNA expression levels were assayed by semi- or real-time quantitative RT-PCR. The expression level of β-actin was used as an endogenous control. Real-time PCR analysis was performed using SYBR Premix Ex Taq kit (Takara, Dalian, China) on an Eppendorf Mastercycler system according to the manufacturer’s protocol. All samples were tested in triplicate, and the results were normalized to β-actin expression.
Flow cytometry. Single-cell suspensions of BM cells, splenocytes or PB cells were stained using standard procedures. Cells were adjusted to 2 × 10^6 cells/ml in PBS with 2% FBS and 0.1% sodium azide, and 1 × 10^6 cells were incubated 30 min on ice with appropriately diluted antibodies (1 ng/µl) in a total volume of 100 µl. After washing three times, the stained cells were analyzed using a flow cytometer (BD FACsAnia, BD Pharmingen). Processed samples were analyzed using CellQuest software (BD Biosciences).

Western blot analysis. Whole-cell lysates from BM cells (1–2 × 10^7) were extracted with RIPA lysis buffer (Thermo Scientific, Waltham, MA, USA). Nuclear protein extracts from spleen were prepared according to the handbook of NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). The protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts of protein (60–80 µg) were separated and transferred onto polyvinylidene fluoride membrane by semi-dry blotting. The membranes were incubated overnight with the antibodies listed in Supplementary Information. Infrared fluorescence on membranes was detected using an Odyssey infrared imaging system (Li-COR). For quantitative determinations, a densitometric analysis of signal bands was performed using Gel-Pro Analyzer software (Media Cybernetics. Bethesda, MD, USA).

Statistical analysis. Quantitative results were presented graphically as mean ± S.E. The statistical differences in the observed data were compared by one-way ANOVA (analysis of variance). P < 0.05 was considered statistically significant.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements
This work was partially supported by the Grants from Ministry of Science and Technology of China (2006BAI23B02 and 2011BAI15B02), the Science and Technology Commission of Shanghai Municipality (11DZ2292400, 12ZR1421100, 13410901400 and 13DZ2280600), and the E-Istitutes of Shanghai Municipal Education Commission (E03003).

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