Heme regulates B-cell differentiation, antibody class switch, and heme oxygenase-1 expression in B cells as a ligand of Bach2

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Heme binds to proteins to modulate their function, thereby functioning as a signaling molecule in a variety of biologic events. We found that heme bound to Bach2, a transcription factor essential for humoral immunity, including antibody class switch. Heme inhibited the DNA binding activity of Bach2 in vitro and reduced its half-life in B cells. When added to B-cell primary cultures, heme enhanced the transcription of Blimp-1, the master regulator of plasma cells, and skewed plasma cell differentiation toward the IgM isotype, decreasing the IgG levels in vitro. Intraperitoneal injection of heme in mice inhibited the production of antigen-specific IgM when heme was administered simultaneously with the antigen but not when it was administered after antigen exposure, suggesting that heme also modulates the early phase of B-cell responses to antigen. Heme oxygenase-1, which is known to be regulated by heme, was repressed by both Bach2 and Bach1 in B cells. Furthermore, the expression of genes for heme uptake changed in response to B-cell activation and heme administration. Our results reveal a new function for heme as a ligand of Bach2 and as a modulatory signal involved in plasma cell differentiation.

Introduction

Heme is an essential molecule for diverse living organisms, including human beings. It plays a variety of roles in oxygen transport, electron transfer, and catalytic reactions.1 In addition to these well-established functions as a prosthetic group, recent reports have revealed that heme regulates several transcription factors, including Bach1,2-5 NPAS2,6 and REV-ERBs,7 and modulates gene expression as an inter- and intracellular signaling molecule in mammals. Several observations have suggested a regulatory role for heme in the immune system. For example, heme oxygenase-1 (HO-1), an enzyme that degrades heme, inhibits dendritic cell maturation.8 Greater serum immunoglobulin M levels are observed in HO-1–deficient mice compared with wild-type (WT) mice,9 suggesting that intracellular heme levels may regulate antibody production. Moreover, suppression of heme synthesis by iron depletion impairs the blastogenic response of splenic lymphocytes.10 These observations collectively suggest that heme may play a critical regulatory role in the immune system. However, little is known about the mechanism of the heme-mediated responses or changes in heme metabolism in the immune system.

Bach1 and Bach2 constitute a subfamily of the basic region-leucine zipper (bZip) family.11 They form heterodimers with small Maf proteins, bind to the Maf recognition element (MARE), and repress target gene expression. Bach2 is specifically expressed in hematopoietic cells of the B-cell lineage, where it is expressed from pro-B to mature-B cell stages.12

Resting mature-B cells resume proliferation and then differentiate into antibody-secreting plasma cells in response to antigen stimulation or polyclonal stimulation. A default pathway for plasma cells is to become IgM-secreting plasma cell. An alternative pathway is to undergo class switch recombination (CSR) and somatic hypermutation (SHM) to become plasma cells, secreting isotypes of Igs such as IgG or IgA with greater affinity for a specific antigen.13 Bach2 plays 2 critical roles in the process of plasma cell differentiation. First, it is required for both CSR and SHM of immunoglobulin genes.14,15 Bach2-deficient (Bach2−/−) B cells preferentially differentiate into IgM-secreting plasma cells, compared with control WT cells.15 Second, Bach2 represses the expression of the transcription factor B lymphocyte-induced maturation protein 1 (Blimp-1),16,17 which is expressed in plasma cells but not in mature-B cells,18 and is essential for plasmacytic differentiation.18,19 Bach2 postpones terminal differentiation of B cells to plasma cells by inhibiting Blimp-1 and hence secures enough time for CSR to occur (a delay-driven diversity model for CSR).15 Such critical roles of Bach2 suggest that Bach2 would be precisely regulated during B-cell activation and plasma cell differentiation, but little is known about the control of Bach2.20

A salient feature of Bach1 is its regulation by heme. Bach1 possesses at least 4 Cys-Pro motifs (CP motifs)21,22 involved in heme-binding. Heme binds to Bach1 to inhibit its DNA binding, to induce its nuclear export, and to induce its polyubiquitination and proteasome-dependent degradation.23,24 As a net effect, heme achieves derepression of Bach1 target genes, including globin and Hmox1, which encodes HO-1, and allows their induction by competing transcription activators such as NF-E2 and Nrf2.2,25-29
Bach1 deficiency in mice results in derepression of HO-1 in multiple tissues and cell types. Although we have previously reported that Bach2 might be a repressor of HO-1 in transformed B cells, this regulation has not been proven in normal B cells. Noting that Bach2 also possesses CP motifs, we explored the possibility that heme may control the Bach2 function in B cells. We also examined the expression of genes involved in the heme metabolism in B cells. The results suggest a cross-regulation of heme metabolism and B-cell activation by Bach2.

Methods

Plasmids

A Bach2 cDNA fragment encoding residues 331-761 was amplified from the pBSF69-J plasmid template by PCR with the following primers (Invitrogen): 5'-CTTGGATCCCCAGGAGTGGTCCCTCGCC-3', which also contained a BamHI site; and 5'-CTTGGATCCCCGGGGTTATCATC-CTACTGCACAGGAGGAG-3', which contained a NoI site next to the termination codon. The amplified fragment was then digested with BamHI and NoI and cloned into pGEX-6P-1 (GE Healthcare). The GST-fusion Bach2 protein was named GST-Bach2. Mutations were created with Bach2 cDNA cloned into the pAlter1 vector (Promega) as described by the manufacturer. A Prdm1 reporter plasmid (promoter-MARE-luc) and pCMVBach2 have been described previously.

Expression and purification of GST-Bach2 and GST-Bach2mCP

Escherichia coli Rosetta (DE3) cells (Merck) transformed with the expression constructs were grown in LB media at 37°C, and the recombinant proteins were induced as described previously. Soluble protein fractions were purified with glutathione-sepharose 4B (GE Healthcare) and heparin-sepharose CL-6B (Amersham Biosciences) columns. Proteins were finally concentrated with the use of an Amicon Ultra-15 membrane (Millipore).

Spectroscopic analysis for the heme-binding assay

The heme-binding was estimated by optical titration in 50mM Tris-HCl at Spectroscopic analysis for the heme-binding assay (Millipore). The heme titration of GST-Bach2 was carried out in the finally concentrated with the use of an Amicon Ultra-15 membrane (Millipore).

EMSA

An oligonucleotide designed based on previously reported (5'GAGTATCCGTTGATCATCAACATTCCGAGC-3') was labeled with [γ-32P]ATP (PerkinElmer) with T4 poly nucleotid kinase (Takara). GST-Bach2 protein or MBP-MafK (60 ng) was incubated with the probe in 10 μL of gel shift buffer. After the addition of heme, the reaction mixtures were left on ice for 10 minutes. Electrophoresis and image analysis were performed as described previously.

Luciferase reporter assay

The 18-81 pre-B cells were transiently transfected with reporter and effector plasmids as described previously. Because heme has a decreased effect in the presence of FBS, 1 or 5μM heme was added to each cell culture medium without 10% FBS.

Mice

C57BL/6J mice were purchased from Charles River Laboratories. The Bach1 and Bach2 genes were generated by crossing respective heterozygous mutant mice. Detailed phenotypic evaluations of the double deficient mice will be reported elsewhere (A.I.N., A.M., K.I., manuscript in preparation). All experiments involving mice were approved by Tohoku University.

Cell lines, preparation of primary B cells, and heme treatment

The 18-81 pre-B cells were maintained in IMDM medium (Invitrogen). B cells were purified from the single-cell suspensions of spleens as described previously. The cells were plated at 1 × 10^6 cells/mL in 24-well plates containing RPMI 1640 medium and 20 μg/mL LPS with or without 20μM heme. Deferoxamine mesylate salt (Sigma-Aldrich) was used as an iron chelator. ELISA and ELISPOT were performed as described previously.

Bach2 protein stability

The 18-81 pre-B cells were centrifuged and resuspended in medium containing 20 μg/mL cycloheximide (Chx) with or without 5μM heme. Primary B cells were incubated with 20 μg/mL LPS with or without 20μM heme for 4 days. The Bach2 protein was detected by a Western blotting assay with the use of MafK and α-tubulin as internal controls. The band intensities of Western blots were measured by a densitometric analysis (National Institutes of Health imaging analyzer). The primary antibodies used were anti-Bach2 antiserum (F69-1) and anti-MafK antiserum (A-1).

Quantitative PCR

RNA isolation and qPCR were carried out as described previously. The sequences of the qPCR primers for Blimp-1, ALAS-N, AID, HO-1, Tfrc, CD91, CD163, ferroportin-1, and β-actin are available from the authors on request.

FACS analysis

The FACS analyses were performed with EGFP and antibodies against mouse CD45R/B220 and CD138 (BD). The cells were analyzed on a FACScalibur instrument with the CellQuest software package (BD).

Intraperitoneal injection of heme

Heme (hemin; Frontier Scientific) was dissolved in 10% ammonium hydroxide in 0.15M NaCl to prepare a stock solution of 100 mg/mL, then was further diluted 1:40 with sterile 0.15M NaCl and injected intraperitoneally into mice (10 μL/mouse). 2,4-dinitrophenyl-conjugated Ficoll (DNP-Ficoll) was injected intraperitoneally into mice (100 μg/mouse).

Results

Heme directly binds to Bach2

Bach2 is structurally related to Bach1 and possesses 5 CP motifs (Figure 1A). To investigate the possibility that Bach2 is a heme-binding protein, we over-expressed a Bach2 fragment spanning residues 331-761, (called GST-Bach), containing the 5 CP motifs and the bZip domain (DNA binding domain) in E.coli. GST-Bach2 was purified to > 85% purity as judged by SDS-PAGE (data not shown). The pale brown color of the purified protein solution (λmax: ~ 420 nm) was suggestive of substoichiometric heme-binding to the Bach2 fragment expressed in E.coli.

The heme titration of GST-Bach2 was carried out in the presence of excess BSA, which is known to bind heme weakly, to suppress nonspecific binding of heme to Bach2. Because the spectroscopic features of heme are sensitive to its environment, the heme-binding to Bach2 can be assessed directly by spectrometric measurements. Absorption spectra during the heme titration were recorded in the presence and absence of the GST-Bach2 protein to

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calculate the differences in the absorption spectra, as shown in Figure 1B. The positive peaks in both the Soret and visible regions represent the formation of a Bach2-heme complex, and 2 intense Soret peaks at 366 and 432 nm are indicative of at least 2 distinct
heme-binding modes in the GST-Bach2 protein. The negative peaks at 401 and 615 nm are because of the decreased accumulation of the BSA-heme complex in the presence of GST-Bach2. The titration curve observed at 401 nm reveals that Bach2 can bind 4 or 5 mole equivalents of heme (Figure 1C). These spectral features and the heme-binding capacity of Bach2 are essentially the same as those observed for Bach1.

The far blue-shifted positive peak at 366 nm is typical of a 5-coordinate ferric high-spin heme with coordination of a thiolate ligand in the CP motif cysteine. In fact, substitution of all of the cysteine residues in the 5 CP motifs of Bach2 by alanine (GST-Bach2mCP) resulted in the disappearance of the 366-nm peak (Figure 1A,D). The peak at 432 nm shifted to 426 nm with these mutations, but it was not lost, suggesting that the 4 CP motifs were not essential for this mode of heme binding. Similar results were obtained by the use of at least 3 independent preparations of the recombinant proteins. These findings unequivocally show that heme binds Bach2 with 2 modes, similar to Bach1.

**Heme regulates both the DNA binding and protein stability of Bach2**

The effect of heme on the DNA binding activity of Bach2 was examined by EMSA. A homodimer of Bach2, as well as its heterodimer with Maf proteins, can bind to DNA containing a MARE sequence. In the presence of various concentrations of heme, a MARE-containing DNA probe was incubated with GST-Bach2 or maltose binding protein-MafK (MBP-MafK) (Figure 1E-F). The DNA-binding activity of the GST-Bach2 was markedly inhibited by heme (Figure 1E lanes 6 and 7, G). This finding is in contrast to the marginal inhibition of the DNA-binding activity of the MBP-MafK by heme (Figure 1F-G). These results clearly indicate that heme regulates the DNA binding activity of Bach2. The DNA binding activity of GST-Bach2mCP was still inhibited by heme in vitro (data not shown), thus suggesting that the remaining heme binding mode was also involved in the regulation by heme.

**Heme induces Bach2 degradation**

To examine the effects of heme on endogenous Bach2 in B cells, we determined the levels of Bach2 in the 18-81 pre-B cell line treated with heme. Chx was added to the culture medium to inhibit de novo protein synthesis. The 18-81 pre-B cells were incubated in medium containing Chx with or without heme. Endogenous Bach2 was relatively stable in the absence of heme, but it disappeared more rapidly in the presence of heme (Figure 2A-B). In contrast to Bach2, the half-lives of MafK and α-tubulin were not markedly affected by heme (Figure 2B). We obtained similar results from 3 independent experiments.

We next examined the effect of heme on the protein level of Bach2 in primary B cells, which can be induced to differentiate to plasma cells in vitro with lipopolysaccharide (LPS). Splenic B220-positive B cells were stimulated with LPS in the presence or absence of heme for up to 4 days, as indicated in Figure 2C. The levels of Bach2 protein remained high for 2 days, and then decreased between 2 to 4 days in response to LPS (Figure 2C). In contrast, in the presence of heme, the levels of Bach2 started to decrease at 1 and 2 days after LPS stimulation (Figure 2C). Although the MafK protein level remained high for the first 2 days, it decreased on days 3 and 4. The addition of heme resulted in a reduction of MafK on days 1 and 2, suggesting that heme may also affect the MafK protein via the change in the Bach2 level in primary B cells. Heme did not cause any gross change in other protein levels, as revealed by Coomassie blue staining of the gel (Figure 2C bottom panel). Similar results were obtained in 3 independent experiments. On the basis of these observations, we concluded that heme induced the degradation of Bach2 in B cells.

**Heme increases the population of Blimp-1–expressing cells through Bach2**

To explore the regulatory significance of the effects of heme on Bach2 in B cells, we examined the effect of heme on the transcriptional repression activity of Bach2 in the 18-81 pre-B cell line (Figure 3A). We used a luciferase reporter plasmid with a single MARE sequence derived from the Prdm1 gene encoding Blimp-1. As reported previously, Bach2 repressed the Prdm1 MARE reporter in untreated cells (Figure 3A). Bach2-mediated repression of the reporter was alleviated by adding heme in the medium for the last 4 hours of cell culture (Figure 3A). These results suggested that heme inhibited the repressor activity of Bach2 in the 18-81 pre-B cells.

To investigate the effect of heme on gene expression during the plasma cell differentiation, we isolated B220-positive B cells and then stimulated them with LPS alone or with LPS and heme. We measured the time course of mRNAs for Blimp-1 and activation-induced cytidine deaminase (AID) by using qPCR. Blimp-1 is not only essential for plasma cell differentiation, but it also suppresses CSR by repressing AID, and an enzyme essential for CSR. The level of Blimp-1 mRNA increased between day 0 and day 2 after LPS stimulation. In the presence of heme, its induction became more prominent (Figure 3B), consistent with the results of the reporter assays. Although the levels of AID mRNA were increased in response to LPS treatment, the level was decreased in the presence of heme to ~50% (Figure 3C).

Nonspecific 5-aminolevulinate synthase (ALAS1 or ALAS-N) is the rate-limiting enzyme of heme synthesis in diverse types of cells, and its expression correlates well with the intracellular levels of heme. The expression of ALAS-N was induced on B-cell activation by LPS in vitro (Figure 3D), thus suggesting that the synthesis of heme was induced on B-cell activation by LPS. However, it was repressed when B cells were treated with both LPS and heme (Figure 3D), confirming that the heme treatment resulted in an increase in the intracellular heme levels. Heme is known to inhibit ALAS-N expression. These results suggest that the increased levels of heme in B cells affect the expression of the genes involved in CSR and plasma cell differentiation, and that heme synthesis increases during B-cell activation because of increased ALAS-N expression.

To examine the effect of heme on Blimp-1 expression in B cells at the single-cell level, we used an EGFP reporter transgenic mouse for the Prdm1 locus. We isolated splenic B220-positive B cells, stimulated them with LPS for 2 days to induce plasma cell differentiation, and determined the percentage of the Blimp-1–EGFP–positive cell population by using FACS. In the WT background, 6.6% ± 1.5% of cells became EGFP-positive in response to LPS (Figure 4A). Additional treatment with heme increased the frequency of EGFP-positive cells by 2.4-fold (15.7% ± 2.66%) compared with cells treated with LPS alone (Figure 4A-B). Most of the EGFP-positive cells expressed CD138, which is a surface marker of plasma cells (Figure 4A). We confirmed that the effect of heme on the frequency of EGFP-positive cells was concentration dependent (data not shown). These results suggested that heme acted as a costimulatory factor for
Blimp-1 expression, regulating the frequency of its expression in activated B cells.

To investigate whether the heme effects involved Bach2, we next performed the same experiments by using Bach2−/− B cells. We interbred Bach2-deficient (Bach2−/−) and Blimp-1-EGFP reporter mice to generate Bach2−/−/Blimp-1-EGFP mice. We first confirmed that Bach2−/− B cells stimulated with LPS alone showed a greater EGFP-positive population (32.0% ± 6.78%) compared with Bach2+/− B cells stimulated with LPS alone (6.6% ± 1.57%; Figure 4A). This observation reiterates that Bach2 is important for the inhibition of Prdm1 gene.14-16

In the Bach2−/− B cells, additional treatment with heme showed only a marginal effect on the percentage of EGFP-positive cells (40.8% ± 7.33% and 1.3-fold; Figure 4A-B). These results suggested that the enhancement of Blimp-1-EGFP expression by heme involved the inactivation of Bach2. The residual effect of heme in Bach2−/− B cells may reflect the derepression of Blimp-1 by the inactivation of Bach1 because we have recently found that Bach1 binds to a Prdm1 gene regulatory region and represses its expression (unpublished observation).

**Heme inhibits CSR in activated B cells**

Heme is expected to affect CSR because Bach2 is required for CSR. To examine the possibility, mouse splenic B220-positive B cells were stimulated with LPS and measured for Ig secretion by isotype-specific ELISA. Heme augmented the IgM secretion induced by LPS (Figure 4C). In contrast, heme inhibited IgG3 secretion (Figure 4C). To detect and quantify individual antibody-secreting B cells, we performed an ELISPOT assay (Figure 4D). When B cells were stimulated with LPS and heme, the number of IgM-secreting cells increased roughly 2-fold compared with cells treated with LPS alone (Figure 4E). Taken together, these results indicate that heme promotes plasma cell differentiation toward IgM-secreting plasma cell differentiation rather than CSR.

**Heme-derived iron is not involved in the regulation of B cells**

It is possible that iron, a product of heme degradation by HO-1 and/or HO-2, but rather than heme itself, might have affected Bach2 and its downstream effects on gene expression. Indeed, we found that heme treatment of splenic B cells resulted in decreases...
in transferrin receptor mRNA levels (data not shown), indicating an increase in the intracellular iron level on the heme treatment. To examine the possibility that iron was responsible for the effects of heme, we stimulated splenic B cells from WT mice with LPS and heme in the presence or absence of an iron chelator deferoxamine, and the cells were analyzed by FACS for CD138 expression (Figure 4G). Although heme enhanced the plasma cell differentiation, the additional treatment with deferoxamine did not reverse the stimulatory effect of heme. Blimp-1 induction was not affected by the deferoxamine treatment (Figure 4G). These results support the interpretation that heme, but not iron, stimulated the plasma cell differentiation.

The effects of heme on antigen stimulation in vivo

To examine the effects of heme on antibody production in vivo, we performed intraperitoneal administration of heme in mice. We first confirmed that heme induced the HO-1 levels in the liver (Figure 5A-B), indicating that exogenous heme was taken up by surrounding cells and organs, and that it induced HO-1 expression. WT mice were immunized with the T cell–independent antigen DNP-Ficoll with or without heme (Figure 5C-D). Heme was also injected intraperitoneally 2 additional times (Figure 5C). The production of antigen-specific IgM was reduced in the presence of heme (Figure 5D). In contrast, when heme was administered only after the immunization of DNP-Ficoll, the production of specific IgM antibodies remained unchanged (Figure 5E-F). These observations suggest that heme may affect some of the initial processes of B-cell differentiation. One possibility was that heme inhibited B-cell proliferation. However, heme did not show any significant inhibition of proliferation of primary B cells activated with LPS in vitro (Figure 5G). Thus, heme may affect the early events driven by antigen stimulation. These results suggest that heme modulates the production of antibodies in mice depending on the timing of the B-cell encounter.

Regulation Hmox1 by Bach2 and Bach1 in B cells

Although HO-1 is critical for proper antibody production, little is known about its expression or regulation in B cells. To examine the correlation between HO-1 expression and B-cell differentiation, pre-B cells (B220low, CD43– IgM– cells in BM), immature-B cells (B220low, IgM+ cells in BM), mature-B cells (B220hi, IgM+ cells in BM), and plasma cells (B220low+, CD138+ cells in spleen) were sorted and purified, and the HO-1 mRNA expression was analyzed (Figure 6A). Compared with B cells in immature stages of differentiation, HO-1 expression was increased dramatically in plasma cells. Because the expression of HO-1 is induced by heme, these results also suggested that there was an increase in the intracellular heme levels in plasma cells. The expression profile of HO-1 in B and plasma cells is opposite that of Bach2, raising the possibility that Bach2 may be involved in the repression of HO-1 in B cells. To investigate this possibility, we isolated a B220low population containing both pro-B and pre-B cells from the BM of control WT or Bach2−/− mice. We also examined the B220low population from Bach1−/− mice and Bach1/Bach2 double-deficient mice, considering their possible redundancy (Figure 6B). The expression of HO-1 was increased 2-fold by the Bach1 deficiency but not by the Bach2 deficiency. HO-1 expression was further increased in the double-deficient B cells (Figure 6B). We concluded that Bach1 and Bach2 redundantly repressed the expression of HO-1 in B cells.

Dynamic expression of genes for heme and iron transport in B cells

The aforementioned results revealed 2 unique aspects of heme in B cells: heme regulates B-cell responses, whereas B-cell activation...
Figure 4. Effects of heme on plasma cell differentiation. (A) The expression of the Blimp-1-EGFP reporter gene determined by a FACS analysis. B220-positive cells from WT (Bach2+/+) Blimp-1-EGFP mice and Bach2-deficient Blimp-1-EGFP (Bach2−/−) mice were stimulated with 20 µg/mL LPS in the presence or absence of 20 µM heme on day 2. Each gate shows the percentage of EGFP-positive cells. (B) The ratio of EGFP-positive cells cultured with LPS + heme or LPS alone in Bach2+/+ (left) or Bach2−/− (right) cells. The data are presented as the means ± SD of triplicate determinations. The statistical analyses were performed by the use of the Student t test. (C) The IgM and IgG3 secretion from splenic B220-positive B cells was analyzed by ELISA. B220-positive B cells were cultured with 20 µg/mL LPS alone (open bars) or LPS and 20 µM heme (filled bars) for 7 days, and the secreted immunoglobulin levels were measured. The data are presented as the means ± SD of triplicate determinations. Groups of 3-5 mice were used for the statistical analysis. P values (⁎ P < .05) were calculated by use of the Student t test. (D) The differentiation of IgM-producing plasma cells in mouse splenic B220-positive B cells stimulated with 20 µg/mL LPS in the presence or absence of 20 µM heme for 2 days. IgM-producing cells were detected by ELISPOT assay. (E) The percentage of IgM-secreting cells within the B-cell population. Each bar indicates the average percentage of IgM-secreting cells in 8 x 10⁶ B cells (open bars: stimulation with LPS alone, filled bars: stimulation with LPS and heme). The data are presented as the means ± SD of triplicate determinations. Groups of 3-5 mice were used for the statistical analysis. P values were calculated with use of the Student t test. (F-G) The percentages of cells expressing CD138 (F) and relative expression levels of Blimp-1 mRNA (G) in splenic B cells from in vitro culture treated with LPS for 2 days. Heme and deferroxamine (µM) were added as indicated. Mean of 2 independent experiments are shown.
regulates heme metabolism. As indicated in Figures 3D and 6A, the expression of ALAS-N and HO-1 was increased on B-cell activation, suggesting a dynamic change in heme metabolism. Consistent with this idea, the mRNA expression levels of hemopexin receptor CD91 and haptoglobin receptor CD163, both involved in protein-bound heme uptake, were reduced dramatically on B-cell activation (Figure 6C). In contrast, the mRNA expression of the HRG1 heme transporter remained unchanged on B-cell activation (Figure 6D). Therefore, B cells appear to use distinct mechanisms for heme uptake before and after activation. Interestingly, HRG1 expression was increased on heme treatment (Figure 6D), suggesting that extracellular heme stimulates heme uptake by activated B cells. Ferroportin mRNA was reduced on B-cell activation by LPS (Figure 6E). This change may also contribute to the changes in heme and iron metabolism in activated B cells.

**Discussion**

To the best of our knowledge, this study is the first report suggesting a molecular mechanism by which heme regulates humoral immunity. Together with our recent report, our present results provide strong evidence that heme augments plasma cell differentiation by inhibiting Bach2, and thus increasing the population of Blimp-1–expressing cells. Because Blimp-1 is the master regulator of plasma cell differentiation, its derepression by heme through Bach2 inactivation may fine-tune the response of humoral immunity. In addition, heme may regulate the B-cell responses by inducing the expression of HO-1, which possesses antioxidant and immunomodulatory functions.9
In the spectral analyses, the 432-nm absorption band (6-coordinate heme-binding mode) and the 366-nm absorption band (5-coordinate heme-binding mode) both became apparent on addition of heme (Figure 1B). These results suggested that Bach2 bound to heme directly and had at least 2 distinct binding modes. Moreover, the spectral changes of GST-Bach2mCP showed that the
366-nm absorption band was because of heme-binding by the CP motifs (Figure 1D). The presence of 2 heme-binding modes is similar to Bach1. Although amino acid residues involved in the heme binding represented by the 432-nm peak remain to be identified, our observation clearly indicates that Bach2 is an intracellular heme receptor in B cells.

We have previously reported that Bach2−/− B cells show profound up-regulation of Blimp-1 and a severe reduction in the CSR and SHM of immunoglobulin genes. When WT B cells carrying the Blimp-1-EGFP reporter gene were stimulated with LPS and heme, we found that the frequency of EGFP-positive cells was increased compared with cells treated with LPS alone (Figure 4A-B). Hence, heme may increase the population of Blimp-1-expressing cells by inactivating Bach2. Interestingly, the exposure of B cells to heme resulted in a 2-fold increase in IgM secretion and decreased IgG secretion (Figure 4C). Concomitantly, the AID mRNA level decreased in the presence of heme to half of the level present in cells with LPS stimulation alone (Figure 3C). Considering the fact that Blimp-1 inhibits AID expression and promotes plasma cell differentiation, our data suggest that heme-mediated induction of Blimp-1 resulted in reduced CSR and enhanced plasma cell differentiation. The metabolism and transport of heme in B cells are rate-limiting enzyme of heme synthesis. The biochemical reactions involved in heme synthesis and degradation enzymes in B cells will be informative. Alternatively, B cells may uptake heme from the microenvironment of lymphoid organs. The dynamic changes in the expression of genes involved in heme transport in B cells (Figure 6) are consistent with this hypothesis. Heme could be released in large amounts from dying cells as a result of many pathologic conditions such as hemoglobinopathies and infection. In addition, macrophages uptake hemoglobin and secrete heme to the microenvironment. Thus, heme trafficking at the site of infection and lymphoid tissues may reveal a novel communication network between macrophages and B cells mediated by heme.

The identification of Bach2 as an intracellular heme receptor will help in investigation of the regulation of the immune system by heme. The metabolism and transport of heme in B cells are interesting issues for future studies. Elucidating the entire picture of regulation by heme in plasma cell differentiation will provide valuable information for our understanding of the complex immune system and its dysfunction, including the initiation and development of various diseases.

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Authorship

Contribution: M.W.-M., A.M., and A.I.-N. performed the experiments; T.M. and K.M. assisted with the experiments; T.M. and K.M. assisted with the experiments; K.I. designed the manuscript. All authors made comments on the manuscript.

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References


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