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NFκB induces overexpression of bovine FcRn

A novel mechanism that further contributes to the enhanced immune response in genetically modified animals carrying extra copies of FcRn

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Abbreviations: FcRn, neonatal Fc receptor; FCGRT, FcRn α-chain gene; LPS, bacterial lipopolysaccharide; NFκB, nuclear transcription factor κB; EMSA, electrophoretic mobility gel shift assay

Among the many functions of the neonatal Fc receptor (FcRn) for IgG, it binds to IgG-opsonized antigen complexes and propagates their traffic into lysosomes where antigen processing occurs. We previously reported that transgenic (Tg) mice and rabbits that carry multiple copies and overexpress FcRn have augmented humoral immune responses. Nuclear factor-kappa B (NFκB) is a critical molecule in the signaling cascade in the immune response. NFκB induces human FcRn expression and our previous *in silico* analysis suggested NFκB binding sites in the promoter region of the bovine (b) FcRn α-chain gene (FCGRT). Here, we report the identification of three NFκB transcription factor binding sites in the promoter region of this gene using luciferase reporter gene technology, electromobility shift assay and supershift analysis. Stimulation of primary bovine endothelial cells with the Toll-like receptor-4 ligand lipopolysaccharide (LPS), which mediates its effect via NFκB, resulted in rapid upregulation of the bFcRn expression and a control gene, bovine E-selectin. This rapid bFcRn gene induction was also observed in the spleen of bFcRn Tg mice treated with intraperitoneally injected LPS, analyzed by northern blot analysis. Finally, NFκB-mediated bFcRn upregulation was confirmed at the protein level in macrophages isolated from the bFcRn Tg mice using flow cytometry with a newly developed FcRn specific monoclonal antibody that does not cross-react with the mouse FcRn. We conclude that NFκB regulates bFcRn expression and thus optimizes its functions, e.g., in the professional antigen presenting cells, and contributes to the much augmented humoral immune response in the bFcRn Tg mice.

Introduction

The history of the neonatal Fc receptor (FcRn) dates to 1958 when Brambell described a saturable receptor that mediates the transport of gamma-globulin (IgG) from mother to fetus.¹ Some years later, he inferred the presence of a similar receptor that protected IgG from catabolism, which would explain why it has the longest half-life of plasma proteins.² FcRn was subsequently identified as the protein that mediates transfer of IgGs from milk in the gut of the neonate in rodents.³ Over time, research showed that FcRn binds IgG at the CH2-CH3 interface in a pH-dependent way, i.e., there is no detectable binding at pH 7.4; binding

only occurs at a slightly acidic pH.^{4–6} FcRn was also shown to be a heterodimer of two polypeptides: an MHC class-I like α-chain and β 2-microglobulin (β2 m).⁷ IgG protection was originally thought to be mediated by capillary endothelial cells;⁸ however, the receptor was also detected in hematopoietic cells, in particular monocytes, macrophages and dendritic cells⁹ that were shown to be also involved in IgG protection.^{10,11} FcRn orthologs have been isolated from mouse, rat, human, sheep, cow, possum, pig, and camel, suggesting that this receptor is present in essentially all mammalian species.¹² The unique ability of this receptor to modulate the half-life of IgG and albumin has guided engineering of novel therapeutics.^{13,14} More recently, several publications

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indicated that FcRn plays major roles in antigen-IgG immune-complex (IC) phagocytosis by neutrophil granulocytes,¹⁵ and also in antigen (Ag) presentation of IgG ICs by professional antigen presenting cells (APC).¹⁶⁻¹⁹

We previously reported the effects of FcRn overexpression accomplished by Tg modification in mice and rabbits.²⁰⁻²⁵ These effects include quantitative as well as qualitative augmentation in the humoral immune response of these animals. More specifically, FcRn overexpression increases the number of Ag-specific B cells in the spleen generated in response to immunization, and increases antibody diversity. Among the mechanisms that contribute to the boosted immune response, the augmented Ag-IgG IC presentation via professional APCs such as dendritic cells that express bFcRn seems to be especially important.^{21,26} It is known that FcRn prevents degradation of circulating IgG (both Ag-specific and Ag-non-specific) and extends its half-life, but, because of the many more Ag-specific IgG producing B cells, the Ag-specific IgG fraction is disproportionately high relative to total IgG in these Tg animals.²⁰ Therefore, these FcRn Tg animals mount an enhanced immune response against epitopes that are otherwise weakly immunogenic or evade recognition by the immune system. This observation has practical usefulness for the discovery of novel antibodies, including both polyclonal and monoclonal Abs (mAbs).²⁶

This study was undertaken to analyze additional mechanisms that contribute to the immune capabilities observed in the Tg mice that overexpress bFcRn. Our earlier *in silico* analysis suggested the nuclear factor-kappa B (NFκB) binding site in the promoter of the bFCGRT,²⁷ and it has also been reported that NFκB signaling regulates functional expression and function of the human FcRn.²⁸ The NFκB/REL family of transcription factors has a central role in coordinating the expression of a wide variety of genes that control immune responses.²⁹

In this paper, we report on studies in which we investigated the NFκB responsiveness of the bFcRn that is expressed, among many other cells, in macrophages and dendritic cells of bFcRn Tg mice and significantly enhance their Ag-presenting capability.²¹ Using several complementary strategies, we could show that bFcRn harbors three NFκB binding sites and that the bFcRn expression can be triggered by exposure to lipopolysaccharide (LPS), which causes toll-like receptor (TLR)-4-dependent, NFκB-mediated stimulation³⁰ *in vitro* in primary bovine aorta endothelial cells (BAECs) or peritoneal macrophages derived from Tg mice that carry and express bFcRn. We could also show bFcRn upregulation in the spleen of bFcRn Tg mice that were treated by intraperitoneally (i.p.) injected LPS.

In addition to the effect on the transcription of bFcRn via the multiple copies of the transgene, this study shows that the level of bFcRn can be further increased in these Tg mice by NFκB induction. This pathway of bFcRn induction is triggered upon stimulation with proinflammatory agents, or adjuvants during immunization and thus represents a physiologic response to such conditions in mice. This regulation optimizes bFcRn expression and function in the professional APCs and contributes to the much augmented humoral immune response in bFcRn Tg mice.

Results

Three NFκB binding sites in the bFCGRT promoter as identified by luciferase reporter gene expression assays. To study whether NFκB transcription factors bind to and induce the bFCGRT promoter (bFP) regions, a luciferase reporter gene construct pGL3-bFP2 (-1787 + 92) was first created that harbors a 1787 bp long segment of the promoter and a 92 bp long segment of the non-coding exon1 of the bFCGRT (Fig. S1; numbers are relative to the first nucleotide of exon1 of the bFCGRT³¹). Human p65 overexpression resulted in 4-, 12-, and 10-fold increased luciferase activity of the pGL3-bFP2 construct in HEK-293, HC11 and JAR cell lines, respectively, compared with the activity of the empty vector, indicating that this DNA segment can be induced by NFκB (Fig. 1A and 1B).

We created two other luciferase reporter gene constructs with sequentially shortened fragments of the promoter region: pGL3-bFP3 (-1112+92) and pGL3-bFP4 (-525 + 92) (Fig. 1A; Fig. S1). Luciferase reporter gene assays were performed in HC11 cells using pGL3-bFP2, -bFP3 and -bFP4, respectively. Co-transfection of these cells with the human p65 expression vector (the most prevalent member of NFκB/REL family that harbors transactivation domain) resulted in similarly high luciferase activity using the pGL3-bFP2 and pGL3-bFP3 constructs compared with the unstimulated constructs, while the luciferase reporter construct harboring the shortest fragment of bFP (pGL3-bFP4) failed to be induced by human p65 (Fig. 1C). These data suggested that the NFκB sensitive region is located in the sequence between -1112 and -525 of the bFCGRT promoter.

In silico promoter analysis within this promoter region revealed three potential NFκB-specific binding sites: NFκB-612: (₋₆₂₁TGGGATCTCC₋₆₁₂); NFκB-758: (₋₇₆₇GAAAAACCCC₋₇₅₈), and NFκB-840: (₋₈₄₉GGAAATTCCC₋₈₄₀) that showed similarity to the consensus NFκB sequence (5'-GGGRNYYYCC-3' where R is an A or G; N is any nucleotide; W is A or T; Y is C or T)³² (Fig. S1).

Based on the *in silico* findings, we created pGL3 luciferase gene constructs that contain mutated NFκB sites in the bFP3 segment (mκB-612, -758, -840) to analyze the NFκB induction of these sites independently (Fig. 1A). The p65 sensitivity of pGL3-bFP3-mκB-840 containing a mutation in the highly consensus κB site was significantly reduced compared with the luciferase activity of the original pGL3-bFP3, but the luciferase activity was not completely abolished. Comparable data were obtained with two other reporter gene constructs (pGL3-bFP3-mκB-612 and pGL3-bFP3-mκB-758) containing mutations in κB sites with lower similarity to the consensus κB site, suggesting that a cumulative effect of the three NFκB binding sequences caused the p65 responsiveness of the bFP. Mutagenesis of all three NFκB sites in one luciferase reporter gene vector construct (pGL3-bFP3-mκB-612-758-840) abolished the p65 sensitivity, suggesting that three NFκB sites contribute to the p65 sensitivity of the bFcRn cis-regulatory region spanning from -1787 bp to +92 bp (Fig. 1D).

We also analyzed the 5'-flanking regions (1500 nt related to the first exon) of the rabbit and mouse FCGRT genes to identify

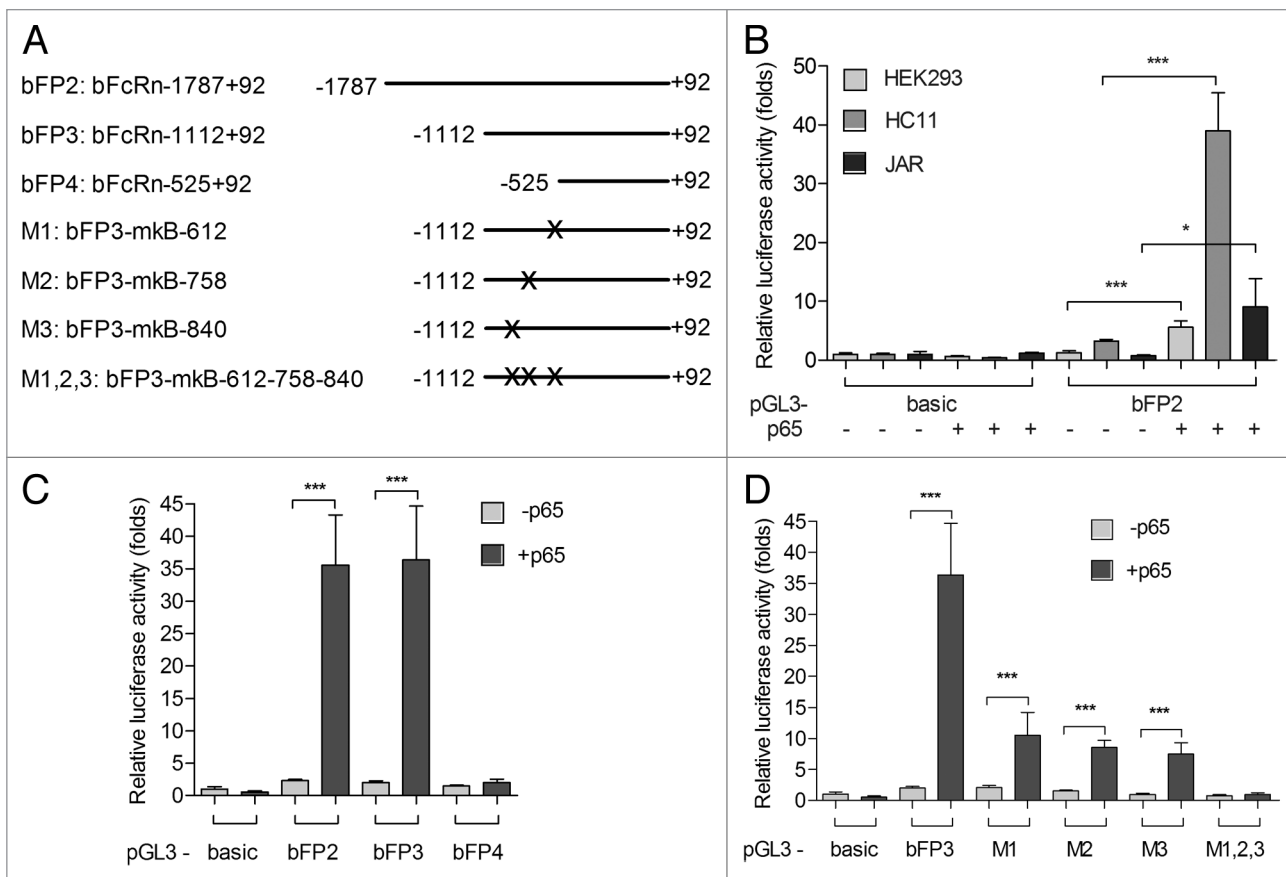


Figure 1. Three NF κ B binding sites in the bFCGRT promoter were identified by luciferase reporter gene expression assays. Cell lines were transiently transfected in the presence or absence of a CMV promoter-driven human p65 expression vector (p65) with pGL3 luciferase reporter vectors containing either sequentially shortened fragments of the bFCGRT promoter (bFP2 harbors a 1787 bp long segment of the promoter and a 92 bp long segment of the non-coding exon1 of the bFCGRT) or mutated bFP3 fragments. (A) Designations of bFP fragments with the positions of the mutated bFP NF κ B binding sites are marked with X. (B) Luciferase activity of the pGL3-bFP2 construct in the HEK-293, HC11 and JAR cell lines. (C) Luciferase activity of pGL3-reporter gene constructs containing sequentially shortened fragments of bFP (pGL3-bFP2, pGL3-bFP3 and pGL3-bFP4) in HC11 cell line. (D) Luciferase activity of bFP3 constructs that have single NF κ B mutation (pGL3-bFP3-m κ B-612 [M1], pGL3-bFP3-m κ B-758 [M2] and pGL3-bFP3-m κ B-840 [M3]) or a bFP3 construct in which all three NF κ B sites were mutated (pGL3-bFP3-m κ B-612-758-840 [M1,2,3]). Results are expressed as relative luciferase activity and represent the mean value (\pm SEM) from at least 3 experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

putative NF κ B binding sites. Our preliminary analysis indicated a single potential NF κ B binding site in the mouse FCGRT promoter (position: -161–152 nt) (Fig. S2A) and four putative NF κ B binding sites in the rabbit FCGRT promoter (positions: -1301–1292 nt, -907–898 nt, 769–760 nt related to the first exon) (Fig. S2B).

Detection of LPS-induced NF κ B nuclear translocation in bovine endothelial cells. To investigate the LPS-induced NF κ B-mediated effects on bFcRn expression, we first analyzed the kinetics of p65 subunit nuclear translocation in primary BAECs upon LPS induction using immunocytochemistry. Untreated and LPS-treated cells were co-stained for bovine p65 by a commercial antibody recognizing the human p65 protein and showing cross-reaction with mouse, rat and bovine p65 and cell nuclei by Hoechst 33342 dye (Fig. S3A). While cytoplasmic localization of p65 subunit was detected in untreated cells, nuclear translocation of p65 was observed after LPS treatment. Relocation of p65 into the nuclei peaked 2 h after adding LPS; thereafter cytoplasmic restoration was detected (Fig.

S3A). These data were calculated based on the fluorescence ratio of nuclear/cytoplasmic p65 protein (Fig. S3B). Based on this experiment, nuclear translocation of the p65 subunit was routinely monitored and the optimal 2-h LPS stimulus was used on BAECs to generate nuclear extract for the electromobility shift assays (EMSA) and supershift analysis.

EMSA and supershift assays confirmed the three NF κ B binding sites in the bFCGRT promoter. To confirm that the bovine p65 subunit of NF κ B directly binds to the NF κ B sites in the promoter of the bFCGRT that we identified by the luciferase reporter gene expression assays, EMSA and supershift assays were performed using nuclear extracts of untreated and LPS-treated BAECs and radiolabeled oligonucleotides representing the κ B-612, κ B-758, κ B-840 and consensus κ B binding sequences. Distinct protein-DNA complexes were detected in the EMSA of LPS-treated BAECs with bFcRn κ B and consensus NF κ B oligonucleotides, though the complex of the κ B-612 oligonucleotide appeared after a longer exposition time (Fig. 2, lower arrow, NF κ B-specific complex, lanes 2, 8, 11, and 14). These complexes

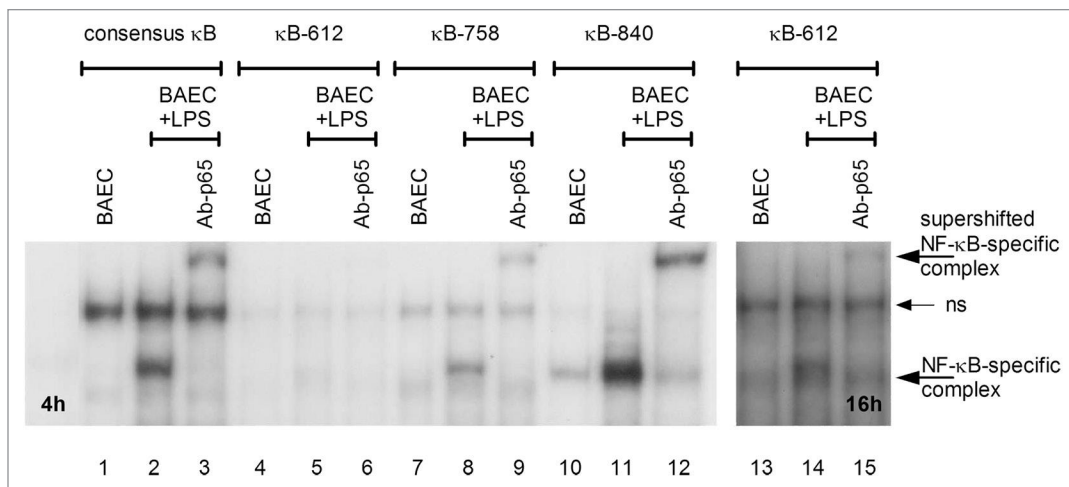


Figure 2. EMSA and supershift assays confirmed three NFκB binding sites in the bFCGR1 promoter. Nuclear extracts (NEs) prepared from untreated and LPS-treated BAECs were incubated with ³²P-oligonucleotides representing the κB-612, κB-758, κB-840 and consensus κB sites in the absence or presence of p65 antibody (Ab-p65). Distinct κB-specific protein-DNA complexes (lower arrow, κB-specific complex) were detected using NEs from LPS-treated BAECs (lane 2, 8, 11), and these complexes could be further shifted by p65 antibodies (upper arrow, supershifted κB-specific complex, lanes 3, 9, 12). In the case of the κB-612 oligonucleotide, the NFκB-specific complex and its supershifted version appeared only after 16 h exposition time (lane 14, 15). ns, non-specific bands.

were further shifted by a p65 specific mAb, indicating that the complexes were indeed NFκB-specific and contained p65 (Fig. 2, upper arrow, supershifted NFκB-specific complex, lanes 3, 9, 12, and 15). No NFκB-specific bands were observed in EMSA of untreated BAECs (lanes 1, 7, and 13) indicating that no p65 subunit was present in the nucleus of untreated cells. We could also observe a band in LPS-treated and untreated samples representing a non-specific complex. Taken together, these results indicate that the oligonucleotides representing the bFCRn NFκB sites identified with luciferase reporter gene assay were able to be bound by the bovine p65.

Exposure of BAECs with LPS upregulates the expression of the bFCRn and E-selectin. To determine whether NFκB induces bFCRn gene expression via an endogenous cell signaling cascade, we treated BAECs with LPS and analyzed the expression of the bFCRn and the bovine E-selectin as control with quantitative real-time PCR. We found that the level of the bFCRn mRNA expression was 2-fold higher after a two-hour LPS treatment after which it returned to its basal level (Fig. 3A). The expression level of the bovine E-selectin reached its maximum (~90-fold higher than its basal level) after 2 h LPS treatment and remained high during the study (Fig. 3B) as described earlier.³³ This experiment confirmed that the bFCRn expression can be upregulated via a cellular signaling pathway that involves NFκB.

LPS treatment of bFCRn Tg mice induces elevated bFCRn mRNA expression in spleen cells. bFCRn expression has been reported in different cells and tissues, including the spleen of bFCRn Tg mice.²¹ To investigate whether LPS treatment upregulates bFCRn expression in vivo we injected LPS i.p. and analyzed the bFCRn mRNA expression using a bFCRn specific northern blot. Data indicated significant and rapid upregulation of the bFCRn in the spleen with a 4-fold increased level, 4 h after LPS treatment after which the bFCRn expression returned

to the basal level (Fig. 4A). LPS treatment reduced the body temperature of the mice (Fig. 4B) as described earlier.³⁴

To exclude the possibility that the increased level of the bFCRn expression is the result of massive bFCRn positive cell influx into spleen during the LPS treatment, we analyzed the ratio of the major spleen cell populations during this study. We found that the LPS treatment did not change the proportion of B cells (B220⁺), T cells (CD3⁺), neutrophil granulocytes (CD11b⁺/Gr1⁺) or dendritic cells (CD11b⁺/CD11c⁺) within the first 8 h (Fig. 5A–D). Because the proportion of CD11b⁺ cells did not change within the first 8 h, we could conclude that there was no change in the proportion of macrophages, the third major CD11b⁺ population. We also detected increased expression of CD11b on the surface of neutrophil granulocytes one hour after LPS treatment and a new population of neutrophils with basal CD11b expression level that influxed the spleen 12 h post treatment (Fig. 5D and 5E). Our data confirmed that the increased level of bFCRn expression we observed during LPS treatment (Fig. 4A) depends on the bFCRn gene upregulation in some splenocytes.

LPS treatment of bFCRn Tg mice increases bFCRn expression at protein level in peritoneal macrophages. To investigate whether an elevated bFCRn mRNA level that occurs after i.p. LPS treatment is followed by increased bFCRn expression at protein level, we generated an FcRn-specific mAb (Z15_6D5/8 mAb) that binds specifically to bFCRn and does not cross-react with the mouse FcRn, based on flow cytometry using peritoneal macrophages (Gr1⁺, CD11b⁺) from bFCRn Tg (that expresses bFCRn based on our previous observation²¹) and wild type (wt) mice (Fig. 6A). We also analyzed the binding of this mAb to monocyte-derived human macrophages and human intestinal T84 epithelial cells and found that it specifically recognizes the human FcRn α-chain protein (Fig. S3) that was identified in these cells.^{9,35}

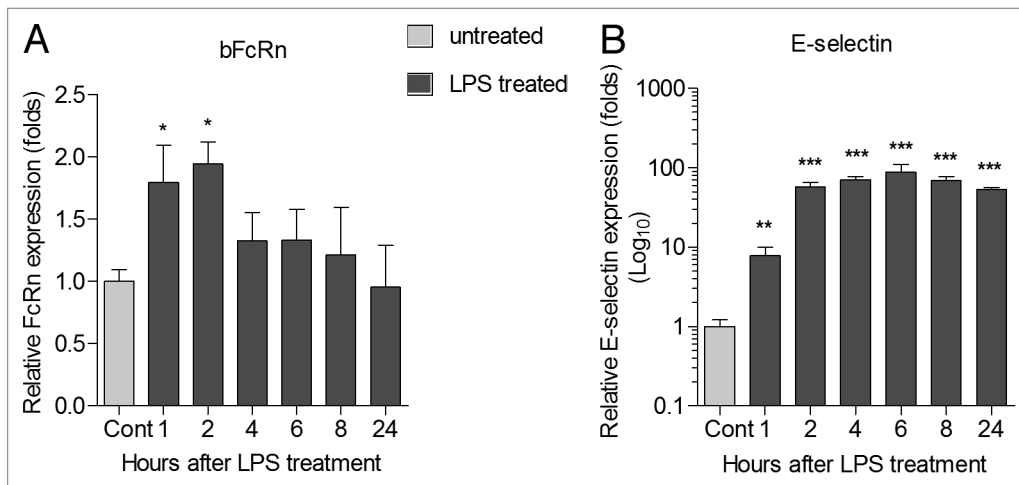


Figure 3. Exposure of BAECs with LPS upregulates the expression of the bovine FcRn and E-selectin. The mRNA levels of bFcRn (A) and E-selectin (B) were determined with quantitative real-time PCR using mRNA extracted from untreated and LPS-treated BAECs. Gene expression is normalized against ubiquitin as housekeeping gene and is shown as relative to the mean of untreated cells. Each bar represents the mean \pm SEM of triplicate samples. The *, **, and *** indicate the significant differences according to the Student *t*-test, $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared with the untreated samples.

We then examined the bFcRn expression of Gr1⁺ CD11b⁺ macrophages isolated from the peritoneal cavity of untreated and LPS-treated bFcRn Tg mice. We observed at least two subsets of macrophages based on their basic bFcRn expressional level and found that, except for a small subset of cells, LPS increased bFcRn expression by ~90% in these macrophages compared with the expression level in cells not treated with LPS (Fig. 6B and C).

Discussion

FcRn is subject to both tissue-specific and developmental regulation. The promoter regions for human and rodent FcRn have been analyzed, and the regulation of expression has been shown to be mediated with Sp-like transcription factors, AP-1, Ets or NFIL6.³⁶⁻³⁸ The expression and function of the human FcRn can be induced by TNF and IL-1 β through NF κ B binding to intronic sequences,²⁸ or downregulated by IFN- γ effect via JAK-STAT-1 signaling.³⁹ Given that FcRn is expressed in APCs where it carries multivalent IgG-based immune complexes to lysosomes and affects Ag processing, presentation and adaptive immune responses, it is reasonable to assume that transcriptional regulation of this receptor and consequently its function is controlled by inflammatory mediators.¹²

Our earlier in silico analysis showed that, similar to its mouse and human homologs, the 5'-flanking region of the bFCGRT does not harbor an obvious TATA box, but instead contains a potential Sp1 site close to the transcription start location (Fig. S1).²⁷ This factor stimulates constitutive promoter activity as previously demonstrated for the rat FcRn.³⁶

In this study, an extended search was performed on the bFCGRT using TESS and TFSEARCH programs^{40,41} revealing, among several other potential candidate sequences that may indicate responsiveness of the bFcRn in inflammatory reactions

that is known to have NF κ B-mediated immunostimulatory effects and analyzed the expression of the bFcRn. In agreement with our previous studies, LPS induced prompt NF κ B nuclear translocation in BAECs (Fig. S3)⁴² and resulted in rapid and significant upregulation of the bFcRn mRNA expression, which returned to its basal level within a couple of hours (Fig. 3A). The proper LPS-induced, NF κ B-mediated stimulus was confirmed by analyzing the expression of the control gene bovine E-selectin that showed a robust increase (Fig. 3B) as described earlier.³³ Vascular endothelium is proposed to be a major site at which FcRn protects serum IgG and albumin from lysosomal degradation and it is known that FcRn overexpression enhances IgG recycling and elongates its biological half-life.^{31,43} However, a local inflammation that may lead to the upregulation of bFcRn affects only a relatively small portion of blood vessels, and thus one may not expect that it would meaningfully influence the biological half-life of IgG. On the other hand, FcRn upregulation in capillary endothelial cells may increase local IgG tissue distribution via IgG transcytosis in inflamed tissues, and thus may contribute in local, IgG mediated protection. This hypothesis should be further analyzed as a role of FcRn in endothelial transcytosis has been already shown.⁴⁴

Recently, we presented data indicating that the bFcRn α -chain is expressed at a high level in dendritic cells and macrophages and spleen of Tg mice that carry and overexpress the transgene bFcRn.²¹ We also showed that dendritic cells from the bFcRn Tg mice have a significantly improved Ag presentation capability compared with dendritic cells from wild-type mice analyzed by T-helper cell proliferation assay.²¹ More recently, we demonstrated that these bFcRn Tg mice are capable of producing substantially greater numbers of activated Ag-specific T cells and larger germinal centers in the spleen compared with immunized wild-type mice (unpublished data). These findings indicate that

(e.g., NF1, c-Rel, Stat5, AP-1, IRF1, IRF2, c/EBP β), three additional NF κ B-specific binding sites (Fig. S1). First, we confirmed that these potential NF κ B binding sites can be induced with NF κ B transcription factor p65 in luciferase reporter gene assays (Fig. 1). As a next step, the sequence-specific binding of the bFcRn κ B sites was verified by EMSA and supershift analysis (Fig. 2). These findings indicated strong and effective molecular interactions between NF κ B p65 and the selected transcription binding sites of the bFCGRT promoter.

As a next step, we stimulated the well-characterized primary BAECs by LPS

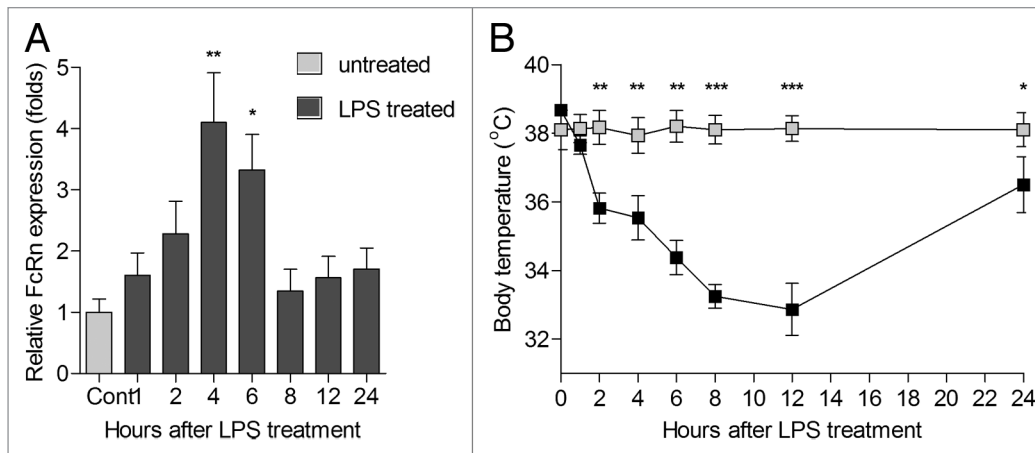


Figure 4. LPS treatment of bFcRn Tg mice induces elevated bFcRn mRNA expression in spleen. LPS was intraperitoneally injected into bFcRn Tg mice and the level of the bFcRn mRNA was analyzed with a bFcRn specific northern blot (A). Body temperatures were measured in LPS-treated (black rectangles) and untreated animals (empty rectangles) (B). Values shown are the mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

a higher expressional level of FcRn in APCs significantly augments Ag presentation to T-helper cells, and consequently boosts the humoral immune response. Based on these facts, we were interested in analyzing whether the transgenic bFcRn α -chain that can be induced by endogenous NF κ B signaling in the Tg mice might provide yet another mechanism for an increase in bFcRn expression, in addition to the multicopy effect of the integrated 5 bFcRn genes. First, we injected LPS into bFcRn Tg mice intraperitoneally and found that this treatment resulted in a 3- to 4-fold upregulation of the bFcRn mRNA level in spleen and this effect lasted for several hours (Fig. 4A). The efficiency of the LPS treatment was confirmed by perceiving a dramatic reduction in body temperatures (Fig. 4B) as described earlier.³⁴ To exclude the possibility that the increased level of bFcRn in the spleen was the result of LPS-induced substantial influx bFcRn positive cells, we also analyzed the ratio of the major cell types in the spleen during LPS treatment. This analysis confirmed that cellular composition of the spleen did not change within the first 8-h period when we detected increased bFcRn expression (Fig. 5), and thus we could conclude that LPS indeed stimulated bFcRn expression in vivo in the spleen of these animals. It is worth mentioning that we previously showed a high degree of homology of the bovine p65 with its human and mouse orthologs at the amino acid level in both the DNA-binding domain, known as the Rel homology domain (RHD), and the transactivation domain (TAD).⁴² Thus, cross-species activation is expected and explains the efficient bFcRn induction with the mouse NF κ B in these Tg mice. During LPS treatment we detected an increased expression of CD11b on neutrophil granulocytes one hour after LPS treatment (Fig. 5E), a phenotype that has been reported previously⁴⁵ and observed a new population of neutrophil granulocytes with a basic CD11b expression level 12 h after LPS treatment, probably due to cytokine-mediated recruitment (Fig. 5D and E).

As a final step, we confirmed that LPS induces bFcRn upregulation in most of the macrophages in bFcRn Tg mice with a newly developed FcRn-specific mAb that does not cross-react with the

mouse FcRn (Fig. 6), although it specifically recognizes human FcRn (Fig. S4). Further analysis is ongoing in our laboratory to analyze if this mAb interferes with binding of IgG or albumin to FcRn. In conclusion, NF κ B regulates bFcRn expression and optimizes its function in professional APCs. We also observed a subset of macrophages that express a lower level of bFcRn and does not show any increase of bFcRn expression above basal level when subjected to LPS stimulus. This observation may corroborate a recent study that identified two physically, functionally, and developmentally distinct peritoneal macrophage subsets that show critical differences based on their responses to LPS.⁴⁶ This observation warrants further analysis.

Because β 2 min is an essential component for assembly of functional FcRn⁷ and secretion from the endoplasmic reticulum,⁴⁷ it is important that the basal level of β 2 min expression can be enhanced by cytokines to meet local requirements for an adequate immune response and possibly also to fulfil its other functions. The transcriptional regulation of β 2 min is similar to that of MHC class I genes and previous reports have indicated that there is a conserved NF κ B transcription factor binding site (in addition to interferon regulatory elements) in the promoter of the human and mouse β 2 min genes and that is important for the cytokine-induced β 2 min expression in lymphoid and myeloid cell types.^{48,49} This suggests that the NF κ B-mediated induction of the bFcRn α -chain is accompanied with the activation of the mouse β 2 min in the APCs and other cytokine-sensitive cells of the bFcRn Tg mice allowing functional overexpression of this interspecies heterodimer receptor. In line with this, our preliminary analysis did not show altered subcellular distribution of the bFcRn in immunized vs. non-immunized spleen sections of Tg mice, although we observed increased bFcRn α -chain staining in different cell types using fluorescence microscopy (data not shown).

We conclude that NF κ B signaling is an important regulatory factor for bFcRn, a finding that corroborates with previous observation on the human FcRn.²⁸ Therefore, it is interesting that no NF κ B-mediated induction was previously reported in

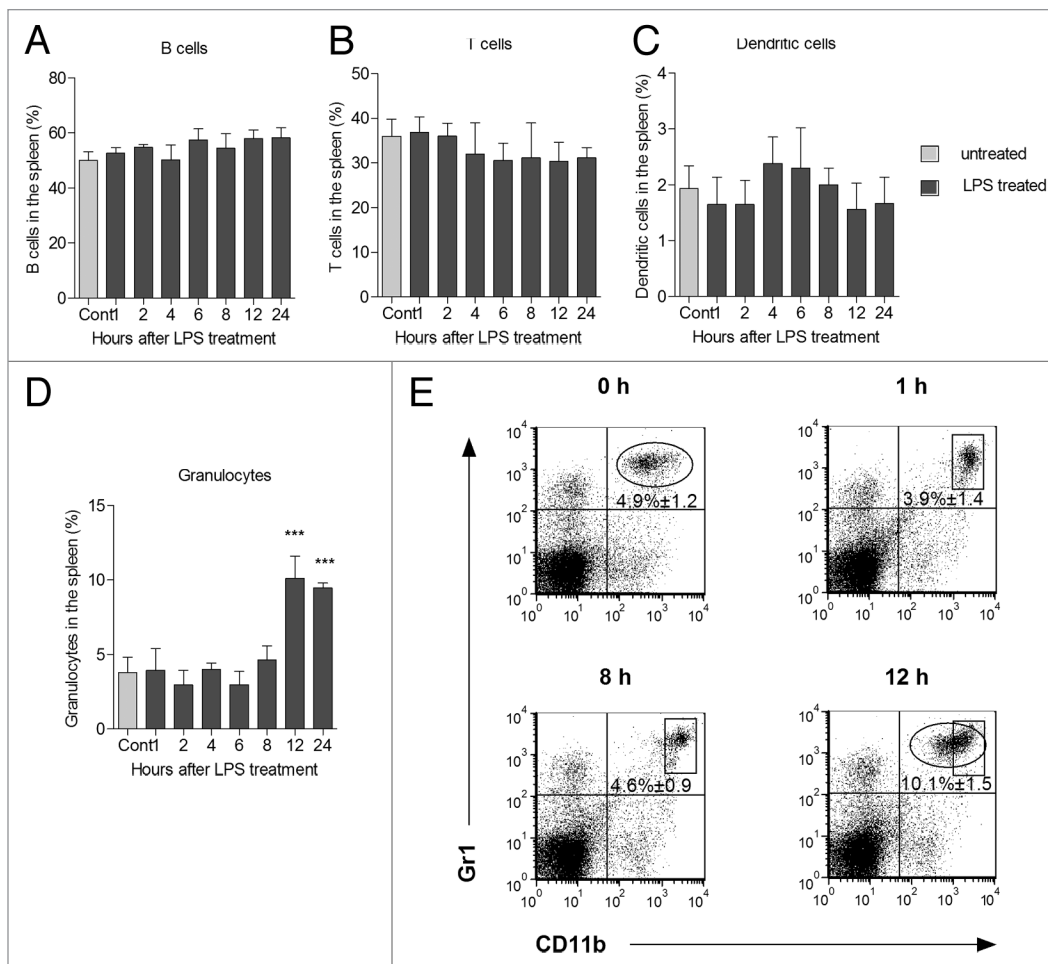


Figure 5. Analysis of spleen cell populations of bFcRn Tg mice before and after LPS treatment. LPS was intraperitoneally injected into bFcRn Tg mice and cytofluorimetric analysis of the major spleen cell populations were performed 1, 2, 4, 6, 8, 12, and 24 h afterwards. Proportions of B220⁺ B cells, CD3⁺ T cells, CD11b⁺/CD11c⁺ dendritic cells and CD11b⁺/Gr1⁺ neutrophil granulocytes are shown in inserts (A–D) respectively. CD11b expression of neutrophil granulocytes were analyzed by flow cytometry after LPS treatment (oval and rectangle shapes refer to the basal and increased expression levels of CD11b, respectively) (E). Bar graph values shown are the mean ± SD (***, $P < 0.001$).

the rodent FCGRT promoter, although the analyzed promoter segments were relatively short.^{36,38} We have searched for putative NFκB binding sites in the 5'-flanking region (1500 nt related to the first exon) of the mouse FCGRT promoter and identified a putative one in the close proximity to the transcription start site³⁷ (Fig. S2A). We were also interested in analyzing the rabbit FCGRT promoter because our Tg rabbits, which overexpress the rabbit FcRn, also show an augmented immune response.^{20,22} Based on a similar in silico analysis, we identified four NFκB binding sites in the rabbit FCGRT promoter that have been predicted with high likelihood (Fig. S2B). The validation of these predicted binding sites requires careful in vitro and in vivo studies. These findings suggests that FCGRT is stimulated by NFκB in these species although the promoter sequences of the human, bovine, mouse and rabbit FCGRT show considerable divergence and the NFκB binding sites differ in numbers and locations with respect to the transcription initiation sites in these species.

In addition to the effect on the transcription of bFcRn via the multiple copies of the transgene, this study shows that the level

of bFcRn can be further increased in these Tg mice by NFκB induction. This pathway of bFcRn induction is triggered upon stimulation with proinflammatory agents (e.g., TNF), or adjuvants during immunization, and thus represents a physiologic response to such conditions in mice. This regulation optimizes bFcRn expression and function in the professional APCs and contributes to the much augmented humoral immune response in the bFcRn Tg mice.

Materials and Methods

Animals and ethics statement. We used hemizygous Tg mice that carry five copies of the bFCGRT in addition to the endogenous mouse FCGRT gene on BALB/c genetic background (BALB/c_Tg5_bFCGRT(19); 19 refers to the founder line) that we previously generated.²⁵ Mice were kept in a specified pathogen-free (SPF) condition in individual ventilation cages (IVC) in the animal house of the Department of Immunology, Eötvös Loránd University, Budapest.

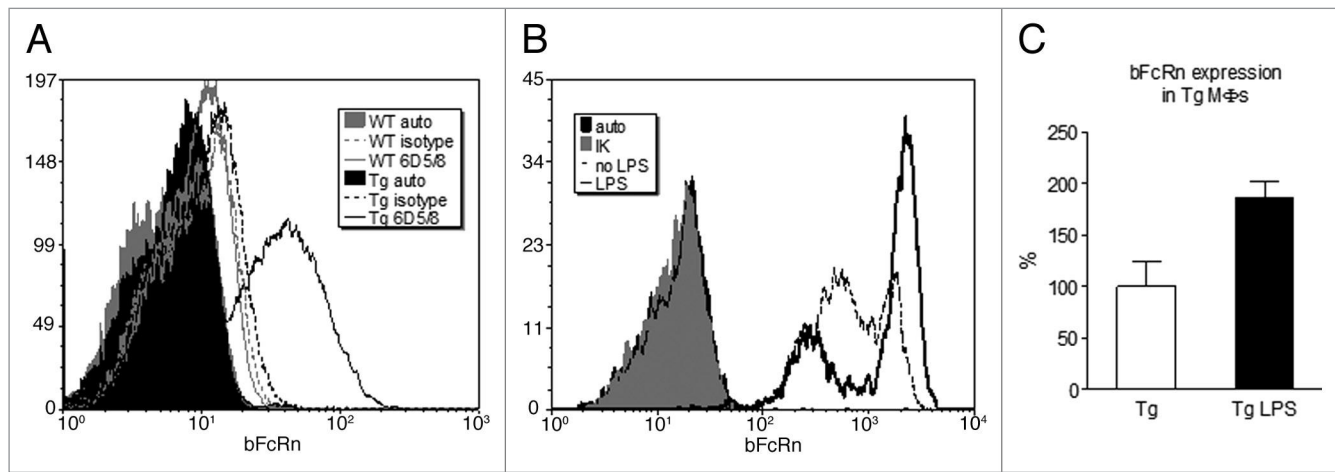


Figure 6. LPS increases bFcRn expression at protein level in peritoneal macrophages of bFcRn Tg mice. **(A)** bFcRn-specific monoclonal antibody (Z15_6D5/8 mAb), that does not cross react with the mouse FcRn, was analyzed in thioglycollate-induced peritoneal cells of wt and bFcRn Tg mice. **(B and C)** Flow cytometric analysis with Z15_6D5/8 mAb showed that the bFcRn expression increased by app. 90% after 24 h of LPS injection in Gr1⁺CD11b⁺ macrophages compared with its expressional level in non-treated cells (average ΔRMFI ratio of untreated mice is shown as 100%).

Treatments of mice in this study were performed in strict accordance with the recommendations in the Guide of the Institutional Animal Care and Ethics Committee at Eötvös Loránd University that operated in accordance with permissions 22.1/828/003/2007 and XIV-I-001/517-4/2012 issued by the Food Chain Safety and Animal Health Directorate of the Government Office of Pest County, Hungary.

Cell lines, primary cells. The mouse mammary epithelial cell line HC11 (ATCC) was cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% FCS (Invitrogen), penicillin (50 U/ml), streptomycin (50 µg/ml), mouse epidermal growth factor (10 ng/ml, Sigma-Aldrich Co.) and insulin (5 µg/ml, Sigma-Aldrich). After transfection for the luciferase reporter gene assay, HC11 cells were maintained in medium with FCS reduced to 2% and without mouse epidermal growth factor.⁵⁰ The JAR cell line (human placental choriocarcinoma) was kept in RPMI-1640 medium supplemented with 10% FCS, penicillin (50 U/ml) and streptomycin (50 µg/ml). The HEK293 (human embryonic kidney epithelial cells) cell line was cultured in the Dulbecco modified Eagle medium (DMEM) supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 µg/ml) and sodium pyruvate (1 mM, Sigma-Aldrich). Primary bovine aortic endothelial cells (BAEC, Cambrex Bio Science Walkersville Inc.) were maintained in EGMTM-MV medium, supplemented with bovine brain extract, hydrocortisone, human epidermal growth factor, 5% FBS and gentamicin/amphotericin-B (according to the manufacturer's instructions, Cambrex). All cells were cultured in a humidified atmosphere of 5% CO₂ in an incubator at 37 °C.

In silico transcription factor binding site analysis. Transcription Element Search System (TESS) software⁵¹ was used for searching putative binding sites in the 5'-flanking region of the bFCGRT. The searches were performed by matrix-based search using mammalian position weight matrices⁵² and relatively high thresholds (minimum lg likelihood ratio: 6, maximum lg likelihood deficit: 6).²⁷ A second search was accomplished where the initial threshold was reduced (minimum lg likelihood ratio:

6, maximum lg likelihood deficit: 10), and the putative binding sites were confirmed by other computer programs, such as TFSEARCH.⁴¹

MatchTM software (BIOBASE Biological Databases GmbH) using a library of positional weight matrices from TRANSFAC® Public 6.0 was used for searching NFκB putative binding sites in the 5'-flanking region of the rabbit and mouse FCGRT with the following parameters: cut-offs: matrix similarity = 0.85, core similarity = 0.85.

DNA cloning and substitution mutagenesis. Three fragments of the 5'-flanking region of the bFCGRT were PCR sub-cloned into the luciferase expression vector pGL3 (Promega, Madison, WI, USA). The following constructs were generated by PCR amplifications: pGL3-bFP2, which contains the segment between -1787 + 92 and was amplified by bFcFU-Mlu: 5'-CCCACGCGTC AGTGCCTGGA TGTTTGGTC-3' and bFcL-Hind: 5'-TTTAAGCTTG CCGGATCCC TTCCCTCTG-3'; pGL3-bFP3, which contains the segment between -1112 and +92 and was amplified by bFcMU-Mlu: 5'-CCGACGCGTG ACACGACTGA AGGGTTTA-3' and bFcL-Hind; and pGL3-bFP4, which contains the segment between -527 and +92 and was amplified by bFcSU-Mlu: 5'-CCCAGCCGTC TCCTTTGTCT TGGGCACTT-3' and bFcL-Hind.

To analyze the responsiveness of the predicted NFκB sites, we performed several PCR-based site-directed mutagenesis as previously described.⁵³ The 5'-flanking region primers from -1112 bp to +92 bp (see above) and two mutagenizing primers for each mutation were used (Table 1). Mutagenizing primer pairs contained unique restriction sites that were not present in the pGL3-basic vector to ligate the two halves of the 5'-flanking region fragments. PCR reactions were performed using 1.25 U of Deep Vent proofreading DNA polymerase (New England Biolabs Inc) and 1 ng of pGL3-bFP3 construct as template per reaction. The total reaction volume was 50 µl, and the final concentrations of the dNTP and each primer were 200

Table 1. Mutagenizing oligonucleotides for the predicted NFκB sensitive sequences of the bFCGRT promoter region

Name of the construct	Sequence	Directions and binding positions (bp)	Restriction digestion site	Annealing temperature
pGL3-bFcRn-κB-612	5'-CCCAAG AGCGT <u>CCAT</u> CAGACA CATTAAAGT-3'	as,-633 to -616	<i>AatII</i>	61 °C
	5'-GGGACCACTT AATGTGTCTG GGGG GAGGTC TTG-3'	s, -612 to -585		57 °C
pGL3-bFcRn-κB-758	5'-GGTGCGCGTG GGGT ACTAGT TTTTTT-3'	as, -749 to -774	<i>SpeI</i>	57 °C
	5'-CCC ACTAGTA CTGAACCGTA CACTAAATGA AAG -3'	s, -768 to -739		58 °C
pGL3-bFcRn-κB-840	5'-TGGTTTGCC G AATTC TTTAA CAGCTGCCG-3'	as, -831 to -859	EcoRI	59 °C
	5'- AGGAATTC GCG CAAACCGATG GTTAAAGACT TGCC -3'	s, -840 to -814		60 °C

Mutated bases are indicated by bold letters, restriction digestion sites generated after the mutagenesis are underlined.

Table 2. Transfection parameters for luciferase reporter gene assay

Cell line	N _{PEI} /P _{DNA} ^a	total DNA (ng/well)	pGL3 construct (ng/well)	phRL-hβactin -213+932 (ng/well)	pET-p65 (ng/well)	Time of harvest after transfection (hour)
HC11	16	1400	200	1	100	72
HEK293	35	1400	50	0.1	10	48
JAR	30	2800	400	1	400	48

^aThe molar ratio of PEI amine nitrogen and DNA phosphate; 1 μl of 0,45 mg/ml PEI solution contains 10 nmol of amine nitrogen, 1 μg of DNA contains 3 nmol of phosphate.⁵⁴

μM and 1 pmol/μl, respectively. The PCR temperature profile was as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at the temperature depending on the melting temperature of primers (Table 1) for 30 s, extension at 72 °C for the time depending on the length of the desired PCR products (1 min was calculated per 1000 bp of length) and final extension at 72 °C for 10 min. After the amplifications, mutant PCR products were purified, digested by the unique restriction endonuclease and ligated. The entire mutagenized fragments produced by ligation were amplified by PCR using the flanking region primers. The resultant products that harbored different mutant κB binding sites were purified, and cloned into the pGL3-basic vector.

Transient transfections. Cell lines were transfected based on the polyethylenimine (PEI) method.⁵⁴ The transfection of HC11 and HEK293 cell lines has been described in our previous study.⁴² Briefly, the cells were seeded into 24-well plate (Corning), pGL3 construct were co-transfected with pET-p65⁵⁵ or the same amount of the empty pCI-neo vector (Promega), with 1 ng/well phRL-hβactin-213+932 internal control vector which was described previously.⁴² The total amount of plasmid DNA was adjusted by adding pUC19, lacking a cDNA to be expressed; the exact amounts of transfected vectors and other parameters are shown in Table 2. The JAR cells were transfected in the same way. Cells were harvested according to the manufacturer's instructions of the Dual-Luciferase Reporter Assay System (Promega), and the luciferase activities of three independent transfections were determined in triplicate using a Luminoskan Ascent luminometer (Thermo Labsystem). Luciferase activity was normalized to *Renilla* luciferase activity, and the results expressed relative to normalized activity driven from pGL3-basic.

Preparation of nuclear extracts, EMSA and supershift assay. Nuclear extracts from untreated and LPS-treated BAECs were prepared using CellLytic Nuclear Extraction kit (Sigma-Aldrich) according to the manufacturer's instructions. The single-strand oligonucleotides were labeled with T4 polynucleotide kinase (New England Biolabs) and 1 μl γ-[32]-ATP (-5 μCi, Institute of Isotopes Co.), annealed to form double-stranded oligonucleotides containing consensus NFκB site and the tested NFκB sequences from the bFCGRT promoter: bFcRn κB-612 (5'-AATGTGTCTG GGATCTCCTT TGGGAT-3'), bFcRn κB-758 (5'-CAAAAAAAGA AAAACCCAC CGTACA-3'), bFcRn κB-840 (5'-CGGCATGCTG GGAAATCCCC TGGCAA-3') and a consensus κB site (5'-AGTTGAGGGG ACTTCCCCAG GC-3') as control. The purification of labeled, double-strand oligomer was performed with Sephadex mini Quick Spin Column (Roche). The binding reaction was performed in 10 μl total volume for 20 min at room temperature using 1 μl labeled oligomer, nuclear extracts containing 6 μg of total protein, and in some cases, 1 μg of rabbit anti-p65 antibodies (H-286, Santa Cruz Biotechnology). The final composition of the binding buffer was described previously.⁵⁶ The samples were run on 5% non-denaturing polyacrylamide gel in 0.25× TBE buffer at 200 V for 2 h. The gels were fixed with 10 V/V% acetic acid solution, dried onto Whatmann 3M paper and exposed to Hyperfilm MP (Amersham Biosciences).

In vitro LPS stimulus and real time RT-PCR. BAECs were plated onto 6-well dishes (Nunclone) pre-coated with 0.5% gelatin (Sigma-Aldrich) and after reaching confluence, the cells were stimulated with 100 ng of *Escherichia coli* LPS (026:B6 serotype, Sigma-Aldrich) per ml. Total RNA was isolated from the cells 1, 2, 4, 8, and 24 h after treatment with TRIzol Reagent (Invitrogen)

following the manufacturer's instructions. The M-MLV Reverse Transcriptase (Promega) was used to synthesize first strand cDNA with OLIGO(dT) primer (Sigma-Aldrich) from 1 μ g of total RNA, at 42°C for 90 min. The level of bovine FcRn α -chain and E-selectin transcripts were analyzed by real-time PCR, which was performed using a Roche LightCycler according to the manufacturer's instructions using the LightCycler DNA Master SYBR Green I kit. Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 5 s at 95 °C, 10 s at 65°C, 4 s at 72 °C. All reactions were run in triplicate in three separate experiments using intron spanning primers for bFcRn (5'-GGCGACGAGC ACCACTAC-3'; 5'-GTCAGGAGCA GGAATAAGCC-3'), bovine E-selectin (5'-GATTATTGCC AGAAACTTA C-3'; 5'-CCAGGTCCAT GTACCGT-3') and bovine ubiquitin as an internal control (5'-TTTTTCGTGAA GACCCTGACC G-3'; 5'-TAAATGGCTA GAGTGCAGAA GG-3'). The results are expressed as the fold change of bFcRn; E-selectin and/or ubiquitin mRNA ratios in LPS treated cells compared with that of controls.

Immunocytochemistry. BAECs were seeded onto 96-well plates at 5000 cells per well concentration in 100 μ l medium for 1 d, and were treated with 10 ng/ml LPS for 30 min, 1, 2, 4, or 8 h before fixation. To quantify NF κ B nuclear translocation, cells were stained with both rabbit anti-p65 subunit antibody (Santa Cruz Biotechnology) that was developed with goat anti-rabbit IgG-Alexa 568 (Invitrogen) and Hoechst 33342 nuclear marker (Invitrogen). Samples were observed and images were recorded by an Olympus IX-81 inverted fluorescence microscope mounted with Olympus DP70 digital camera (Olympus Optical Co). Each area of interest was photographed using a red (for p65) and a blue (for Hoechst) filter, then the two photos were overlaid. CellP software (Olympus Soft Imaging Solutions Ltd) were used to calculate the mean red intensity of every nuclear region (where the blue value was above the threshold), and regions of interests inside the cells but outside the nuclei (where the blue value was under the threshold). Then, the ratio of the nuclear mean red intensity and perinuclear red intensity was calculated for each cell of the photograph. Finally, the ratios were normalized to be comparable through different experiments; the values of untreated controls were set to 1. The number of analyzed cells was between 30 and 40 in an experiment, and at least three independent experiments were done. We used the normalized values for statistical analysis and visualized as means (\pm SEM).

In vivo LPS treatment and Northern analysis. Ten week-old male BALB/c (wt) and bFcRn Tg mice were injected i.p. with 250 μ g LPS per 100 g of body weight. 1, 2, 4, 6, 8, 12, and 24 h after LPS treatment mice were sacrificed, spleens were removed and homogenized in Precellys homogenizer using RNazol Bee buffer (AMS Biotechnology). Rectal body temperatures were recorded at every time point of sample collections. Total RNA was isolated from spleens and 3 μ g of total RNA was size fractionated on 1% agarose/2.2 M formaldehyde gel, transferred to Hybond N+ membrane (Amersham) and hybridized with the ³²P-labeled cDNA probe synthesized by PCR primers as described.³¹ The signals obtained were evaluated using Fuji medical X-Ray film

and quantified with GeneTools (SynGene). The results were expressed as fold change of bFcRn mRNA in LPS treated mice compared with that of untreated controls.

Flow cytometry. Single-cell suspensions from spleen were isolated and first incubated with anti-CD32/CD16 (clone 2.4G2) for 30 min. Subsequently the cells were incubated with fluorochrome-conjugated specific Abs at 4 °C for 50 min in staining buffer (PBS with 0.1% BSA and 0.05% sodium azide), washed twice, and then analyzed using a FACSCalibur equipped with CellQuest software (BD Biosciences). Anti-mouse CD45R/B220-PECy5, CD3-PE and CD11b-PerCP-Cy5.5 were obtained from eBioscience (San Diego, CA). Anti-mouse CD11c-PE and Gr-1(Ly-6G)-PE were purchased from BD PharMingen. Isotype controls were obtained from BD PharMingen or eBioscience.

Generation of FcRn-specific mAb. Two month-old female BALB/c mice were immunized with thioglycollate-induced cells isolated from bFcRn Tg mice several times. Three days after the last boost, fusion of spleen cells and selection of hybridomas were performed under the same conditions as described earlier.²⁴ Hybridoma microculture supernatants were first screened in ELISA assay using recombinant soluble bFcRn molecule,⁵⁷ then positive clones were further tested in flow cytometry using the bFcRn-stable transfected mammary epithelial cell line B4,⁵⁷ and then using thioglycollate-induced peritoneal cells from wt and bFcRn Tg mice. Finally, a positive microculture was selected for cloning using limiting dilution method, resulting in FcRn specific clone Z15_6D5/8. The mAb was produced in vitro in cell culture media, purified with protein G (Pierce) and conjugated to AlexaFluor 488 (Invitrogen/Life Technologies) according to the manufacturers' instructions. This mAb was further tested in flow cytometry using monocyte-derived human macrophages and human intestinal T84 cells. Human peripheral monocytes were separated from buffy coats obtained from healthy donors using CD14 MicroBeads (Miltenyi Biotec; cell purity was > 95% when analyzed by flow cytometry). Cells were cultured for 5 d in the presence of 40 ng/ml GM-CSF (R&D Systems) in CellGro Serum Free Medium (CellGenix) to obtain monocyte derived macrophages; cytokine was supplemented every 2 d. T84 cells were cultured as described previously.³⁵

Detection of bFcRn expressional level in peritoneal macrophages by flow cytometry. Peritoneal cavity cells were isolated 24 h after i.p. LPS treatment (250 μ g LPS per 100 body weight gram) or from untreated bFcRn Tg mice and stained with Gr1(Ly6-G)-PE (BD PharMingen) and CD11b-PerCP-Cy5.5 (eBioscience) antibodies or appropriate isotype controls. Cells were fixed with 2% paraformaldehyde and stained with AlexaFluor 488 conjugated bFcRn specific Z15_6D5/8 in PBS containing 0.2% saponine and 0.1% BSA (both purchased from Sigma-Aldrich, Inc.) or appropriate isotype control. Cells were subjected to flow cytometric analysis with FACSCalibur equipped with CellQuest software (BD Biosciences). The ratio of relative mean fluorescence intensity (Δ RMFI) was calculated as $(\text{GeoMean}_{\text{bFcRn}} - \text{GeoMean}_{\text{autofluorescence}}) / (\text{GeoMean}_{\text{isotype control}} - \text{GeoMean}_{\text{autofluorescence}})$.

Statistical analysis. Results are presented as means \pm SE (SEM). Data were entered into and analyzed using SPSS version

11.5 (SPSS Inc). Promoter analysis was statistically evaluated by unpaired *t*-test or one-way ANOVA followed by Newman-Keuls post hoc testing. *P*-values less than 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

Cervenak J and Bender B are scientific researchers; Bosze Z and Oster W are co-founders, Kacsokovics I is co-founder and CEO of ImmunoGenes Ltd, Budakeszi, Hungary a company specialized in the generation of FcRn transgenic animals for the production of polyclonal and monoclonal antibodies (www.immunogenes.com). Eötvös Loránd University, Budapest, Hungary and Agriculture Biotechnology Center, Gödöllo, Hungary have been

granted a European patent for this technology (EP2097444), and seek patent in other major markets. The technology has been licensed exclusively to ImmunoGenes Ltd.

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/mabs/article/26507

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