

BOB.1/OBF.1 controls the balance of TH1 and TH2 immune responses

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BOB.1/OBF.1 is a transcriptional coactivator essential at several stages of B-cell development. In T cells, BOB.1/OBF.1 expression is inducible by co-stimulation. However, a defined role of BOB.1/OBF.1 for T-cell function had not been discovered so far. Here, we show that BOB.1/OBF.1 is critical for T helper cell function. BOB.1/OBF.1^{-/-} mice showed imbalanced immune responses, resulting in increased susceptibility to *Leishmania major* infection. Functional analyses revealed specific defects in TH1 and TH2 cells. Whereas expression levels of TH1 cytokines were reduced, the secretion of TH2 cytokines was increased. BOB.1/OBF.1 directly contributes to the IFN γ and IL2 promoter activities. In contrast, increased TH2 cytokine production is controlled indirectly, probably via the transcription factor PU.1, the expression of which is regulated by BOB.1/OBF.1. Thus, BOB.1/OBF.1 regulates the balance of TH1 versus TH2 mediated immunity.

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Introduction

T helper cells are classified according to their production of defined cytokines. TH1 cells secrete high amounts of IFN γ and IL2, thereby inducing cell-mediated immunity, mainly through the activation of macrophages. TH2 cells secrete IL4, IL5 and IL13, thereby affecting the humoral immune responses (Liew, 2002). Whether a naïve CD4T cell develops into the TH1 or TH2 lineage primarily depends on the signals the cells receive during presentation of the antigen by the APC, as well as on the type of antigen (Murphy *et al*, 2000).

Activation or silencing of specific genes in TH1 or TH2 lineages is controlled by complex mechanisms including

transcriptional regulation and epigenetic modifications (Murphy and Reiner, 2002; Ansel *et al*, 2003). The IL4-induced STAT6 activation leads to the rapid expression of the transcription factor GATA-3, the master regulator of TH2 lineage development. In contrast, the transcription factor T-bet was identified as an important regulator of TH1 development. T-bet is expressed upon IFN γ -induced STAT1 activation and promotes induction of IFN γ expression in TH1 cells. In addition to T-bet and STATs (Soutto *et al*, 2002; Tong *et al*, 2005), several transcription factors were shown to regulate IFN γ promoter activity, like NF-AT (Yang *et al*, 1999), AP-1, CREB, YY1 and NF- κ B (Cippitelli *et al*, 1995; Ye *et al*, 1996; Sica *et al*, 1997; Sweetser *et al*, 1998; Zhang *et al*, 1998). However, IFN γ gene regulation is not yet completely understood.

Several studies described the importance of an efficient TH1 response for host immunity against the protozoan parasite *Leishmania major* (Sacks and Noben-Trauth, 2002). In the murine model, the control of infection with a high dose of *L. major* in genetically resistant strains, such as CBA/J, C57BL/6 and C3H, ultimately depends on the IL12-driven release of TH1-derived IFN γ . This activates infected macrophages to eliminate parasites and thus leads to containment of the parasites. In contrast, infection of susceptible Balb/c mice leads to progressive, non-healing lesions associated with an early and dominant TH2 response (Sacks and Noben-Trauth, 2002).

BOB.1/OBF.1 (also named OCA-B) was originally described as a B-cell-specific transcriptional coactivator (Luo *et al*, 1992; Gstaiger *et al*, 1995; Pfisterer *et al*, 1995; Strubin *et al*, 1995). When recruited to DNA via protein-protein interaction with Oct1 or Oct2 it enhances octamer-dependent transcription. BOB.1/OBF.1 is important at multiple stages of B-cell development, in the bone marrow (Schubart *et al*, 2000; Hess *et al*, 2001; Brunner *et al*, 2003b; Siegel *et al*, 2006), as well as at late stages in secondary lymphoid organs (Nielsen *et al*, 1996; Hess *et al*, 2001; Samardzic *et al*, 2002). BOB.1/OBF.1^{-/-} mice completely fail to form germinal centers upon immunization with thymic-dependent antigens. Consequently, the production of secondary immunoglobulin (Ig) isotypes is massively reduced (Kim *et al*, 1996; Nielsen *et al*, 1996; Schubart *et al*, 1996). Beside the role of BOB.1/OBF.1 as a transcriptional coactivator, a non-transcriptional role was identified. In the cytoplasm, an isoform of BOB.1/OBF.1 is myristoylated and localizes to the membrane (Yu *et al*, 2001). Cytoplasmic BOB.1/OBF.1 interacts with the Src-kinase Syk and directly regulates Syk stability (Siegel *et al*, 2006).

In T cells, BOB.1/OBF.1 expression can be induced by treatment with α CD3 + α CD28 antibodies, or by co-stimulation with phorbol ester and ionomycin (Sauter and Matthias, 1997; Zwilling *et al*, 1997; Moriuchi and Moriuchi, 2001). The transactivation function of BOB.1/OBF.1 is also regulated by co-stimulation (Zwilling *et al*, 1997). BOB.1/OBF.1 expression was also observed in human T-cell lymphomas (Marafioti *et al*, 2003). Recently it was shown that Lck

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promoter-driven expression of BOB.1/OBF.1 in mice leads to increased expression of the transcription factor Spi-B in thymocytes, due to the direct activation of an octamer containing Spi-B promoter (P2) by BOB.1/OBF.1 (Bartholdy *et al*, 2006).

Whereas the role of BOB.1/OBF.1 in B-cell development and function is well documented, the role in T cells remains to be elucidated. Here, we analyzed T-cell development and function in BOB.1/OBF.1^{-/-} mice. We show that BOB.1/OBF.1 is expressed in TH1 as well as in TH2 cells. The cytokine secretion of both subtypes of T helper cells was affected by the BOB.1/OBF.1 deficiency. As a consequence, the resistance in experimental leishmaniasis was severely impaired. We have elucidated the molecular network by which BOB.1/OBF.1 regulates the balance of TH1 versus TH2 activity.

Results

Reduced T-cell numbers in BOB.1/OBF.1-deficient mice

In order to elucidate the function of BOB.1/OBF.1 in T cells, we carefully reanalyzed the T-cell compartment of BOB.1/OBF.1^{-/-} mice. Whereas the cellularity of the thymus was normal and development of thymocytes up to single-positive stages was unaffected (data not shown), splenic T-cell numbers were reduced about two-fold. T-cell subpopulations were also reduced in lymph nodes of BOB.1/OBF.1-deficient animals, albeit less significantly (Figure 1A and B). Both, CD4⁺ and CD8⁺ subpopulations were affected. Therefore, the ratio of CD4⁺ to CD8⁺ subpopulations in lymph nodes and spleen is unaltered (Supplementary Figure S1). The strong reduction of peripheral B cells had been described previously.

To analyze the expression level of BOB.1/OBF.1 in T helper subtypes, immunoblots were performed. Similar to unpolarized CD4⁺ T cells, BOB.1/OBF.1 is induced in both TH1 and TH2 cells by co-stimulation (α CD3 + α CD28), or by treatment with PMA + ionomycin. Stimulation of differentiated T helper cells with IL12 or IL4 alone had no influence on BOB.1/OBF.1 expression (Figure 1C). In contrast to unpolarized CD4⁺ T cells, which do not show detectable BOB.1/OBF.1 expression without stimulation, polarized TH1 and TH2 cells express low levels of BOB.1/OBF.1. Expression levels can be further increased by re-stimulation of the polarized cells. The polarization efficiency was controlled in parallel by ELISA. This analysis revealed that TH1-polarized cells did not produce TH2 cytokines and vice versa (Supplementary Figure S2). Stimulation with α CD3-specific antibodies alone is sufficient to induce BOB.1/OBF.1 expression, both in naïve T cells and polarized TH1 or TH2 cells (Supplementary Figure S3, data not shown).

BOB.1/OBF.1-deficient mice are not able to control *L. major* infection

Since BOB.1/OBF.1 is expressed in both main T helper subpopulations, we wondered whether the BOB.1/OBF.1 deficiency leads to physiological consequences for T helper cell function. The infection of mice with *L. major* is an established and sensitive method to measure the correct TH1/TH2 balance *in vivo*. The control of *L. major* infection in genetically resistant strains depends on the consecutive activation of dendritic cells (DCs), followed by an efficient IFN γ -dominated TH1 response. When BOB.1/OBF.1^{-/-} mice

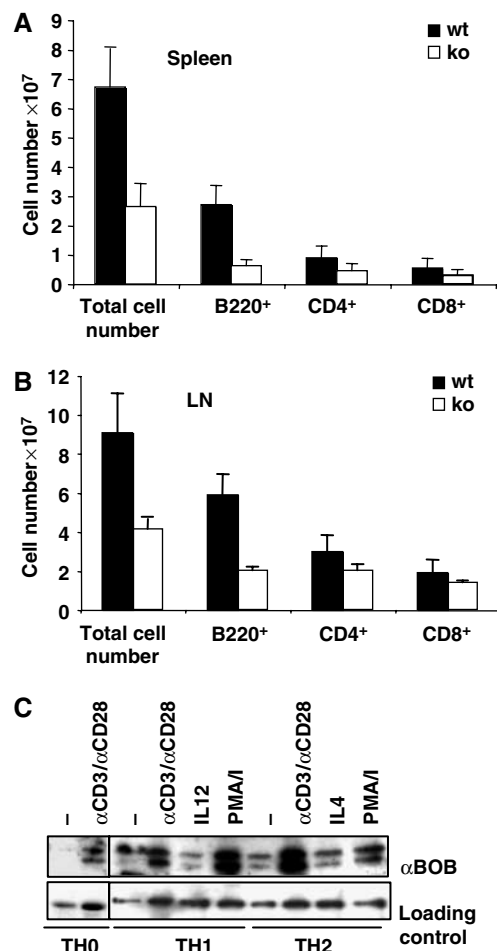


Figure 1 (A, B) T-cell numbers are reduced in BOB.1/OBF.1-deficient mice. (A) Cell suspensions of spleens from wild-type ($n = 9$) and BOB.1/OBF.1^{-/-} mice ($n = 11$) derived from four individual experiments were stained for B220, CD4 or CD8. (B) Analyses of cell suspensions from lymph nodes for B220⁺, CD4⁺ and CD8⁺ cell numbers. Data presented are the mean values (\pm s.d.) of cell numbers positive for the analyzed cell surface markers. (C) BOB.1/OBF.1 expression in TH0, TH1 and TH2 cells increases after TCR stimulation. Lysates of the indicated cell lineages after 6 days of polarization, re-stimulated with plate-bound α CD3 + α CD28, with PMA/ionomycin (PMA/I), IL12 or IL4 or left untreated for 24 h, were resolved by SDS-PAGE and immunoblotted with α BOB.1/OBF.1. For a loading control, the blot was reprobed with α tubulin or α PLC γ 1.

that had been generated on the C57BL/6 background were challenged by infection with *L. major*, they showed significantly increased footpad swelling (Figure 2A) and a 20- to 100-fold higher parasite load in infected footpads and popliteal draining lymph nodes, compared with control mice (Figure 2B and C). Analyses of cytokine production of draining lymph node T cells after re-stimulation with *L. major* antigen revealed that virtually no IFN γ was produced from these cells deficient for BOB.1/OBF.1. In contrast, the TH2 cytokine IL4 was not produced by either wild-type or BOB.1/OBF.1^{-/-} cells upon re-stimulation, whereas the IL13 production was low but detectable and found slightly increased in comparison to wild-type cells (Figure 2D). As BOB.1/OBF.1^{-/-} lymphocytes were able to proliferate in response to *L. major* antigen, they had clearly been sensitized and were not anergic (Supplementary Figure S4). In addition, analyses

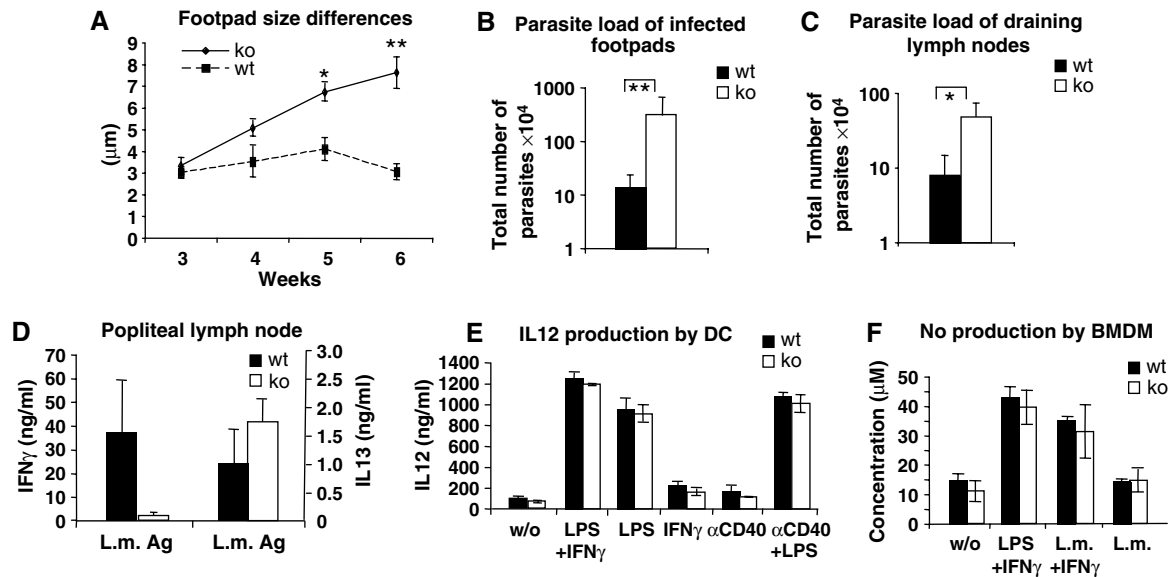


Figure 2 BOB.1/OBF.1^{-/-} mice are not able to contain parasites after infection with *L. major*. (A) Left hind footpads of wild-type ($n = 6$) and BOB.1/OBF.1^{-/-} mice ($n = 6$) were infected with *L. major* parasites. Differences between left and right hind footpad size were measured. $*P \leq 0.05$; $**P \leq 0.005$. After 6 weeks, mice were analyzed for (B) parasite load of infected footpads and for (C) parasite load of draining lymph nodes. (D) CD4⁺ T-cell numbers from draining lymph nodes were adjusted, re-plated and re-stimulated with *L. major* antigen. This experiment was performed with triplicates. After 24 h of stimulation, supernatants were analyzed for IFN γ ($P = 0.0056$) and IL13 ($P = 0.078$) levels by ELISA. Data were analyzed with Student's *t*-test. Infection experiments were performed twice with similar results. Data show mean values \pm s.d. (E) Bone marrow-derived BOB.1/OBF.1^{-/-} DCs produce equal amounts of IL12 compared with wild-type DCs, after stimulation with the indicated inducers for 24 h. (F) BMDM were obtained from BOB.1/OBF.1^{-/-} and wild-type mice killed after 6 weeks of *L. major* infection, and the NO production in response to indicated stimuli was determined. (L.m. = *L. major* antigen). The experiments in (E) and (F) were performed with triplicates and repeated twice. One representative experiment is shown. Data are the means \pm s.d.

of T-cell populations in draining lymph nodes of infected animals revealed again reduced numbers of B as well as T cells in BOB.1/OBF.1^{-/-} mice (Supplementary Figure S5).

As IL12 produced by DCs and macrophages is a major stimulus for generation of the TH1 response, we asked whether DCs and macrophages from BOB.1/OBF.1^{-/-} mice show normal IL12 production. Bone marrow-derived DCs (Figure 2E) and macrophages (data not shown) from BOB.1/OBF.1^{-/-} mice released normal amounts of IL12 in response to different stimuli. Furthermore, wild-type and BOB.1/OBF.1^{-/-} bone marrow-derived macrophages from *L. major* infected animals showed equally effective production of NO after phagocytosis of *L. major* and activation with IFN γ and LPS *in vitro* (Figure 2F).

The inability of BOB.1/OBF.1^{-/-} mice to cure *L. major* infection is intrinsic to CD4⁺ cells

To exclude that the impaired B-cell compartment of BOB.1/OBF.1^{-/-} mice had an impact on susceptibility to *L. major* in our model, Rag2^{-/-} mice were reconstituted with purified CD4⁺ cells from either wild-type or BOB.1/OBF.1^{-/-} mice, together with wild-type B cells, and subsequently infected with *L. major*. Again, after 4 weeks footpad swelling was significantly larger in Rag2^{-/-} mice reconstituted with CD4⁺ T cells from BOB.1/OBF.1^{-/-} mice compared with Rag2^{-/-} mice that had received wild-type CD4⁺ T cells (Figure 3A). This was accompanied by a 10- and 20-fold increase in parasite load of infected footpads and of draining lymph nodes in mice reconstituted with mutant CD4⁺ T cells (Figure 3B and C). Notably, non-reconstituted Rag2^{-/-} mice showed a less than two-fold higher footpad swelling and parasite load in infected footpads than mice substituted with

BOB.1/OBF.1^{-/-} CD4⁺ T cells (Figure 3A and data not shown). In addition, the cell numbers of draining lymph nodes, as well as the ability of these cells to proliferate and to produce cytokines in response to *L. major* antigen *ex vivo*, was analyzed. After 2 weeks of infection, the general cell numbers as well as the numbers of CD4⁺ T cells in draining lymph nodes of infected Rag2^{-/-} mice that received wild-type or BOB.1/OBF.1^{-/-} mice were found to be equal. In addition, there were no differences in the ability of these cells to proliferate in response to *L. major* antigen, albeit these responses were very low. After 4 weeks of infection, we could detect a slight reduction of cell numbers in draining lymph nodes of mice that received BOB.1/OBF.1^{-/-} CD4⁺ T cells (Supplementary Figures S6). In addition, the wild-type cells showed a two-fold higher proliferation potential and an approximately 100-fold increase in IFN γ secretion in comparison to BOB.1/OBF.1^{-/-} cells, upon re-stimulation with *L. major* antigen (Figure 3D and Supplementary Figure S6). Thus, the defect to control *L. major* infection is indeed caused by impaired function of BOB.1/OBF.1^{-/-} CD4⁺ T cells.

T helper cells generated from BOB.1/OBF.1^{-/-} mice show defects in cytokine secretion

To clarify the molecular mechanisms responsible for the severe leishmaniasis observed in BOB.1/OBF.1^{-/-} mice, we first asked whether the imbalanced TH1/TH2 cytokine production observed during infection with *L. major* could be reproduced *in vivo*. Therefore, we analyzed cytokine production in polarized and re-stimulated T helper cell subsets established from purified naive CD4⁺ cells. Naive CD4⁺ lymph node T cells were cultured either under neutral (TH0) conditions or polarized toward TH1 or TH2 lineages,

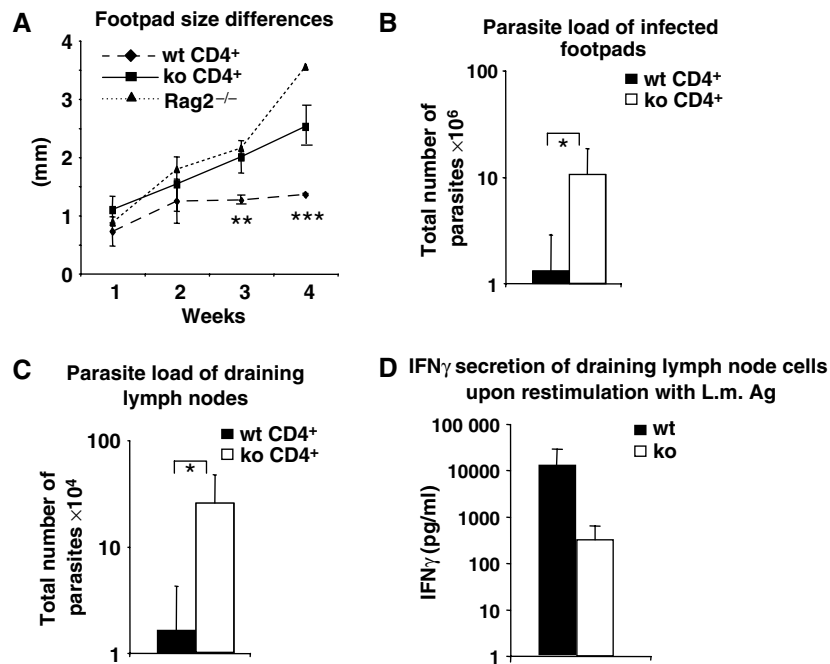


Figure 3 The inability of BOB.1/OBF.1^{-/-} mice to develop an efficient response to *L. major* infection is T-cell intrinsic. Rag2^{-/-} animals were reconstituted with either wild-type ($n = 10$) or BOB.1/OBF.1^{-/-} CD4⁺ T cells ($n = 8$), together with wild-type B cells, and infected the next day with *L. major*. (A) Differences of left and right footpad sizes were measured. After 4 weeks, animals were killed and the parasite load of infected footpads (B) as well as of draining lymph nodes (C) was determined. * $P \leq 0.05$; ** $P = 0.039$; *** $P = 0.009$. Data were analyzed with Student's *t*-test. The results of three independent experiments are shown in (A). In two of three independent experiments the draining lymph nodes as well as the footpads were used for the analyses of parasite load (B, C); in the third experiment, the draining lymph node cells were used for the analysis of IFN γ secretion upon re-stimulation with L.m. antigen. (D) The numbers of draining lymph node CD4⁺ T cells were enumerated, equal amounts of CD4⁺ T cells were reseeded and re-stimulated with *L. major* antigen. After 6 days in culture, IFN γ production was measured by ELISA.

respectively. After re-stimulation with α CD3/ α CD28 antibodies, secreted cytokines were analyzed by ELISA. Like in our *in vivo* experiment, the expression of TH1 cytokines IL2 and IFN γ was reduced in BOB.1/OBF.1^{-/-} cells (Figure 4A and 4B). In contrast, the synthesis of TH2 cytokines IL4 and IL13 was enhanced (Figure 4C and 4D). These differences in cytokine secretion by BOB.1/OBF.1^{-/-} TH1 or Th2 cells were already detectable after 48–72 h of polarization initiation (Supplementary Figure S7).

To address the question whether the reduction in IFN γ levels produced by BOB.1/OBF.1^{-/-} TH1 cells is caused by a lower IFN γ production per cell or by fewer cells producing IFN γ , we performed intracellular cytokine staining experiments. We observed that the number of cells staining positive for IFN γ was reduced. In addition, the amount of IFN γ produced per cell is reduced, as is evident from the lower mean fluorescence intensity. Analyses of TH2 cells established from BOB.1/OBF.1^{-/-} mice revealed corresponding increases in cell numbers that produce IL4 and also in IL4 mean fluorescence intensity (Figure 4E).

The imbalanced secretion of TH1 and TH2 cytokines results from an altered mRNA expression

To analyze whether reduced IFN γ levels are a consequence of reduced RNA levels rather than defects in cytokine secretion, we quantified cytokine RNA levels by RT-PCR. This analysis showed reduced IFN γ RNA levels in polarized and re-stimulated BOB.1/OBF.1^{-/-} TH0 and TH1 cells. In contrast, IL4 RNA levels were slightly increased in BOB.1/OBF.1^{-/-} TH2 cells (Figure 5A). These data were confirmed by real-time

PCR (Figure 5B). In addition, the expression of the key regulatory transcription factors for TH1 and TH2 development, T-bet and GATA-3, was analyzed and found to be unaffected in BOB.1/OBF.1^{-/-} cells (Figure 5B and C). Analysis of the IL12 receptor β 2 (IL12R β 2) mRNA (Figure 5D), necessary for developing TH1 cells to receive the IL12 signal from APCs, also revealed a slight decrease in untreated and re-stimulated TH1 cells generated from BOB.1/OBF.1^{-/-} mice. This reduced IL12R β 2 expression levels might be physiologically relevant, as we saw a reduced polarization frequency toward TH1 cells *in vitro*. In addition, we observed lower IL12-induced IFN γ secretion by BOB.1/OBF.1-deficient TH1 cells in comparison with wild-type cells (data not shown).

BOB.1/OBF.1 directly activates the IFN γ promoter

Since BOB.1/OBF.1 influences as a transcriptional coactivator the activity of gene expression, we asked whether the reduced IFN γ expression observed in BOB.1/OBF.1^{-/-} TH0 and TH1 cells might be due to a direct contribution of BOB.1/OBF.1 to the IFN γ promoter activity. BOB.1/OBF.1 regulates transcription together with Oct factors. Therefore, we searched for octamer motifs in the IFN γ promoter and found five potential sites within the upstream 900 bp (Supplementary Figure S8). All sites were analyzed by electrophoretic mobility shift assay (EMSA) for ternary complex formation with Oct1 and BOB.1/OBF.1. HeLa nuclear extracts supplemented with *in vitro* translated BOB.1/OBF.1 were used. The strongest ternary complex similar to that of a consensus octamer sequence was observed with the octamer site,

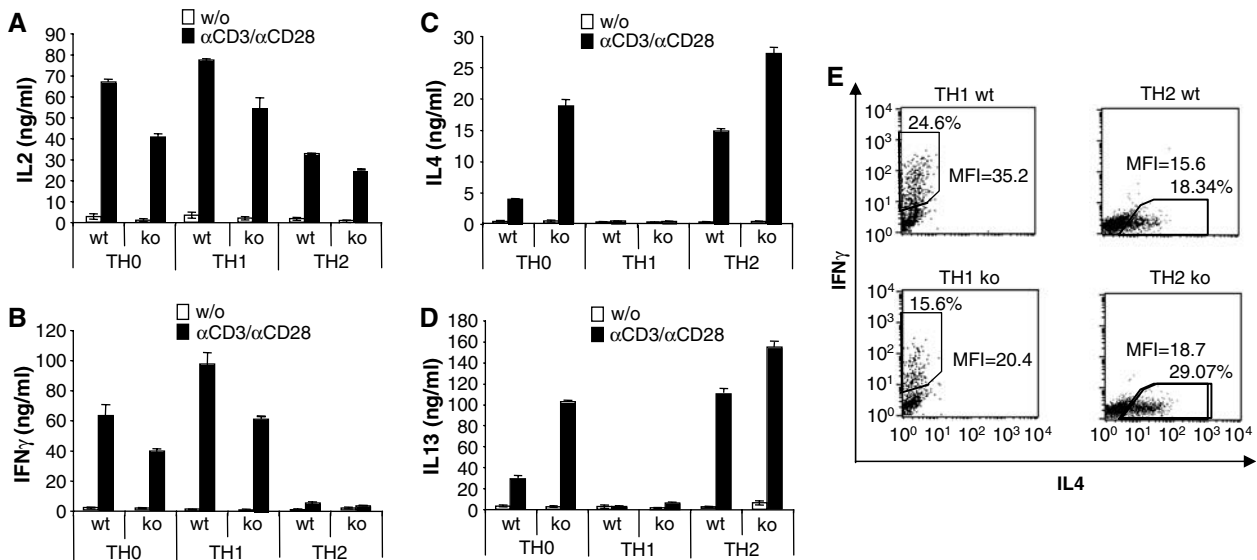


Figure 4 BOB.1/OBF.1 deficiency leads to impaired function of T helper cell subsets. Naïve CD4⁺ T from lymph nodes of wild-type and BOB.1/OBF.1^{-/-} mice were grown either under neutral conditions or under polarization conditions. After 6 days, cells were washed, adjusted in cell numbers, re-plated and either left uninduced or re-stimulated with plate-bound α CD3 + α CD28. After 24 h in culture, cytokine release was measured by ELISA. (A, B) BOB.1/OBF.1^{-/-} TH0 and TH1 cells produce less IL2 and IFN γ in comparison with wild-type cells. (C, D) BOB.1/OBF.1^{-/-} TH0 and TH2 cells produce increased levels of IL4 and IL13 in comparison with wild-type cells. The experiment was performed with triplicates and repeated at least three times. One representative experiment is shown. Data are the means \pm s.d. (E) IFN γ produced by TH1 cells or IL4 produced by TH2 cells established from naïve cells of wild-type (wt) or BOB.1/OBF.1^{-/-} (ko) mice was analyzed by intracellular cytokine FACS analyses. MFI, mean fluorescence intensity.

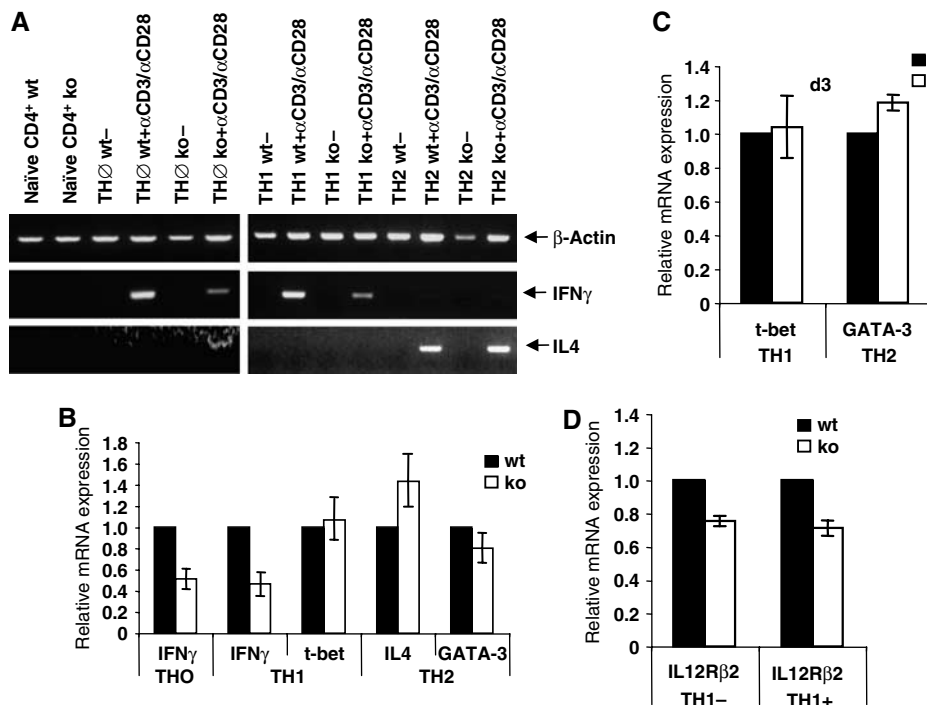


Figure 5 The imbalanced synthesis of TH1 and TH2 cytokines results from an altered mRNA expression. (A) Naïve CD4⁺ T cells were either left untreated or kept for 6 days either under neutral conditions (TH0) or in TH1 or TH2 differentiation medium, re-plated and either left untreated or induced with plate-bound α CD3 + α CD28. After 24 h, RNA was prepared and analyzed by RT-PCR for IFN γ and IL4 mRNA expression. The analysis of β -actin expression served as internal control. (B) TH0, TH1 or TH2 cells were generated from naïve CD4⁺ cells derived from wild-type and BOB.1/OBF.1^{-/-} mice. After re-stimulation of cells for 24 h with plate-bound α CD3 + α CD28, mRNA expression of IFN γ , IL4, T-bet, GATA-3 was analyzed by real-time PCR. (C) Expression of T-bet and GATA-3 was analyzed at day 3 (d3) of T helper cell differentiation. (D) Expression of IL-12R β 2 mRNA was analyzed in differentiated TH1 cells from wild-type and BOB.1/OBF.1^{-/-} cells that were either left untreated (-) or induced with plate-bound α CD3 + α CD28 (+). Relative expression levels are shown. The experiments were performed with triplicates. Data are means \pm s.d.

close to the TATA box (M5; -129 to -136) (Figure 6A and Supplementary Figure S8). In supershift experiments, antibodies against Oct1 and BOB.1/OBF.1 blocked ternary complex formation. Oct2 antibodies did not interfere, since HeLa cells do not express Oct2 protein (Figure 6A). The IFN γ M5 site could also efficiently compete against a labelled consensus octamer site (Figure 6B). In contrast, when the M5 sequence was mutated, no binding of Oct and BOB.1/OBF.1 or competition could be detected (Figure 6B). A second more distal octamer motif (M2) (Figure 6A and Supplementary Figure S8) was also able to form ternary complexes, albeit with a reduced efficiency (Figure 6A). Both sites vary from the consensus Oct binding site at the first or last position, respectively. Oct proteins have also been described to bind to the murine IL2 and IL4 promoters. We therefore analyzed these sites for ternary complex formation. Indeed, weak complexes composed of Oct and BOB.1/OBF.1 proteins were detected with the IL2 promoter site, but no complex was detected at the IL4 promoter (Figure 6A).

To investigate whether BOB.1/OBF.1 binds to the IFN γ promoter *in vivo*, chromatin immunoprecipitation (ChIP) was performed with purified CD4⁺ T cells from wild-type or BOB.1/OBF.1^{-/-} mice. Whereas binding of Oct1 and Oct2 was detectable in wild-type and BOB.1/OBF.1^{-/-} cells, BOB.1/OBF.1 binding to the IFN γ promoter M5 octamer motif could be detected only in α CD3 + α CD28 stimulated wild-type cells (Figure 6C). No binding of Oct proteins and BOB.1/OBF.1 to the IFN γ promoter octamer motif M2 could be detected *in vivo* (data not shown).

As we observed a binding of BOB.1/OBF.1 together with Oct to the IFN γ promoter, we wondered whether this binding results in the activation of IFN γ promoter activity. Therefore, the promoters of the IFN γ -, IL2- and IL4 genes were cloned in front of a luciferase reporter gene and transfected into NIH/3T3 cells, along with Oct2, BOB.1/OBF.1 or a combinations of these expression vectors. Consistent with our binding studies, we observed that BOB.1/OBF.1, together with Oct2, was able to transactivate the murine IFN γ promoter by approximately 20-fold (Figure 6D). The IL2 promoter activity is also stimulated by BOB.1/OBF.1 together with Oct2, albeit to a lesser extent (five-fold). In contrast, IL4 promoter activity was virtually not affected by coexpression of BOB.1/OBF.1 and Oct2 (Figure 6D).

In order to elucidate the importance of the octamer motif for IFN γ promoter activity in T cells, Jurkat cells were transfected with either the wild-type or the mutant IFN γ luciferase reporter construct (M5 mut; Figure 6B). The mutated octamer motif causes a reduction of 35% of the PMA/ionomycin-induced IFN γ promoter activity in Jurkat T cells (Figure 6E). This indicates that this octamer motif and the interacting transcription factors contribute to full IFN γ promoter activity in T cells.

The T-box transcription factor T-bet is the major factor necessary for IFN γ production. Initially, T-bet binding sites were identified about 2300 bp upstream of the IFN γ promoter. Additionally, an alternative region, the T-bet responsive unit (-445 to -415), was described (Soutto *et al*, 2002). Recently, several T-bet binding sites were identified within the proximal IFN γ promoter region (Cho *et al*, 2003). Moreover, one of those sites overlaps with the M5 motif, identified in our study as an Oct/BOB.1/OBF.1 binding site (Supplementary Figure S8). Therefore, we asked, whether Oct and BOB.1/OBF.1

cooperate with T-bet to transactivate the IFN γ promoter. Transfection experiments revealed a synergistic effect of Oct and BOB.1/OBF.1, together with T-bet, on the IFN γ promoter activity when cotransfected into NIH/3T3 cells (Figure 6F).

In conclusion, these findings indicate that BOB.1/OBF.1 directly regulates IFN γ promoter activity in T cells together with Oct2 and T-bet.

BOB.1/OBF.1 influences PU.1 expression in TH2 cells

Given that we could not show a direct regulation of IL4 or IL13 promoter activity by BOB.1/OBF.1 (Figure 6A and D, data not shown), we wondered how the expression of these cytokines might be affected by the loss of BOB.1/OBF.1. Recent experiments demonstrated that the transcription factor PU.1 is expressed in TH2 cells and attenuates TH2 cytokine expression by interfering with GATA-3 function (Chang *et al*, 2005). Previous cotransfection experiments had suggested a direct regulation of PU.1 expression by Oct transcription factors and BOB.1/OBF.1 in B cells (Kistler *et al*, 1995; Chen *et al*, 1996). We therefore analyzed PU.1 expression in wild-type and BOB.1/OBF.1-deficient TH2 cells. PU.1 protein levels (Figure 7A) and RNA levels (Figure 7B) were clearly reduced in BOB.1/OBF.1-deficient cells. The PU.1 promoter contains a well-conserved octamer motif, and we could show that Oct1 and BOB.1/OBF.1 can form ternary complexes at this site (Figure 7C). When nuclear extracts from HeLa cells were incubated with *in vitro* translated BOB.1/OBF.1, a slower migrating ternary complex was detected in an EMSA (Figure 7C, lane 3). Supershift assays revealed that this complex was composed of BOB.1/OBF.1 and Oct1 (Figure 7C, lanes 4 and 5). This complex formation at the octamer motif of the PU.1 promoter was identical to that formed at the consensus octamer site (Figure 7C). In addition, transfection of Oct2 and BOB.1/OBF.1 expression vectors along with a reporter consisting the PU.1 promoter revealed a synergistic stimulation of the PU.1 promoter (Figure 7D). Mutating the octamer motif causes a reduction by 65% of the PMA/ionomycin-induced PU.1 promoter activity in T cells (Figure 7E). Finally, in order to prove the contribution of Octamer factors and BOB.1/OBF.1 in the regulation of the PU.1 promoter *in vivo*, ChIP assays were performed using stimulated TH1 or TH2 cells. In those experiments, a direct binding of Oct factors as well as of BOB.1/OBF.1 to the PU.1 promoter in wild-type TH2 cells could be demonstrated. In contrast, in TH1 cells no binding of the analyzed transcription factors was observed (Figure 7F). Together, these data demonstrate the importance of Oct and BOB.1 proteins for PU.1 promoter activity in T cells. Reduced PU.1 levels most likely result in higher GATA-3 activity and consequently increased TH2 cytokine expression levels.

Discussion

Earlier analyses of BOB.1/OBF.1^{-/-} mice had revealed its importance for effective B-cell immune functions. Here, we show that the lack of the transcriptional coactivator BOB.1/OBF.1 in mice leads to an impaired TH1/TH2 balance. These effects are of decisive importance for the immune response *in vivo*. BOB.1/OBF.1^{-/-} mice are not able to control experimental leishmaniasis, as reflected by increased parasite load of infected footpads and draining lymph nodes. Our new

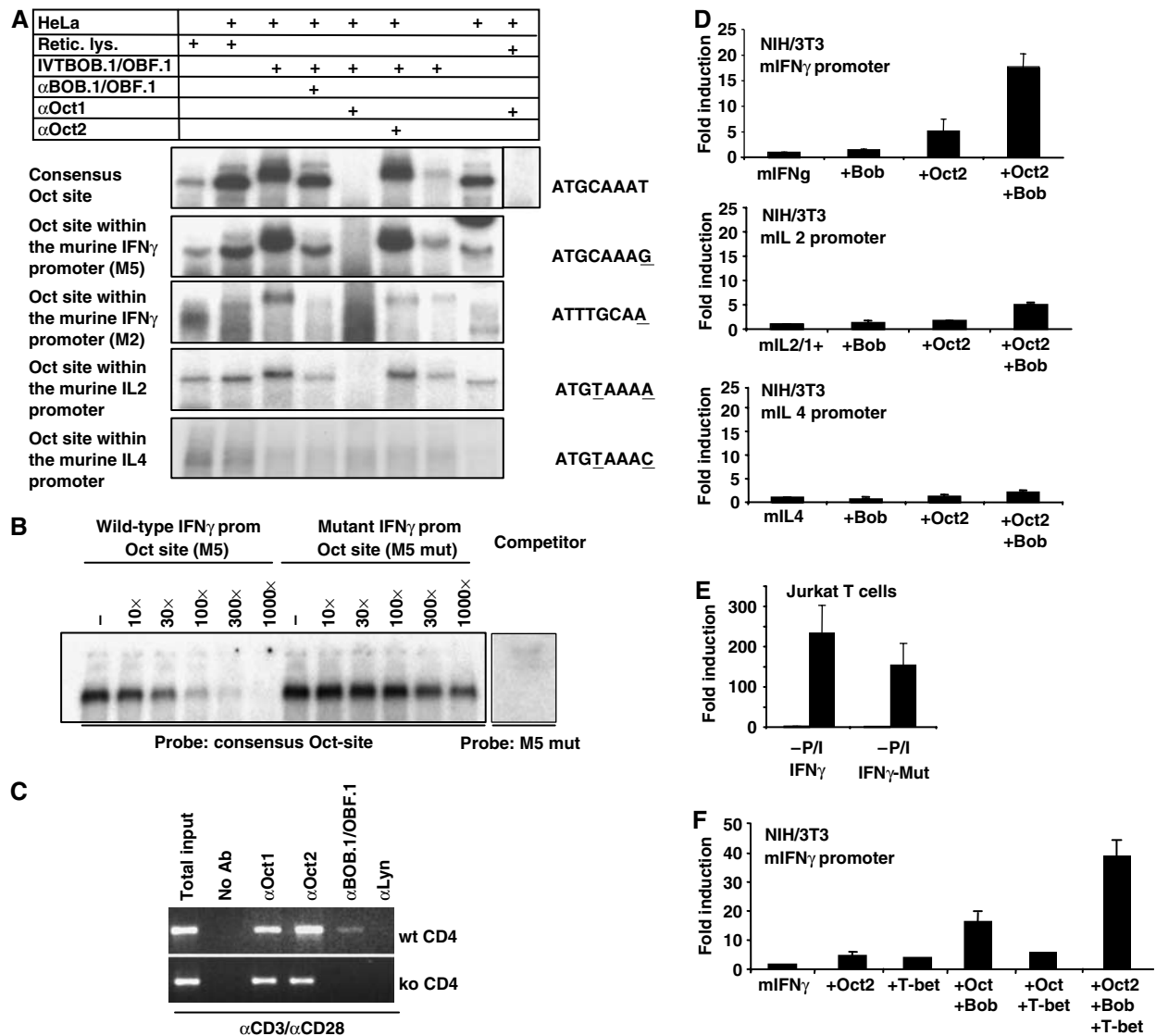


Figure 6 BOB.1/OBF.1 is involved in the regulation of the IFN γ promoter, *in vitro* as well *in vivo*. (A) EMSA experiments with HeLa nuclear extracts were performed using labelled oligonucleotides bearing either a consensus octamer motif or the octamer sequences identified within the IFN γ promoter (M5 and M2), or within the IL2 and IL4 promoter. Unprogrammed reticulocyte lysate or *in vitro* translated BOB.1/OBF.1 protein was included. Complexes were analyzed by adding specific antibodies for Oct1, Oct2 or BOB.1/OBF.1, as indicated. (B) Ternary complexes formed between the consensus octamer site and HeLa nuclear extracts supplemented with *in vitro* translated BOB.1/OBF.1 were challenged with either the wild-type or mutated sites from the IFN γ promoter (the octamer motif ATGCAAAT was mutated to AgtacaAT). The mutated site was also unable to form ternary complexes when tested as labeled fragment (right panel). (C) ChIP assay. The chromatin of α CD3 + α CD28-induced wild-type or BOB.1/OBF.1 $^{-/-}$ CD4 $^{+}$ T cells was analyzed. Immunoprecipitated DNA was PCR amplified using primers specific for the IFN γ promoter M5 octamer motif. Chromatin immunoprecipitated with α Lyn antibodies was included as negative control. (D) Analysis of the dependence of the IFN γ promoter activity on Oct and BOB.1/OBF.1 proteins in transient transfection experiments using NIH/3T3 fibroblasts. The value of the IFN γ promoter activity without cotransfection of Oct2 and BOB.1/OBF.1 expression vectors was determined. (E) Jurkat T cells were transfected with either the wild-type or the octamer-mutated (M5 mut) IFN γ promoter reporter construct. Subsequently, cells were left untreated (–) or induced with PMA/ionomycin (+ P/I). The activity of the unstimulated wild-type promoter was set to 1 and the fold induction was determined. (F) Oct2, BOB.1/OBF.1 and T-bet transactivate the IFN γ promoter in synergy. Expression vectors for Oct2, BOB.1/OBF.1 and T-bet were cotransfected along with the murine IFN γ promoter reporter construct into NIH/3T3 cells. The value of the IFN γ promoter activity without cotransfection was set to 1. The fold induction was determined. (D–F) The experiments were repeated five times and mean values as well as s.d. were determined.

results now document that in addition to the defective B-cell effector functions, T-cell functions are also severely compromised in BOB.1/OBF.1 $^{-/-}$ mice. This emphasizes the critical role that BOB.1/OBF.1 plays for the majority of effector cell populations of the adaptive immune system.

Lymphocytes express two octamer factors, the ubiquitously expressed Oct1 and the lymphocyte-specific Oct2 protein (Kamps *et al*, 1990; Schöler, 1991; Staudt and

Lenardo, 1991). Whereas in B cells, both factors are constitutively expressed, in peripheral T cells as well as in non-transformed T-cell lines, Oct2 expression as well as BOB.1/OBF.1 expression and function are induced upon TCR stimulation (Kang *et al*, 1992; Zwilling *et al*, 1997). Here, we show that BOB.1/OBF.1 expression is detectable in polarized TH1 and TH2 cells, and that its expression levels can be further increased by stimulation of T helper cells.

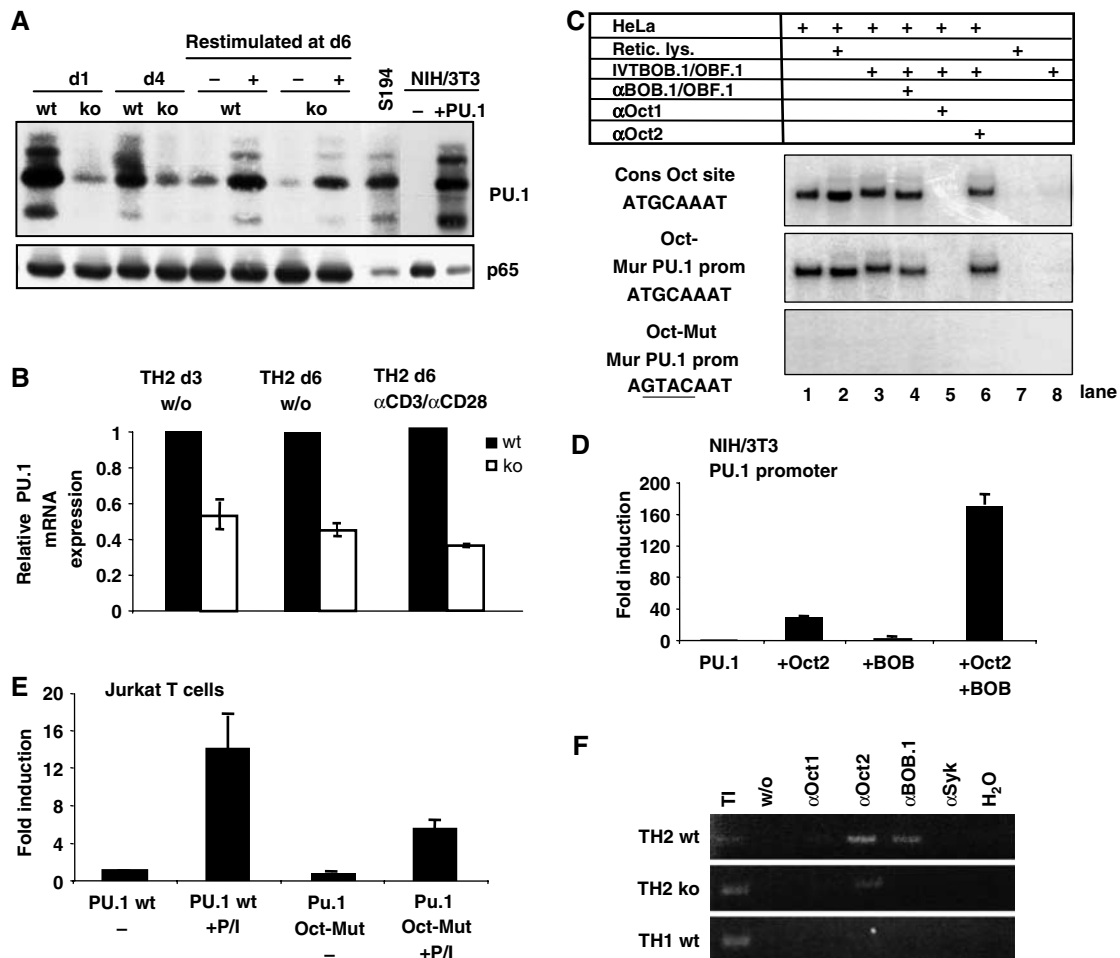


Figure 7 BOB.1/OBF.1 expression is necessary for PU.1 promoter activity in TH2 cells. (A) PU.1 expression is downregulated in developing and established BOB.1/OBF.1^{-/-} TH2. Wild-type or BOB.1/OBF.1^{-/-} developing TH2 cells were analyzed for PU.1 expression at day 1 (d1), day 4 (d4) and day 6 (d6) during differentiation. At day 6, cells were re-stimulated for 24 h with plate-bound α CD3 + α CD28. The analyses of extracts from the S194 B-cell line or from NIH/3T3 cells, either left untreated (-) or transfected with a murine PU.1 expression vector (+PU.1), served as internal controls. Analyses of p65 expression served as loading control. (B) RNA was prepared from developing TH2 cells at day 3 (d3) or differentiated TH2 cells at day 6 (d6), or from re-stimulated, as indicated, at d6 established TH2 cells from either wild-type or BOB.1/OBF.1^{-/-} mice and analyzed by real-time PCR for relative PU.1 expression. The experiments were performed in triplicates. Data are the means \pm s.d. (C) Oct and BOB.1/OBF.1 form ternary complexes at the octamer motif of the PU.1 promoter (see Figure 6A). (D) Oct and BOB.1/OBF.1 synergistically transactivate the PU.1 promoter (see Figure 6D). (E) The octamer motif is essential for the induction of the PU.1 promoter activity in T cells. Reporter constructs bearing either the wild-type or octamer mutant PU.1 promoter were transfected into Jurkat T cells and subsequently stimulated with PMA/ionomycin. The activity of the wild-type promoter without induction was set to 1. The fold induction was determined. (D, E) The experiments were repeated five times and mean values \pm s.d. are shown. (F) ChIP assay. The chromatin of α CD3 + α CD28-induced wild-type or BOB.1/OBF.1^{-/-} TH1 and TH2 cells was analyzed. Immunoprecipitated DNA was PCR amplified using primers specific for the PU.1 promoter octamer motif. Chromatin immunoprecipitated with α Syk antibodies was included as negative control.

Using an *in vivo* model of experimental leishmaniasis we found that BOB.1/OBF.1^{-/-} mice, generated on the resistant C57BL/6 background, developed a severe infection with a drastically reduced capacity to contain the parasites. Importantly, whereas the IFN γ secretion *in vitro* was reduced only 30–40%, popliteal lymph node cells from *L. major*-infected animals were almost unable to produce IFN γ . These T cells from infected BOB.1/OBF.1^{-/-} mice, however, were sensitized in an antigen-specific manner, since they proliferated in response to *L. major* antigen. In parallel, we found low but increased production of the TH2 cytokine IL13 in draining lymph node cells of infected animals, most likely a result of the reduced PU.1 expression found in BOB.1/OBF.1^{-/-} TH2 cells. However, this increase was modest and we were unable to detect IL4 in draining lymph nodes of infected wild-type and BOB.1/OBF.1-deficient animals, or

from reconstituted Rag2^{-/-} mice after re-stimulation *ex vivo* with *L. major* antigen. This indicates that the immune response in BOB.1/OBF.1-deficient mice is not completely shifted toward a TH2 response. Therefore, we conclude that the reason for the severe leishmaniasis in BOB.1/OBF.1-deficient mice is primarily impaired IFN γ production.

No defects were found in DCs and macrophages that are involved in effective immune response against *L. major*. Despite the finding that B cells are not necessary for the elimination of *L. major* after infection with high parasite numbers (Sacks *et al*, 1984; Brown and Reiner, 1999), an effect of B cells in the activation of macrophages could not be excluded *a priori*. However, adoptive transfer experiments confirmed that the defect in BOB.1/OBF.1^{-/-} mice is intrinsic to T cells.

We could show that BOB.1/OBF.1 deficiency had an effect on TH1 cell function. However, a direct regulation of the IFN γ

promoter by Oct/BOB.1/OBF.1 had not been reported before. The promoter region encompassing roughly the first 600 bp upstream of the start site of transcription was identified to be responsible for signal- and tissue-specific control of IFN γ gene expression (Chrivia *et al*, 1990; Ciccarone *et al*, 1990; Penix *et al*, 1993). We identified several potential binding sites for Oct1/2 and BOB.1/OBF.1 within this region. A combination of *in vitro* and *in vivo* experiments documented the importance of these factors for IFN γ promoter activity. Binding of Oct2 and BOB.1/OBF.1 to a non-consensus octamer motif was also recently demonstrated for the Btk promoter (Brunner and Wirth, 2006). Moreover, we showed that Oct and BOB.1/OBF.1 act in synergy with T-bet in the IFN γ promoter activation. Together, our findings suggest that Oct transcription factors and BOB.1/OBF.1 are needed for high levels IFN γ gene expression.

We also saw reduced expression of the IL12R β 2 in BOB.1/OBF.1 $^{-/-}$ TH1 cells. APC-derived IL12 plays a key role in TH1 differentiation, as shown by the fact that mice deficient in either IL12 receptor or in IL12 itself show severe defects in TH1 responses (Magram *et al*, 1996; Wu *et al*, 1997, 2000). Additionally, although IL12 is the major cytokine necessary for TH1 cell generation, IFN γ secreted by CD4 $^{+}$ cells induces TH1 cell development via an autocrine loop, together with IL12 (Bradley *et al*, 1996). Consistent with the reduced expression of the IL12R β 2 chain, we found reduced (40%) levels of IFN γ secretion upon IL12 stimulation (data not shown). As this reduction is similar to that seen after CD3/CD28 stimulation, the specific contribution of the reduced expression of IL12R β chain is unclear.

The importance of the octamer motif for IL2 transcription in T cells is well documented (Brunvand *et al*, 1988; Shibuya and Taniguchi, 1989; Hentsch *et al*, 1992; de Grazia *et al*, 1994; Pfeuffer *et al*, 1994). Octamer transcription factors also bind to the IL4 promoter (Chuvpilo *et al*, 1993; Li-Weber *et al*, 1998). However, they were shown to exhibit only a minor or possibly even an inhibitory effect on IL4 gene transcription (Pfeuffer *et al*, 1994; Cron *et al*, 2001). We confirmed binding of octamer transcription factors to the IL2 promoter octamer motif. Moreover, we found that Oct1/2 and BOB.1/OBF.1 form ternary complexes at the IL2 promoter. Consistent with the reduced expression of IL2 in BOB.1/OBF.1 $^{-/-}$ T cells, we found that BOB.1/OBF.1 induced the IL2 promoter together with Oct2. Reduced expression levels of IL2 might be responsible for the reduced numbers of T cells that we observed in BOB.1/OBF.1 $^{-/-}$ mice.

In contrast, no significant direct contribution of BOB.1/OBF.1 to IL4 gene expression was evident from our analyses. No functional octamer motif was detected within the IL13 promoter. Indeed, we found that the effect of BOB.1/OBF.1 on TH2 cytokines is rather indirect. Here, we show that PU.1 is a direct target of BOB.1/OBF.1 in TH2 cells. PU.1 in turn negatively regulates TH2 cytokine secretion by interfering with GATA-3 binding to promoter DNA. Hence, silencing of PU.1 expression in TH2 cells results in increased TH2 cytokine secretion (Chang *et al*, 2005). Therefore, it is likely that BOB.1/OBF.1 influences TH2 cytokine synthesis indirectly by regulation PU.1 expression. Recently, regulation of the PU.1-related transcription factor Spi-B by BOB.1/OBF.1 was reported (Bartholdy *et al*, 2006). Expression levels of Spi-B were very low in TH2 cells and no clear dependency on BOB.1/OBF.1 could be detected.

In summary, our data demonstrate that in addition to its key role in B lymphocyte effector functions, BOB.1/OBF.1 also plays a critical role in fine-tuning TH1 and TH2 cytokine expression. The relevance of this fine-tuning is evident upon challenge of BOB.1/OBF.1-deficient mice with *L. major*. Therefore, BOB.1/OBF.1 is a pleiotropic regulator of effector functions in the adaptive immune system.

Materials and methods

Mice

C57BL/6 wild-type, BOB.1/OBF.1 $^{-/-}$ or Rag2 $^{-/-}$ mice on the same genetic background (5–8 weeks of age) were obtained from our breeding facility. Infected mice were kept under SPF conditions.

Cell purification of CD4 $^{+}$ T cells

For naïve CD4 $^{+}$ T-cell purification, single cell suspensions of lymph nodes were incubated with antibodies against B220, CD8 (hybridoma supernatants), DX5 and MHCII (BD). After complement lysis, T cells were purified on a lympholyte M gradient. CD44 $^{+}$, Mac1 $^{+}$ (BD) and Gr1 $^{+}$ (hybridoma supernatant) cells were removed by negative selection using magnetic sheep anti-rat IgG beads (Dyna). For purification of CD4 $^{+}$ T cells, lymph node cell suspensions were depleted for B220 $^{+}$ and CD8 $^{+}$ cell by complement lysis. The achieved purity of cell populations was higher than 95%.

Cell purification of B220 $^{+}$ cells

B220 $^{+}$ cells were purified from spleen single cell suspensions by complement lysis of CD4 $^{+}$, CD8 $^{+}$ and DX5 $^{+}$ cells, followed by a lympholyte M gradient. Subsequently, CD44 $^{+}$, Mac1 $^{+}$, Ter119 $^{+}$ and Gr1 $^{+}$ cells were removed by negative selection using magnetic sheep anti-rat IgG beads (Dyna). Antibodies used for purification were purchased from BD.

Cell stimulation

Cells were stimulated as follows: α CD3: 4 μ g/ml (soluble or coated; BD); α CD28: 0.5 μ g/ml (BD); PMA: 50 ng/ml; ionomycin: 500 ng/ml (all from Sigma); DCs: 0.3×10^5 cells/well + α CD3; IFN γ : 2 μ g/ml (Biotrend); IL4: 2% of supernatant of IL4-producing cells.

DCs or macrophages were stimulated as follows: LPS: 10 ng/ml (Invivogene); IFN γ : 50 U/ml; the ratio of *L. major* antigen per cell number was 3:1; α CD40: 10% of supernatant of α CD40-producing hybridoma cells. Cells were stimulated with IFN γ for 48 h before harvesting; all other inducers, including again IFN γ , were added 24 h before harvesting supernatants for cytokine or NO quantification.

TH1/TH2 polarization

Purified naïve CD4 $^{+}$ T cells ($0.5\text{--}1 \times 10^5$ cells/well; purity $\geq 95\%$) were stimulated with coated α CD3 plus soluble α CD28 (BD) in RPMI medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin, 50 μ M β -ME in the presence of IL2 (2% of supernatant of IL2-producing cells; 5 ng/ml) (= TH0 conditions). For TH1 polarization, IFN γ (2 μ g/ml; Biotrend), IL12 (2 μ g/ml; Biotrend), and α IL4 monoclonal antibodies (5 μ g/ml; BD), were added. For TH2 polarization, IL4 (2% supernatant of cells; 5 ng/ml) and α IFN γ (5% supernatant of hybridoma cells) were added. At day 6, cells were washed twice with PBS and re-stimulated, as indicated, for additional 24 h.

Proliferation assay

T-cell proliferation was assessed by ^3H -labelled thymidine incorporation. A total of $0.5 \times 10^6\text{--}1 \times 10^6$ purified CD4 $^{+}$ T cells per well were seeded in a 96-well plate and grown at 37°C and 5% CO $_2$ in complete RPMI medium (\pm stimuli). After 36 h of stimulation, 0.5 μ Ci/well ^3H -labelled thymidine (Amersham) was added for additional 10 h. Cells were harvested using the Inotech cell harvester system. Incorporated radioactivity was measured using the Wallace 1450 Microbeta Plus liquid scintillation counter and expressed as c.p.m. per well.

Intracellular cytokine staining

Cytokine production was determined by intracellular staining as previously described (Bird *et al*, 1998).

Bone marrow-derived DCs and macrophages (BMDM)

Tibias and femurs were flushed using a 25-gauge needle. Bone marrow cells were washed twice and cultured (3×10^6 cells/5 ml) in medium supplemented with granulocyte macrophage-colony stimulating factor (GM-CSF) (5% of supernatant of GM-CSF-producing cells). Non-adherent cells were harvested on day 8 and used for stimulation. BMDM were obtained from femurs, as described previously (Sunderkotter *et al*, 1993).

ELISA

Cytokine release was detected in supernatants by ELISA. All antibodies were purchased from BD, except for α IL13 (R&D). The recombinant mouse cytokine standards were from Cell Concepts (IL2 and IL13), Biotrend (IFN γ), PreproTechEC LTD (IL4) and PreproTech (TEBU) (IL12 p40). Extinction was analyzed at 405/490 nm on microplate ELISA reader, using the SoftMaxPro 4.3 LC software.

NO quantification

Macrophages cultured in 24-well plates were primed for 24 h with 50 U/ml murine IFN γ (Cell Concepts). Subsequently, cells were stimulated with LPS (Sigma) and IFN γ as positive control, and with *L. major* (*L. major*:macrophage ratio 3:1) opsonized with 1 % normal mouse serum (DAKO). Twenty-four hours later, supernatants were analyzed for nitrite concentration at 540 nm in a microtiter plate assay, using Griess reagent (Sigma), as described (Ding *et al*, 1988).

L. major infection

L. major strain MHOM/IL/81/SE/BNI was passaged in BALB/c mice *in vivo* and grown *in vitro* in Schneider's *Drosophila* medium (Cambrex) at 25°C, as previously described (Sunderkotter *et al*, 1993; Schonlau *et al*, 2003). Experimental leishmaniasis was initiated by subcutaneous injection of 2×10^7 *L. major* promastigotes (stationary phase) in 20 μ l PBS into the left hind footpad. The thickness of the infected and the contralateral uninfected footpad were measured weekly using a metric caliper (Oditest).

Determination of parasite load

The number of viable parasites in infected footpads and draining lymph nodes was evaluated by a limiting-dilution assay, as previously described (Titus *et al*, 1985; Schonlau *et al*, 2003).

Adoptive transfer experiments

CD4⁺ T cells or B220⁺ B cells were purified from lymph nodes or spleen, respectively, as described above. Enrichment after depletion was 95–98% for the desired population. Age-matched Rag2^{-/-} mice received 10⁶ purified wild-type or BOB.1/OBF.1^{-/-} CD4⁺ T and 10⁷ wild-type B220⁺ cells intravenously. Twenty-four after the cell transfer into Rag2^{-/-} mice, animals were infected with *L. major*.

EMSA

Whole-cell extract preparation and the EMSA procedures have been described (Lernbecher *et al*, 1993). The sequences of probes used

are available upon request. For supershift experiments, an anti-Oct1 antibody (serum), anti-Oct2 antibody (C-20; Santa Cruz Biotechnology) or anti-BOB.1/OBF.1 antibody (serum) was used. EMSA conditions for detection of specific Oct/BOB.1/OBF.1 complexes were described previously (Luo *et al*, 1992). In these experiments, 2 μ g HeLa extracts and 5 μ l *in vitro* translated BOB.1/OBF.1 protein or the same amount of unprogrammed TNT lysates (Promega) were added where indicated.

Real-time PCR

Total RNA was isolated using the High pure RNA isolation Kit (Roche Diagnostics) and reverse transcribed using M-MLV reverse transcriptase. PCR primer sequences are available upon request. Real-time PCR was performed and analyzed as described (Boehm *et al*, 2001).

Transfection experiments

Transfections of NIH/3T3 cells were performed by electroporation (Bio-Rad) with 450 V and 250 μ F in PBS; transfections of Jurkat cells were performed with 250 V and 975 μ F in PBS.

Plasmids

The murine IFN γ promoter (+73 to -523) was cloned by genomic PCR. The fragment was cloned upstream of the luciferase reporter gene (Brunner *et al*, 2003a). Mutation of the octamer motif (M5; T2G, G3T, C4A, A5C) was generated using the QuickChangeTM Mutagenesis kit (Promega) Expression vectors for BOB.1/OBF.1 have been described (Pfisterer *et al*, 1995). IL2, and IL4 promoter constructs were kindly provided by E Serfling. The cloning of the murine PU.1 promoter construct as well as the octamer mutant PU.1 promoter construct was described (Kistler *et al*, 1995). The expression vector for T-bet was kindly provided by M Boothby.

ChIP assay

The ChIP assay kit was used as described (Brunner *et al*, 2003a). Primers sequences for PCR were as follows:
IFN γ -M5 5': ACAAGAATGGCACAGGTGGGC,
IFN γ -M5 3': CCGAGGAGCTTCGATCAGGTA;
PU.1 5': CAGCCGGCCAGAGACTTCTGT;
PU.1 3': GCCTGCCACTGGGAGATAGTCC.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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