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IBP (IRF-4 Binding Protein) inhibits IL-17 and IL-21 production by controlling IRF-4 function

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Abstract

The TH17 lineage is a novel $CD4^+$ T cell effector subset that plays a key role in inflammatory and autoimmune responses, via its ability to produce IL-17 and IL-21. Given the potentially deleterious effects of TH17 cells, their generation needs to be strictly controlled. The regulatory pathways that prevent the inappropriate development of TH17 cells have not been fully elucidated. IRF-4 is a transcription factor that has recently emerged as a key regulator of TH17 differentiation. Our laboratory has isolated a protein, which interacts with IRF-4, that we have termed IBP (IRF-4 Binding Protein). Our studies previously demonstrated that IBP can act as an activator of Rho GTPases and that mice deficient in IBP develop a lupus-like syndrome upon aging. Here we show that TCR transgenic IBP deficient mice rapidly develop rheumatoid arthritis-like joint disease and large-vessel vasculitis. The pathology observed in the absence of IBP is associated with an enhanced responsiveness of T cells to low-levels of stimulation and with the inappropriate synthesis of IL-17 and IL-21. Furthermore, we demonstrate that the effect of IBP on cytokine production is due to its ability to sequester IRF-4 and prevent it from targeting the transcriptional regulatory regions of the IL-17 and IL-21 genes. Consistent with this finding, the enhanced ability of IBP deficient T cells to produce IL-17 and IL-21 is abolished by the concurrent lack of IRF-4. Taken together these studies suggest that IBP plays a key regulatory role in the prevention of T cell-mediated autoimmunity by ensuring that the production of IL-17 and IL-21 does not occur in response to self-antigens.

INTRODUCTION

Recent studies have uncovered the existence of a novel TH effector subset, the TH17 lineage, whose deregulation has been implicated in the pathogenesis of autoimmunity (Bettelli et al., 2007b; Weaver et al., 2006). In particular, TH17 cells are believed to play a key role in rheumatoid arthritis (RA) (McInnes and Schett, 2007; Toh and Miossec, 2007), a disease characterized by destructive inflammatory lesions affecting the synovial membranes of joints and by aberrant humoral responses that result in the production of autoantibodies like Rheumatoid Factor and anti-cyclic citrullinated peptide (CCP) antibodies. The ability of the TH17 subset to produce IL-17 is critical to their role in RA pathogenesis, since IL-17 can induce the production of proinflammatory cytokines such as TNF-α and IL-1 as well as

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stimulate MMP activity, matrix catabolism, and bone resorption (Koenders et al., 2006; Stamp et al., 2004). TH17 cells have also recently been shown to produce IL-21 (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007), a cytokine that can amplify the differentiation of TH17 cells in an autocrine manner as well as control T-dependent humoral responses (Leonard and Spolski, 2005; Mehta et al., 2004). TH17 cells develop via a pathway distinct from TH1 and TH2 cells. Induction of IL-17 production depends on the presence of Stat3 and RORγt (Ivanov et al., 2006; Laurence et al., 2007; Yang et al., 2007), while IL-21 expression requires the presence of Stat3 but not of RORγt (Nurieva et al., 2007). Given the potentially deleterious effects of the cytokines produced by TH17 cells, their production needs to be strictly controlled so that acquisition of these effector functions occurs only in response to the appropriate antigenic stimuli. The regulatory pathways that prevent the inappropriate production of IL-17 and IL-21 have, however, not been fully elucidated.

Interferon Regulatory Factor 4 (IRF-4) is a member of the IRF family of transcription factors whose absence leads to profound defects in the function and homeostasis of mature T and B cells (Mittrucker et al., 1997). Expression of IRF-4 is upregulated in response to T cell activation and we, as well as others, have shown that IRF-4 can regulate IL-4 production and TH2 differentiation (Hu et al., 2002; Rengarajan et al., 2002a). Interestingly, recent studies have demonstrated that IRF-4 is also a crucial regulator of TH17 differentiation (Brustle et al., 2007). During a yeast two-hybrid screen aimed at identifying proteins interacting with IRF-4, our laboratory isolated a human cDNA encoding a novel protein that we termed IBP (IRF-4 Binding Protein) (Gupta et al., 2003b). IBP shares significant homology with SWAP-70, a novel type of Rac activator. In contrast to SWAP-70 that is expressed mostly in B cells and mast cells, IBP is highly expressed in T cells. In unstimulated T cells, IBP is present in a “dormant“ conformation due to an inhibitory interaction between its N- and C-termini. TCR engagement leads to the tyrosine phosphorylation of the N-terminus of IBP, disrupting the autoinhibitory interaction and enabling IBP to be recruited to the immunological synapse (IS) where IBP activates Rac and Cdc42 (Gupta et al., 2003a). Our previous studies in mice deficient for IBP ($IBP^{trap/trap}$ mice) have revealed that lack of IBP, with age, leads to the development of a lupus-like syndrome, which is characterized by the accumulation of $CD44^{hi}CD62L^{lo}$ T cells and IgG^{+} B cells, profound hypergammaglobulinemia, autoantibody production, proteinuria, and glomerulonephritis (Fanzo et al., 2006). Consistent with the ability of IBP to act as an activator of Rho GTPases, IBP deficient T cells exhibited defects in ERK1/2 activation and in cytoskeletal reorganization including impaired assembly of the IS and actin polymerization.

We have recently generated TCR transgenic (DO11.10) IBP deficient mice. Surprisingly, these mice rapidly develop, with a high degree of penetrance, an autoimmune condition marked by the presence of rheumatoid arthritis-like joint disease and large-vessel vasculitis. In contrast to the SKG mouse, in which spontaneous development of arthritis is linked to the emergence of autoreactive T cell clones secondary to impairments in negative selection (Sakaguchi et al., 2003), thymocyte negative selection is not affected by the lack of IBP. The pathology observed in the absence of IBP, is instead, associated with an accumulation of TCR transgene positive T cells, which exhibit an altered pattern of responsiveness to pMHC complexes and aberrantly express both IL-17 and IL-21. Importantly, our studies demonstrate that the aberrant cytokine production observed in the absence of IBP is due to its ability to regulate IRF-4. These findings thus suggest that absence of IBP leads to autoimmunity due to the ability of IBP to control both the TCR signaling threshold as well as the acquisition of pathogenic TH effector functions.

RESULTS

Spontaneous development of rheumatoid arthritis-like joint disease and large-vessel vasculitis in IBP$^{trap/trap}$ DO11.10 mice

To systematically analyze the effects of IBP deficiency on TCR-mediated signaling pathways, IBP$^{trap/trap}$ mice were backcrossed onto a BALB/c background and then crossed to DO11.10 mice, which carry a I-A^{d}-restricted transgenic T cell receptor that recognizes a specific peptide (OVA$_{323-339}$) derived from ovalbumin (Murphy et al., 1990; Robertson JM, 2000). Surprisingly, beginning at about 7 weeks of age, IBP$^{trap/trap}$ DO11.10 mice but none of the IBP$^{+/+}$ DO11.10 mice, started spontaneously developing joint erythema and swelling, which affected the wrist joints and fingers and, less frequently, the ankle joints and toes in a symmetrical manner (Fig. 1A). No obvious swelling of the knees, elbows, shoulders, or vertebral joints was observed. Approximately 90% of IBP$^{trap/trap}$ DO11.10 mice developed a chronic progressive arthritis, which tended to be more severe in females and occasionally resulted in joint deformities and impaired mobility (Supplementary Fig. 1). Histopathologic analysis revealed synovitis with pannus formation, composed predominantly of fibroblasts, monocytes, lymphocytes, and a variable number of plasma cells and neutrophils (Fig. 1B and data not shown). The infiltrate invaded the adjacent joint structure with destruction of cartilage and increased osteoclastic activity leading to resorption of the subchondral bone and bone erosions. Serologic analysis demonstrated that IBP$^{trap/trap}$ DO11.10 mice exhibited elevated titers of Rheumatoid Factor (RF), anti-Collagen II antibodies, and anti-cyclic citrullinated peptide antibodies (anti-CCP) in their serum but only a small increase in anti-dsDNA antibodies (Fig. 1C and Supplementary Fig. 1). IBP$^{trap/trap}$ DO11.10 mice thus develop an inflammatory arthropathy that shares many clinical, histological, and serological features with human rheumatoid arthritis.

Beginning at ~3 months of age, IBP$^{trap/trap}$ DO11.10 mice also started dying suddenly. Over 90% of IBP$^{trap/trap}$ DO11.10 mice had died by 12 months of age with female IBP$^{trap/trap}$ DO11.10 mice dying more rapidly than male IBP$^{trap/trap}$ DO11.10 mice (Supplementary Fig. 1). Consistent with the idea that the sudden death of the IBP$^{trap/trap}$ DO11.10 mice might be due to abnormalities of their cardiovascular system, histologic analysis revealed that all of the arthritic IBP$^{trap/trap}$ DO11.10 mice also had developed severe inflammation of the root of the aorta with massive transmural infiltration of lymphocytes, monocytes, including occasional multinucleated giant cells, variable numbers of plasma cells and neutrophils, and reduplication or destruction of the internal elastic lamina (Fig. 1D and Supplementary Fig. 1). Occasionally, inflammation was also observed in the medium-sized arteries of the kidney and of the lung, but not veins or capillaries. No significant inflammation of other organs was noted, including the small and large intestine, pancreas, and liver.

IBP deficiency leads to abnormal TCR responsiveness and the spontaneous activation of TCR transgenic T cells

To explore the pathways underlying the pathology associated with IBP deficiency, we first investigated whether lack of IBP leads to abnormalities in central tolerance. Unlike what has been described for previous murine models, the emergence of spontaneous arthritis and vasculitis in the IBP$^{trap/trap}$ DO11.10 mice was not due to a failure of eliminating autoreactive T cells within the thymus, as assessed by injecting IBP$^{+/+}$ and IBP$^{trap/trap}$ mice with high doses of anti-CD3 Ab (Supplementary Fig. 2) or by crossing IBP$^{trap/trap}$ mice with HY transgenic mice, a well-characterized model of negative selection (Supplementary Fig. 2). Furthermore, no defects in the development or function of regulatory T cells could be observed in the absence of IBP (Supplementary Figs. 3 and 4).

These findings raised the possibility that the immunopathology observed in the $IBP^{trap/trap}$ DO11.10 mice might be mediated by mature $CD4^+$ T cells secondary to an enhanced recognition of self-peptides by the $IBP^{trap/trap}$ DO11.10 TCR in the periphery. Consistent with this idea, expression of CD5, a gauge of the strength of TCR signaling upon interaction with self-ligands (Kieper et al., 2004), was higher on $IBP^{trap/trap}$ DO11.10 $CD4^+$ T cells than on $IBP^{+/+}$ DO11.10 $CD4^+$ T cells. Increased CD5 expression was observed in peripheral $CD4^+$ T cells from adult mice as well as in thymic CD4SP cells from newborn $IBP^{trap/trap}$ DO11.10 mice (Supplementary Fig. 5). To directly evaluate whether IBP deficiency leads to abnormalities in the recognition of pMHC complexes by the DO11.10 TCR, the responsiveness of purified naïve $IBP^{trap/trap}$ DO11.10 $CD4^+$ T cells to different doses of $OVA_{323\text{-}339}$ peptide was assayed *in vitro* (Fig. 2A). As expected, the proliferative responses of the $IBP^{+/+}$ DO11.10 $CD4^+$ T cells increased upon exposure to increasing doses of $OVA_{323\text{-}339}$ peptide. In contrast, $IBP^{trap/trap}$ DO11.10 $CD4^+$ T cells displayed a markedly abnormal pattern of proliferation, which was characterized by hyperresponsiveness to low-doses of $OVA_{323\text{-}339}$ peptide but hyporesponsiveness to high-doses of the same peptide. Thus, in the absence of IBP, mature $CD4^+$ T cells become hyperresponsive to low-levels of stimulation.

The abnormal responsiveness exhibited by the $IBP^{trap/trap}$ DO11.10 T cells to pMHC complexes suggested that, in the absence of IBP, TCR transgenic T cells might become spontaneously activated in response to endogenous peptides. Indeed, by 6 weeks of age (before the onset of the autoimmune phenotype), ~30% of $CD4^+$ T cells from $IBP^{trap/trap}$ DO11.10 mice exhibited an effector phenotype (Fig. 2B). Most of the $CD62L^{lo}CD44^{hi}$ T cells were $KJ1\text{-}26^{high}$ and thus expressed the TCR transgene (Supplementary Fig. 6). Furthermore, already by 6 weeks of age, and even more markedly by 15 weeks of age, $KJ1\text{-}26^{high}$ $IBP^{trap/trap}$ $CD4^+$ T cells spontaneously upregulated the expression of CD69 and ICOS (Fig. 2C). Although an increase in $KJ1\text{-}26^{low}$ T cells was also observed in the $IBP^{trap/trap}$ DO11.10 mice, these $KJ1\text{-}26^{low}$ T cells expressed a naïve phenotype (Supplementary Fig. 6).

To investigate whether transfer of the $IBP^{trap/trap}$ DO11.10 T cells could mediate pathogenic effector functions, total splenocytes from the arthritic $IBP^{trap/trap}$ DO11.10 mice were transferred into syngeneic SCID mice. These transfers were accompanied by the sudden demise of 6 out of 14 recipient mice by 3 months while no deaths were recorded in mice receiving $IBP^{+/+}$ DO11.10 T cells (0/9 recipient mice). No premonitory signs of morbidity were evident in the recipients of $IBP^{trap/trap}$ DO11.10 lymphocytes that suddenly died leading us to suspect that these mice succumbed to the profound cardiovascular abnormalities associated with the absence of IBP. Transfer of serum from arthritic $IBP^{trap/trap}$ DO11.10 mice instead did not lead to any pathology (data not shown). Consistent with the finding that the abnormal acquisition of effector markers and costimulatory molecules mapped to the $KJ1\text{-}26^{high}$ rather than to the $KJ1\text{-}26^{low}$ T cell compartment, transfer of purified $KJ1\text{-}26^{high}$ $IBP^{trap/trap}$ DO11.10 T cells but not of purified $KJ1\text{-}26^{low}$ $IBP^{trap/trap}$ DO11.10 T cells into nude Balb/c mice also led to the sudden death of the recipient mice (Table I).

IBP deficiency leads to the aberrant production of IL-17 and IL-21

The distinctive pathophysiology observed in the absence of IBP indicated that IBP, besides regulating TCR responsiveness, could also control T cell effector function. In particular, the presence of both inflammatory lesions and aberrant humoral responses in the $IBP^{trap/trap}$ DO11.10 mice raised the possibility that IBP could regulate the production of IL-17 and IL-21. To investigate this hypothesis, cytokine production was assessed after obtaining naïve $CD4^+$ T cells from 5-week old $IBP^{+/+}$ DO11.10 or $IBP^{trap/trap}$ DO11.10 mice by negative depletion (Lin et al., 2004) and culturing them with APCs pulsed with $OVA_{323\text{-}339}$ peptide. When compared to wt T cells, $IBP^{trap/trap}$ DO11.10 T cells exhibited an increased ability to produce IL-17 (Fig. 3A). Furthermore, in contrast to $IBP^{+/+}$ DO11.10 T cells, $IBP^{trap/trap}$ DO11.10 T

cells also synthesized significant amounts of IL-21 (Fig. 3B). Deregulated production of IL-17 and IL-21 was not due to global upregulation in cytokine production since $IBP^{trap/trap}$ DO11.10 $CD4^+$ T cells produced less IL-2 than $IBP^{+/+}$ DO11.10 $CD4^+$ T cells (Fig. 3C) and variable levels of IL-4 and IFN-γ (Supplementary Fig. 7). A similar increase in IL-17 and IL-21 production was also detected with FACS sorted $CD44^{lo}CD62L^{hi}CD25^{-}CD4^+$ $IBP^{trap/trap}$ DO11.10 cells (Supplementary Fig. 8). The deregulated production of IL-17 and IL-21 observed upon stimulation of $IBP^{trap/trap}$ DO11.10 $CD4^+$ T cells under neutral conditions was accompanied by increased expression of RORγt but not by elevation of IL-22 (Fig. 3D).

To assess whether the aberrant synthesis of IL-17 and IL-21 by $IBP^{trap/trap}$ DO11.10 cells detected *in vitro* led to deregulated production of IL-17 and IL-21 *in vivo* as well, we evaluated the levels of IL-17 and IL-21 in the sera of arthritic $IBP^{trap/trap}$ DO11.10 mice (Fig. 3E). As compared to $IBP^{+/+}$ DO11.10, $IBP^{trap/trap}$ DO11.10 mice exhibited increased serum levels of both IL-17 and IL-21. Increased expression of IL-17 and IL-21 was also observed in the joints of the arthritic $IBP^{trap/trap}$ DO11.10 mice (Fig. 3F). Furthermore, consistent with the fact that aberrant production of IL-21 by IBP deficient $CD4^+$ T cells might enable these T cells to inappropriately drive the terminal differentiation of B cells, immunohistochemical staining of spleen sections revealed that, in the absence of IBP, numerous plasma cells could be observed within the T cell zones or perivascular lymphoid sheaths (Fig. 3G). Taken together, these data thus indicate that lack of IBP leads to the inappropriate synthesis of IL-17 and IL-21, whose production should be tightly controlled and occur only upon exposure to selected pathogens.

IRF-4 controls both IL-17 and IL-21 production

The deregulated production of IL-17 and IL-21 by $IBP^{trap/trap}$ DO11.10 $CD4^+$ T cells could be detected even under conditions where these cells displayed proliferative responses identical to those exhibited by the $IBP^{+/+}$ DO11.10 $CD4^+$ T cells (i.e. with 1μM $OVA_{323-339}$ peptide) suggesting that the abnormal cytokine production was not simply the result of the aberrant TCR responsiveness displayed by these cells. Given that we had originally cloned IBP as a protein interacting with the transcription factor IRF-4 (Gupta et al., 2003b), we hypothesized that these effects might be linked to the ability of IBP to modulate IRF-4 function. To start exploring this possibility we first investigated whether IRF-4 could regulate not only the synthesis of IL-17 but also that of IL-21. For these studies we took advantage of previously generated IRF-4 deficient mice (Mittrucker et al., 1997). Naïve $CD4^+$ T cells were purified from wt and $IRF\text{-}4^{-/-}$ mice and cultured *in vitro* under TH0 and TH17 conditions. Consistent with recent observations (Brustle et al., 2007), wt $CD4^+$ T cells produced substantial amounts of IL-17 when cultured under TH17 conditions, but $CD4^+$ T cells derived from IRF-4 deficient mice completely lost their ability to synthesize IL-17 (Fig. 4A). These results were further confirmed by performing intracellular FACS (Fig. 4B). In contrast to wt T cells, no IL-17 producing cells could be detected upon stimulation in the absence of IRF-4. The lack of IRF-4 instead resulted in an enhanced ability to produce IFN-γ under TH17 conditions. Importantly, when the production of IL-21 was investigated, no IL-21 synthesis could be observed when IRF-4 deficient T cells were cultured under TH17 conditions and then restimulated (Fig. 4C). Since the absence of IL-21 production in this setting could be secondary to the defective synthesis of IL-17, and since IL-21 can also be produced by TH2 cells (Leonard and Spolski, 2005; Mehta et al., 2004), we also investigated the effects of IRF-4 deficiency on IL-21 synthesis when $CD4^+$ T cells were differentiated under TH2 conditions. As shown in Fig. 4D, production of IL-21 under these culture conditions was also abrogated by the absence of IRF-4.

A survey of the IL-21 promoter revealed that it contains potential IRF-4 binding elements, we thus proceeded to directly test whether IRF-4 could function as a transactivator of the IL-21 promoter. To this end, we performed transient transfection assays in Jurkat cells that either lacked or contained IRF-4, which we had utilized in previous studies (Hu et al., 2002).

Consistent with studies demonstrating that NFAT proteins participate in the regulation of the IL-21 promoter (Kim et al., 2005; Mehta et al., 2005), inducibility of a luciferase reporter construct driven by the IL-21 promoter could be observed in Jurkat cells lacking IRF-4 upon stimulation with PMA and ionomycin (Fig. 4E). Importantly however, under these stimulatory conditions, Jurkat cells expressing IRF-4 demonstrated a greater level of luciferase activity when transfected with the IL-21 promoter construct (IL-21 LUC) but not with an IL-21 promoter construct containing mutations within the IRF-4 binding site (IL-21 MUT LUC). These findings thus indicate that IRF-4 can act as a transactivator of the IL-21 promoter and suggest that NFAT proteins and IRF-4 cooperate in the transcriptional regulation of this gene.

Absence of IBP leads to enhanced targeting of the IL-17 and IL-21 promoters by IRF-4

We had previously reported that IBP can be found in the cytoplasm and can be rapidly recruited to the immunological synapse (Gupta et al., 2003a). IBP, however, also contains putative NLS motifs (Gupta et al., 2003b) raising the possibility that it could also translocate to the nucleus. Subcellular fractionation experiments indeed demonstrated that IBP could be detected not only in the cytoplasm but also within the nuclear compartment of primary $CD4^+$ T cells (Fig. 5A). Coimmunoprecipitation experiments confirmed that nuclear IBP and IRF-4 can physically interact (Fig. 5B).

Since we had observed that, in addition to the IL-21 promoter, the IL-17 promoter also contains a potential binding site for IRF-4, ChIP assays with an anti-IRF-4 Ab were conducted next to examine whether the absence of IBP could lead to deregulated recruitment of IRF-4 containing complexes to the IL-17 and IL-21 promoters *in vivo*. As shown in Fig. 5C, when T cells were cultured under unskewed conditions, the lack of IBP resulted in markedly enhanced targeting of these regulatory regions by IRF-4 containing complexes. To further confirm these findings, we utilized an oligonucleotide corresponding to the IRF-4 binding element within the IL-21 promoter to perform oligonucleotide precipitation assays (ONPs) on extracts obtained from wt or $IBP^{trap/trap}$ $CD4^+$ T cells that had either been left unstimulated or stimulated under neutral conditions (Fig. 5D). Consistent with the ChIP findings, the levels of IRF-4 that were precipitated from stimulated $IBP^{trap/trap}$ $CD4^+$ T cells were much greater than those precipitated from stimulated wt $CD4^+$ T cells despite similar input levels of IRF-4 (Figure 5D, upper panel). Interestingly, reblotting with an IBP antibody did not demonstrate any binding of IBP to this oligonucleotide in wt T cells (Figure 5D, lower panel). Taken all together these data thus support the notion that nuclear IBP can regulate the ability of IRF-4 to access the transcriptional regulatory regions of the IL-17 and IL-21 genes.

IBP prevents the ability of IRF-4 to bind and transactivate the IL-21 promoter

To explore the mechanisms by which IBP controls IRF-4 we first investigated whether IBP could regulate the translocation of IRF-4. Subcellular fractionation experiments (Fig. 6A) failed to demonstrate any effect of the absence of IBP on the cellular localization of IRF-4 suggesting that the differential ability of IRF-4 to target the IL-17 and IL-21 promoters was not simply the result of changes in its subcellular localization.

We next examined the possibility that nuclear IBP may be able to sequester IRF-4 by determining whether IBP could prevent IRF-4 from targeting its DNA binding site within the IL-21 promoter. To facilitate these experiments we first conducted a mutational analysis to better define the region of IBP involved in the interaction with IRF-4 (Fig. 6B). This analysis revealed that a mutant of IBP (IBP 1-385) lacking a large portion of the carboxy-terminus of IBP (between aa 386 and 631) lost its ability to interact with IRF-4. Consistent with the finding that the N-terminal domain can exert an autoinhibitory effect, a mutant lacking this region (IBPΔN) instead exhibited an enhanced interaction with IRF-4. Oligonucleotide precipitation (ONP) experiments with an oligonucleotide containing the IRF-4 binding element within the

IL-21 promoter were next performed on extracts of 293T cells transfected with IRF-4 in the presence or absence of FL IBP, IBPΔN, or IBP 1-385 (Figure 6C). IRF-4 was robustly precipitated from 293T cells transfected with IRF-4 alone but not from mock transfected 293T cells. The presence of either FL IBP or of IBPΔN markedly diminished the amount of precipitated IRF-4. In contrast, strong binding of IRF-4 to this site could still be detected in 293T cells cotransfected with IBP 1-385. Input levels of IRF-4 in the different samples were similar. IBP can thus directly inhibit the ability of IRF-4 to bind to the IL-21 promoter.

The effects of IBP on the transactivating activity of IRF-4 were also examined. Jurkat cells that either lack or contain IRF-4 were transiently transfected with the IL-21 luciferase reporter construct in the presence or absence of FL IBP, IBPΔN, or IBP 1-385 (Figure 6D). Cells were then either left unstimulated or stimulated with PMA and ionomycin. Similar to the results in Figure 4, presence of IRF-4 augmented the inducibility of this construct upon PMA and ionomycin stimulation. Consistent with our previous findings that, in T cells, FL IBP needs to be posttransationally modified in order to become activated, coexpression of FL IBP was unable to affect the transactivating ability of IRF-4, while cotranfection of a mutant lacking the autoinhibitory domain (IBPΔN) blocked the IRF-4-mediated transactivation. Importantly, the IBP 1-385 mutant that loses its ability to physically interact with IRF-4 was unable to significantly affect the transactivating ability of IRF-4 confirming the functional relevance of the IBP-IRF-4 interaction. To ensure that the inability of the IBP 1-385 mutant to interfere with the transactivating activity of IRF-4 was not due to the presence of the N-terminal autoinhibitory domain, an IBP mutant lacking both the autoinhibitory domain and the IRF-4 interacting domain was also generated (ΔN-385). Cotransfection of (ΔN-385) with IRF-4 also failed to block the IRF-4-mediated transactivation of the IL-21 luciferase reporter construct (Supplementary Fig. 9), confirming that the ability of IBP to interfere with the ability of IRF-4 to drive the expression of IL-21 is dependent on its physical interaction with IRF-4.

To finally ascertain whether the ability of IBP to regulate IRF-4 was indeed responsible for the aberrant production of IL-17 and IL-21 observed in $IBP^{trap/trap}$ mice, we generated mice deficient in both IBP and IRF-4. As expected from previous studies demonstrating that neither a deficiency of IRF-4 nor a lack of IBP leads to obvious developmental defects (Fanzo et al., 2006; Mittrucker et al., 1997), $IBP^{trap/trap}$IRF-$4^{-/-}$ mice were viable, fertile and did not exhibit any significant defects in the maturation of the immune system (Supplementary Table I). To assess whether lack of IRF-4 could alter the deregulated production of IL-17 observed in the absence of IBP, naïve $CD4^+$ T cells were cultured under unskewed conditions and IL-17 production in the supernatants was assessed by ELISA. The lack of IRF-4 completely abolished the increased ability of $IBP^{trap/trap}$ T cells to synthesize IL-17 (Fig. 7A). In line with prior observations that IRF-4 controls the expression of RORγt, the aberrant RORγt expression detected in the absence of IBP was also dependent on the presence of IRF-4 (Fig. 7B). Consistent with a critical role for IRF-4 not only in the control of IL-17 but also in the regulation of IL-21, absence of IRF-4 also prevented the enhanced ability of $IBP^{trap/trap}$ T cells to produce IL-21 when the T cells were cultured under either unskewed conditions (Supplementary Figure 10) or TH2 conditions (Fig. 7C). These defects were not due to abnormalities in proliferation, since $CD4^+$ T cells from mice of all four genotypes exhibited similar CFSE profiles (Fig. 7D). Furthermore, differences in IL-17 production under these conditions did not simply correlate with differential ability of the four T cell populations to produce TGF-β or IL-6 (Supplementary Fig. 10). Interestingly, in contrast to the findings with IL-17 and IL-21, neither the absence of IBP nor the lack of IRF-4 affected the induction of ICOS upon T cell stimulation *in vitro* (Supplementary Fig. 10). These results thus indicate that the enhanced ability of IBP deficient T cells to aberrantly produce IL-17 and IL-21 is critically dependent on the presence of IRF-4.

DISCUSSION

Although T cells play a key role in the pathogenesis of many autoimmune diseases such as RA and SLE, the molecular machinery preventing the emergence of autoreactive T cells has not been fully elucidated. Our studies suggest that deregulation of IBP-controlled pathways represents an important pathogenic mechanism leading to the spontaneous development of autoimmunity. The ability of IBP to play a critical role in this process is likely due to its capacity to control the responsiveness of T cells to pMHC complexes and to ensure that the production of IL-17 and IL-21 does not occur in the absence of the proinflammatory conditions known to drive TH17 differentiation.

One of the crucial aspects underlying the spontaneous development of autoimmunity in IBP deficient mice is the inability of $IBP^{trap/trap}$ T cells to accurately sense the strength of TCR engagement. As shown in Fig. 2A, $IBP^{trap/trap}$ DO11.10 T cells exhibit a highly abnormal pattern of responsiveness. Consistent with our previous findings that polyclonal IBP deficient T cells exposed to strong stimulatory conditions proliferate to a lesser extent than wt T cells (Fanzo et al., 2006), $IBP^{trap/trap}$ DO11.10 T cells exhibit defective proliferative responses upon exposure to high doses of $OVA_{323-339}$ peptide. $IBP^{trap/trap}$ DO11.10 T cells, however, exhibit enhanced proliferative responses to low-levels of stimulation, an effect that we have also observed with polyclonal IBP deficient T cells (unpublished observations). The hyperresponsiveness of the $IBP^{trap/trap}$ DO11.10 $CD4^+$ T cells to low-levels of stimulation was further corroborated by their spontaneous activation *in vivo* in the absence of any exposure to ovalbumin and by their enhanced expression of CD5. Increased CD5 expression was also noted on CD4SP cells from newborn $IBP^{trap/trap}$ DO11.10 mice suggesting that this phenotype was the consequence of intrinsic changes in the recognition of the DO11.10 TCR for its ligands rather than to exposure of these T cells to a pathogenic environment. Interestingly, preliminary studies from $IBP^{trap/trap}$ $RAG^{-/-}$ DO11.10 mice that we have recently generated reveal that the TCR transgenic T cells from these mice still retain the observed abnormalities indicating that the arthritis and vasculitis occurring in $IBP^{trap/trap}$ DO11.10 TCR transgenic mice are likely due to the inappropriate recognition of cross-reactive epitopes by the DO11.10 TCR rather than to the expression of a second TCR by these cells.

Importantly, our studies indicate that IBP, in addition to regulating the responsiveness of T cells to TCR engagement, controls the capacity of T cells to acquire a potentially pathogenic effector program. Indeed, one of the crucial abnormalities observed in the absence of IBP was increased production of IL-17, a cytokine with known proinflammatory effects relevant to the pathophysiology of rheumatoid arthritis in humans (Koenders et al., 2006; Stamp et al., 2004). We furthermore have demonstrated that the deregulated IL-17 production observed in the absence of IBP is critically dependent on IRF-4, a transcription factor recently shown to be necessary for TH17 differentiation (Brustle et al., 2007). Interestingly, IRF-4 is an important cellular target of the HTLV-I Tax oncoprotein (Mamane et al., 2002) and it is intriguing to speculate that deregulation of IRF-4 may also play a role in the autoimmune arthritis associated with the overexpression of Tax in mice (Habu et al., 1999). Consistent with previous studies showing that IRF-4 can regulate the expression of ROR-γt (Brustle et al., 2007), the enhanced production of IL-17 observed in the absence of IBP was accompanied by increased expression of ROR-γt, an effect that was dependent on the presence of IRF-4. It is, however, important to note that reconstitution of RORγt expression in IRF-4 deficient T cells only partially restores the deficient IL-17 production exhibited by these cells (Brustle et al., 2007). Thus our finding that IRF-4 containing complexes can also target the regulatory regions of the IL-17 gene indicates that the role of IRF-4 in TH17 differentiation is multifaceted and includes both direct effects on the transcription of IL-17 gene as well as indirect effects on the expression of other IL-17 transactivators.

The acquisition of a TH17 phenotype normally requires progression through a series of developmental stages during which TH17 cells are sequentially exposed to key cytokines (Bettelli et al., 2007a; Ivanov et al., 2007). Presence of IL-6 is crucial for the initiation of this process and leads to the induction of IL-21 production, which, in turn, acts in an autocrine manner and further amplifies commitment of TH cells toward the TH17 lineage. Our studies demonstrate that IBP deficient T cells can produce IL-21 even during the initial stimulation by antigen and in the absence of any TH17 skewing conditions. This abnormality may have profound pathophysiologic consequences since aberrant synthesis of IL-21 may not only reinforce the abnormal IL-17 production exhibited by these cells, but may also enable these $CD4^+$ T cells to inappropriately promote the terminal differentiation of B cells at extrafollicular sites and, thus, provide help to autoreactive B cells (William et al., 2002). Consistent with this notion, the absence of IBP leads to the aberrant localization of Blimp1 positive plasma cells within peripheral lymphoid organs and the presence of autoantibodies. By controlling IL-21 production, IBP may thus play a critical role in ensuring that T cell help to B cells is provided only to appropriately selected B cell populations (Vinuesa et al., 2005). Unlike what we observed with IL-17 and IL-21, IBP deficiency did not affect the production of IL-22 in agreement with studies demonstrating that the regulation of IL-22 differs from that of IL-17 and that IL-22 may primarily function as a downstream target of IL-23 (Ouyang et al., 2008).

Similarly to IL-17, the ability of IBP to control IL-21 production is dependent on its interaction with IRF-4. At a mechanistic level, IBP does not affect either the expression or the subcellular localization of IRF-4. Consistent with the finding that SWAP-70 contains three nuclear localization signals and can be detected in the nucleus of activated B cells (Masat et al., 2000), IBP could be detected in the nuclear compartment of T cells where it “sequestered” IRF-4, preventing it from binding and transactivating the IL-21 promoter. The interaction of IBP and IRF-4 requires its carboxy-terminus, which is also important for the ability of IBP to activate Rho GTPases (Gupta et al., 2003a). Experiments are now in progress to determine whether the two functions of IBP map to distinct subdomains within its carboxy-terminal region. Unlike other master regulators of TH differentiation like GATA3 and RORγt, the expression of IRF-4 is upregulated by TCR stimulation and is not restricted to a specific TH effector lineage (Matsuyama et al., 1995; Rengarajan et al., 2002b). The presence of mechanisms that restrain the access of IRF-4 to selected regulatory regions may thus be particularly important to ensure that its activity can be controlled in a TH lineage-specific manner.

In contrast to the $IBP^{trap/trap}$ DO11.10 mice, young $IBP^{trap/trap}$ Balb/c mice do not develop obvious signs of arthritis, although evidence of a systemic autoimmune disease can be observed in these mice upon aging (unpublished observations). Interestingly, however, T cells derived from $IBP^{trap/trap}$ Balb/c mice do exhibit abnormal production of both IL-21 and IL-17 upon *in vitro* stimulation (unpublished observations). These findings suggest that the pathogenic effector function observed in the absence of IBP becomes unmasked in the setting of a restricted TCR repertoire (i.e. in the DO11.10 TCR transgenic background) and/or of declining T cell numbers (i.e. upon aging). Given that the absence of IBP leads to an exaggerated ability to undergo homeostatic proliferation (unpublished observations), the pathogenicity of the IBP deficient T cells may become evident only after these T cells receive a signal to expand in order to replenish or maintain the mature T cell pool. It is also likely that interactions with other cellular constituents are required for IBP deficient T cells to fully acquire their pathogenic potential. Indeed, we have found that IBP deficient T cells exhibit markedly elevated ICOS expression *in vivo* but not *in vitro* suggesting that the deregulated ICOS expression observed in these mice is under complex regulation. We thus envision a scenario whereby the dysregulated production of IL-17 and IL-21 by IBP deficient T cells, via the effects of these cytokines on other cellular compartments, leads to additional aberrations within T cells eventually resulting in the development of autoimmunity.

Absence of IBP can thus result in the development of either a lupus-like syndrome or rheumatoid arthritis-like joint disease. The finding that IBP deficiency can lead to these distinct autoimmune manifestations is consistent with the fact that familial aggregation of RA and SLE has been reported (Alarcon-Segovia et al., 2005; Criswell et al., 2005). Deregulation of IL-17 and IL-21 production furthermore has been detected in murine models of RA and SLE as well as in patients affected by these two disorders (Afzali et al., 2007; Herber et al., 2007; McInnes and Schett, 2007; Sawalha et al., 2007). Blockade of the IL-21/IL-21R pathway has recently been reported to be efficacious in ameliorating disease in murine models of both lupus and RA (Herber et al., 2007; Young et al., 2007). The capacity of IBP to control both TCR responsiveness and the production of IL-17 and IL-21 thus suggest that deregulation of IBP-mediated pathways could function as a common pathogenic mechanism involved in the development of these two distinct autoimmune disorders.

MATERIALS AND METHODS

Mice

IBP deficient mice were generated by Lexicon Pharmaceuticals (Omnibank) utilizing a gene trapping strategy, and hence have been termed $IBP^{trap/trap}$ mice (Fanzo et al., 2006). The original mice (on a mixed 129XC57BL/6 background) were backcrossed into either Balb/c mice or into C57BL/6 mice for >6 generations. $IBP^{trap/trap}$ mice on a Balb/c background were then crossed to DO11.10 TCR transgenic mice (Jackson Laboratory) to generate $IBP^{trap/trap}$DO11.10 mice; C57BL/6 background were crossed to HY-TCR transgenic mice (Taconic) to generate $IBP^{trap/trap}$HY mice. IRF-$4^{-/-}$ mice on a C57BL6 background were obtained from Dr. T. Mak at Departments of Immunology and Medical Biophysics, University of Toronto and the Amgen Institute (Mittrucker et al., 1997). To generate $IBP^{trap/trap}$IRF-$4^{-/-}$ mice, IRF-$4^{-/-}$ mice were crossed with $IBP^{trap/trap}$ that had been backcrossed onto the C57BL/6 background. Syngeneic SCID, $Rag^{-/-}$, and nude mice were obtained from Jackson Laboratory. All mice used in the experiment were kept under specific pathogen-free conditions. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Columbia University.

Flow Cytometry

Single cell suspensions from thymus, spleen, and lymph nodes were isolated, stained with fluorochrome-conjugated KJ1-26 (Caltag laboratories), CD3ε, CD4, CD5, CD8, B220, CD25, CD44, CD62L, CD69, (Pharmingen), T3.70, and ICOS antibodies (eBiosciences) and analyzed by FACS. Data were analyzed using FlowJo (Treestar) software.

Clinical assessment of arthritis

Joint swelling was monitored by inspection and scored as follows: 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the wrist or ankle; 2, erythema and mild swelling extending from the wrist or ankle to the mid-paw; 3, erythema and moderate swelling extending from the wrist or ankle to the mid-paw; 4, erythema and severe swelling encompassing the wrist or ankle, paws, and digits. Score for all 4 paws were totaled for each mouse (Brand et al., 2004).

Histopathology and immunohistochemical staining

Tissue specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E) and analyzed by light microscopy. Joints were fixed in 10% phosphate-buffered formalin, decalcified in 10% EDTA-4Na, and embedded in paraffin. Immunostaining of spleen sections with antibodies

against CD3, Blimp-1, and Syndecan-1 was performed as previously described (Angelin-Duclos et al., 2000; Fanzo et al., 2006).

Serum autoantibodies

Serum levels of Rheumatoid Factor (RF) and of antibodies against Collagen II, cyclic citrullinated peptide (CCP), and double-stranded DNA (dsDNA) were determined by ELISA (Alpha Diagnostics for RF, CII, and dsDNA Abs and Axis-Shield Diagnostics for CCP Abs) as described (Kuhn et al., 2006; Sakaguchi et al., 2003). Data were analyzed using Student's t-test.

In vivo thymocyte deletion

6-week-old mice were injected with 250 μg of anti-CD3 antibody i.p. as previously described (Sakaguchi et al., 2003). 72 hours later, mice were sacrificed and the thymus collected. Total thymocytes were counted, stained for CD4 and CD8, and analyzed by FACS.

Proliferation studies and cytokine production

Naïve $CD4^+$ T cells were purified as previously described (Lin et al., 2004) or, in selected experiments, by sorting $CD44^{lo}CD62L^{hi}CD25^{-}CD4^+$ T cells. For proliferation assays, cells were cultured at 1×10^5 per well in 96-well-plate together with 4×10^5 syngeneic $IBP^{+/+}$ APCs loaded with increasing doses (0, 0.1, 1, 10 μM) of $OVA_{323\text{-}339}$ peptide for 48 hours as described (Song et al., 2005) and then pulsed with BrdU for 18 hours. Incorporated BrdU was measured by BrdU ELISA (Roche-Applied-Science). For cytokine analysis 1×10^6 cells/ml were stimulated with 4×10^6 APC loaded with $OVA_{323\text{-}339}$ peptide at 1μM concentration in 24-well-plates. TH0 and TH2 differentiation was conducted as previously described (Fanzo et al., 2006). For TH17 differentiation, naïve purified CD4 T cells were activated by plate-bound anti-CD3ε and soluble anti-CD28 in the presence of anti-IFNγ (10μg/ml, BD Biosciences), anti-IL4 (10μg/ml, BD Biosciences), IL6 (20ng/ml, Peprotech) and TGF-β (5ng/ml, Peprotech) as previously described (Ivanov et al., 2006). Supernatants were analyzed for IL-21 (R&D Systems), IL-17, IFN-γ (eBioscience), TGF-β (eBioscience) and IL-2 (eBioscience) production by ELISA.

Transient transfection assays

Transient transfection assays were performed as previously described (Hu et al., 2002). Briefly, 10×10^6 control or IRF4-expressing Jurkat cells were transfected with 5 μg of a murine IL-21 promoter luciferase reporter plasmid (Mehta et al., 2005) by electroporation at 260V and 960μF with a BTX electroporator. 100-200 ng of the pRL-TK reporter plasmid expressing renilla luciferase under the control of the thymidine kinase promoter was added to each transfection as a transfection efficiency control. After transfection, the cells were allowed to recover for 16hr at 37°C, 6% CO_2. The cells were then collected and resuspended in 3 ml of media and equally split into two 1.5 ml aliquots. The aliquots were then cultured in the presence or absence of PMA (50 ng/ml) and ionomycin (1 μM) for 4 hrs. The transfected cells were then harvested, lysed, and assayed for luciferase activities with the Dual Luciferase Assay System (Promega) according to the manufacturer's instructions. The firefly luciferase activity was normalized on the basis of renilla luciferase activity.

Cell extracts, protein assays

Nuclear and cytoplasmic extracts were prepared utilizing the NE-PER Nuclear and Cytoplasmic extraction reagent kit as previously described (So et al., 2006). The purity of the nuclear and cytoplasmic fractions was verified by probing with antibodies against Lamin B (sc-6217, Santa Cruz Biotechnology) and b-tubulin (clone D66, Sigma-Aldrich). Cell lysates were immunoprecipitated with an anti-IBP antibody or anti-IRF-4 Ab as previously described

(Gupta et al., 2003a). The immunoprecipitates were resolved by 8% SDS-PAGE, transferred to a nitrocellulose membrane, and then immunoblotted with an anti-IRF-4 antibody or the anti-IBP antiserum. The protein bands were visualized by ECL (Amersham). Chromatin immunoprecipitation (ChIP) assays were performed as previously described (Sciammas et al., 2006). Briefly, $CD4^+$ T cells were purified from wt mice and $IBP^{trap/trap}$ mice and either left unstimulated or stimulated for 24 hrs under unskewed conditions. After harvesting, the cells were cross-linked with formaldehyde and chromatin extracts were prepared by standard methods. The DNA/protein complexes were immunoprecipitated with an IRF-4 specific antibody or a control antibody. After cross-linking was reversed and proteins digested, the DNA was purified from the immunoprecipitates as well as from input extracts, and then analyzed by PCR using primers specific for the murine IL-21 promoter (5′ CCCTTGTGAATGCTGAAAACTG3′ and 5′GGCCTTGGTCTGGTTCTCACT3′), the IL-17 promoter (5′GCGGTACTCAGTTAAATAGAACGT3′ and 5′ ATTAGTGCAGGACTCACCACAGA3′) or β-actin (5′GCTCCTCCTGAGCGCAAGT3′ and 5′TCGTCATACTCCTGCTTGCTGAT3′). Oligonucleotide precipitation (ONP) assays were conducted as previously described (Azam et al., 1997). Briefly, nuclear extracts were precleared with streptavidin-agarose beads and then incubated with biotinylated double-stranded oligonucleotide corresponding to the trimerized IRF-4 binding site within the IL-21 promoter (5′CCTTGGTGAATGCTGAAAACTGGAATTCACCCAT3′). Proteins bound to the biotin-labeled DNA were collected by streptavidin-agarose beads, separated by 8% SDS-PAGE and then analyzed by Western blotting.

Real-time RT-PCR

Total RNA was isolated from cells or organs using RNeasy Mini Kit (Qiagen GmbH). cDNAs were prepared and analyzed for the expression of the gene of interest by real-time PCR using a Sybr-Green PCR master mix kit (Applied Biosystems). The primers for IL-17, IL-21, IL-22, and RORγt genes have been previously described (Chung et al., 2006; Lubberts et al., 2005; Wurster et al., 2002; Xi et al., 2006). The expression of each gene was normalized to the expression of β-Actin.

Adoptive transfers

For splenocyte transfers, cells suspensions were obtained from either arthritic $IBP^{trap/trap}$DO11.10 mice or age- and sex- matched $IBP^{+/+}$DO11.10 mice, and 2×10^7 cells were injected i.v. into syngeneic SCID mice. For T cell transfers, lymphocytes were pooled from peripheral lymph nodes and spleen, total $CD4^+$ T cells were positively selected by MACS magnetic beads sorting (Miltenyl Biotec) and then stained with the KJ1-26 Ab (Caltag laboratories). KJ1-26^{high} and KJ1-26^{low} cells were sorted by FACSDiva (BD Biosciences). The sorted cells were then washed and 2×10^6 cells were injected i.v. into syngeneic nude mice.

Regulatory T cell suppression assays

$CD4^+$ T cells (5×10^4) from DO11.10 $IBP^{+/+}$ mice were cultured in 96-well plates with T cell depleted Mitomycin C (Sigma) treated APCs (2×10^5) pulsed with $OVA_{323\text{-}339}$ (1 μM) in the presence/absence of increasing numbers of FACS sorted $CD4^+CD25^+$ regulatory T cells from $IBP^{+/+}$ DO11.10 or $IBP^{trap/trap}$ DO11.10 mice for 72 hrs as previously described (Thornton and Shevach, 2000). For proliferation assays, cultures were pulsed with [^{3}H]-thymidine (PerkinElmer, MA) for the last 12 h of culture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IRF-4
: Interferon Regulatory Factor 4

IBP
: IRF-4 Binding Protein

SLE
: Systemic Lupus Erythematosus

RA
: Rheumatoid Arthritis

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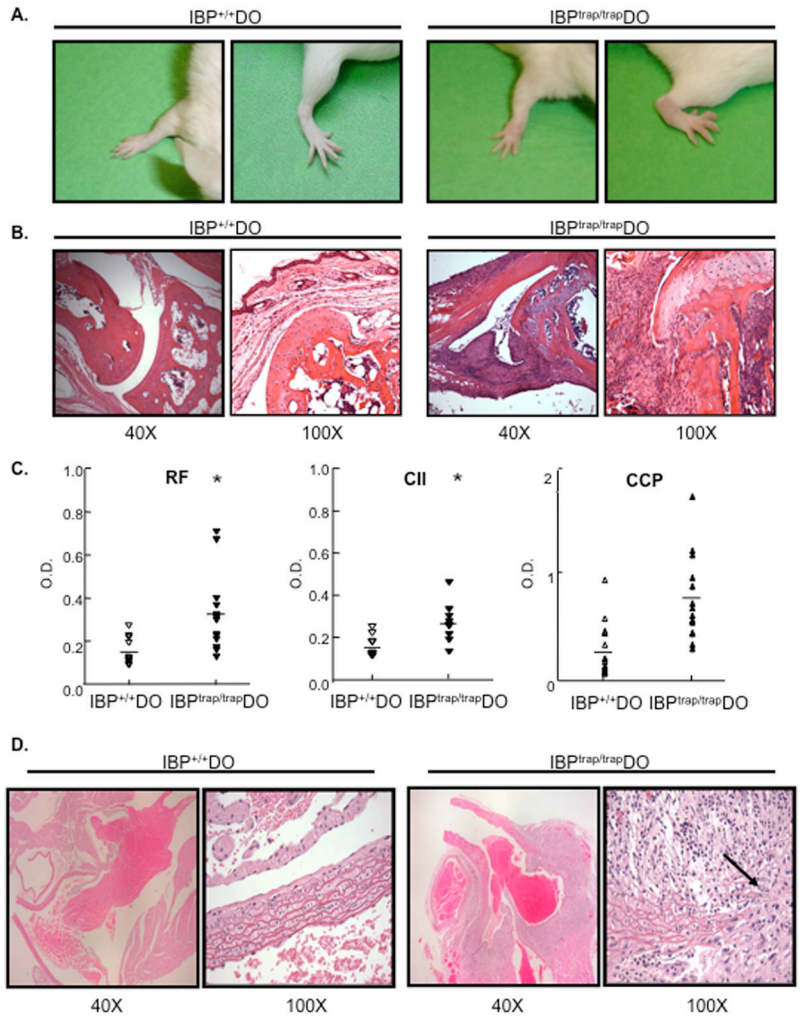


Figure 1.

Development of arthritis and large-vessel vasculitis in IBP$^{trap/trap}$ DO11.10 mice. **A.** Shown are the wrist and ankle of a normal 16 wks old IBP$^{+/+}$ DO11.10 (IBP$^{+/+}$ DO) female mouse (left) and of an affected IBP$^{trap/trap}$ DO11.10 (IBP$^{trap/trap}$ DO) female mouse (right) that had developed swelling and erythema of the joints. **B.** Histopathologic analysis (hematoxylin/eosin staining) of wrist joints of a 12-week-old female IBP$^{+/+}$ DO11.10 mouse (left) and an age- and sex- matched IBP$^{trap/trap}$ DO11.10 mouse (right). Light microscopy images (magnification of 40 and 100×, as indicated) are shown. These findings are representative of 6 mice for each group. **C.** Serological analysis. Sera from IBP$^{+/+}$ DO11.10 and IBP$^{trap/trap}$ DO11.10 mice (6–25 weeks old, male and female, n=6–12) were collected and levels of rheumatoid factor (RF), anti-collagen II (CII) antibodies, and anti-cyclic citrullinated peptide (CCP) antibodies were analyzed by ELISA. *p<0.05. **D.** Histopathologic analysis (hematoxylin/eosin staining) of the root of the aorta of a 16 wk-old IBP$^{+/+}$ DO11.10 female mouse (left panels) and an age-matched IBP$^{trap/trap}$ DO11.10 female mouse (right panels). Light microscopy images (magnification of 40× and 100×, as indicated) are shown. These findings are representative of 6 mice for each group.

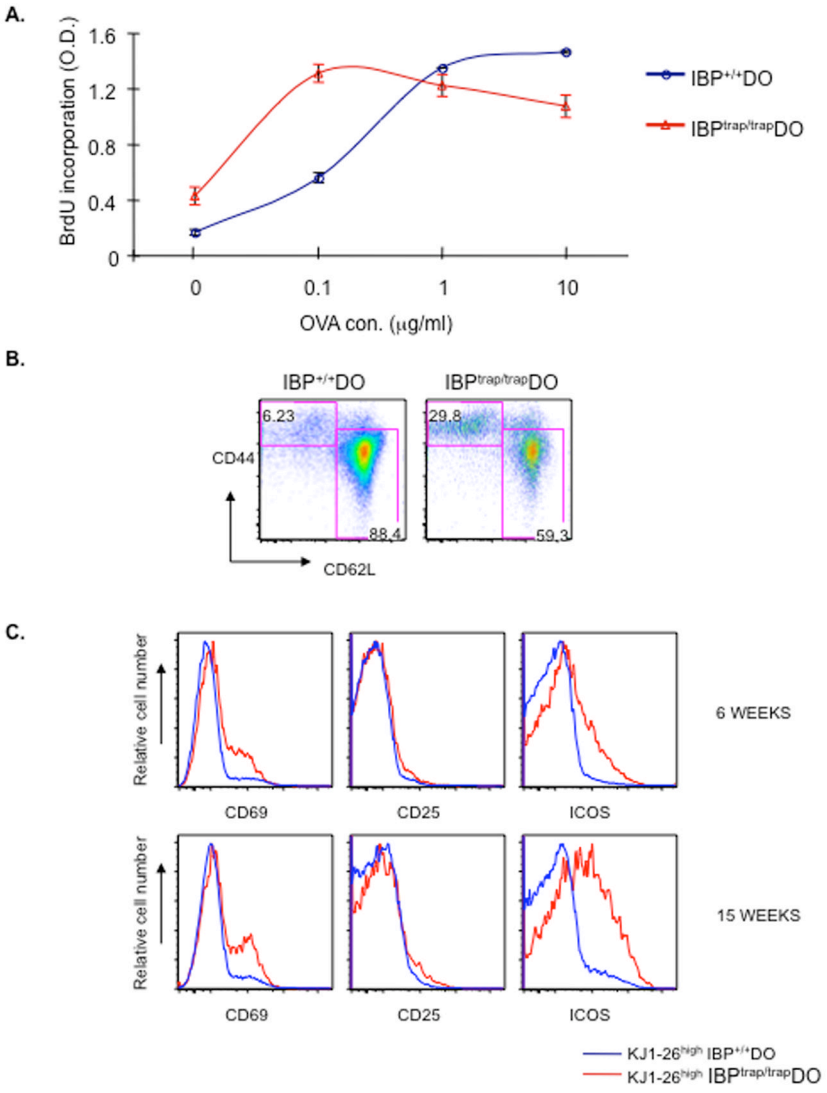
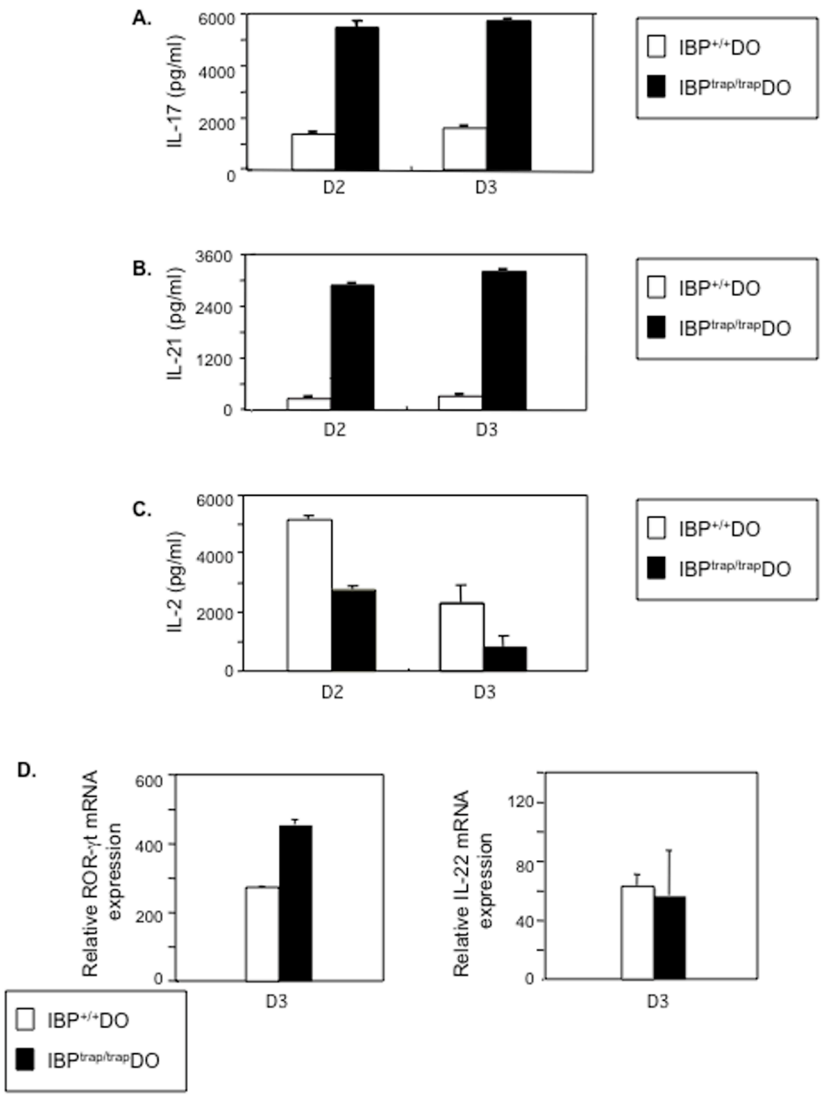


Figure 2.

Lack of IBP leads to abnormalities in the responsiveness of DO11.10 $CD4^+$ T cells. **A.** Proliferative responses to $OVA_{323-339}$ peptide. Naïve $CD4^+$ T cells ($CD44^{low}CD62L^{high}$) were isolated from both $IBP^{+/+}$ DO11.10 (blue) and $IBP^{trap/trap}$ DO11.10 (red) mice and stimulated for 3 days with $IBP^{+/+}$ APCs pulsed with increasing doses (0, 0.1, 1, 10 μM) of $OVA_{323-339}$ peptide. Cell proliferation was assayed by BrdU ELISA. Experiment shown is representative of 3 independent experiments. **B.** Spontaneous acquisition of an effector phenotype *in vivo* in the absence of IBP. FACS analysis of CD44 and CD62L expression on $CD4^+$ splenic T cells from a 6-week-old female $IBP^{+/+}$ DO11.10 (left) and a sex- and age- matched $IBP^{trap/trap}$ DO11.10 (right) mouse. **C.** Spontaneous activation of $IBP^{trap/trap}$ DO11.10 T cells *in vivo*. The surface expression level of CD69, CD25, and ICOS on KJ1-26^{high} $IBP^{+/+}$ DO11.10 (blue) and KJ1-26^{high} $IBP^{trap/trap}$ DO11.10 (red) was analyzed by FACS and overlaid histograms are shown. Data are representative of three 6 week-old mice/group.



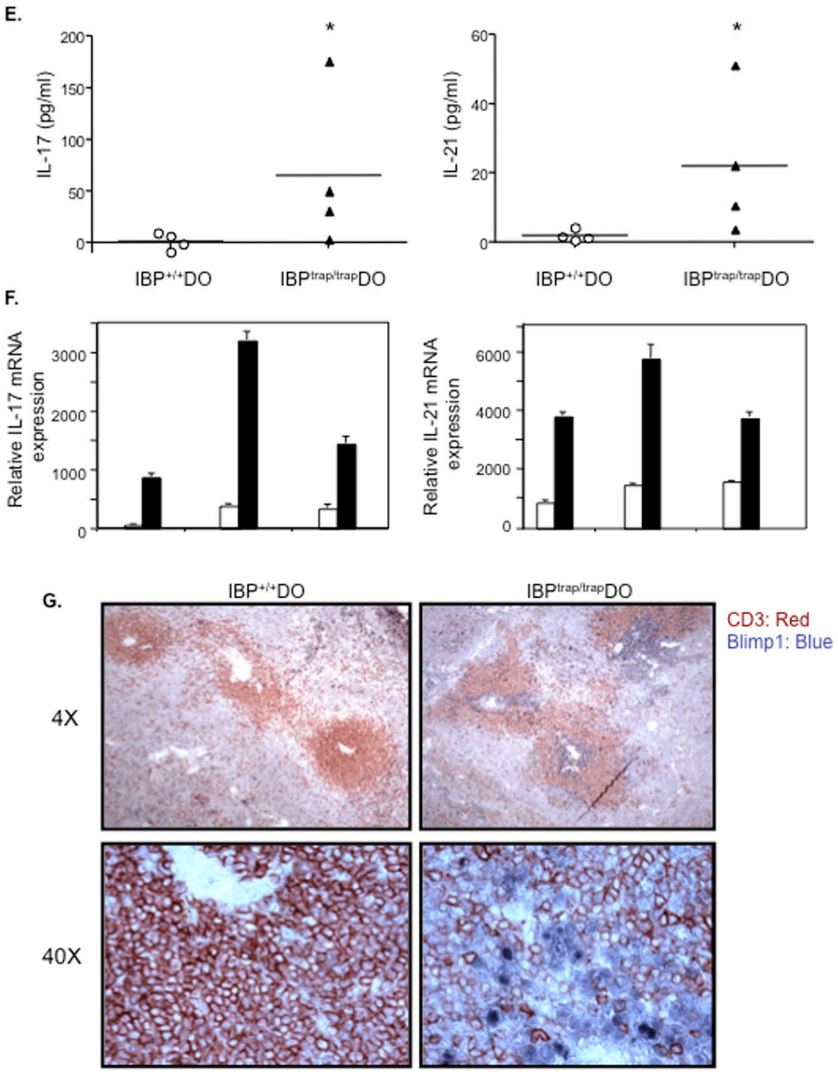


Figure 3.

Lack of IBP leads to aberrant production of IL-17 and IL-21 *in vitro* and *in vivo*. **A.** Naïve $CD4^+$ T cells derived from 6 wks. old $IBP^{+/+}$ DO11.10 (white bars) or $IBP^{trap/trap}$ DO11.10 (black bars) mice were cultured with $IBP^{+/+}$ APCs pulsed with 1 μM $OVA_{323-339}$ peptide for the times indicated. The production of IL-17 in the supernatants was measured by ELISA. Experiment shown is representative of 3 independent experiments. **B.** Naïve $CD4^+$ T cells derived from 6 wks. old $IBP^{+/+}$ DO11.10 (white bars) or $IBP^{trap/trap}$ DO11.10 (black bars) mice were cultured as above. The production of IL-21 in the supernatants was measured by ELISA. Experiment shown is representative of 3 independent experiments. **C.** Naïve $CD4^+$ T cells derived from 6 wks. old $IBP^{+/+}$ DO11.10 (white bars) or $IBP^{trap/trap}$ DO11.10 (black bars) mice were cultured as above. The production of IL-2 in the supernatants was measured by ELISA. Experiment shown is representative of 3 independent experiments. **D.** Naïve $CD4^+$ T cells derived from 6 wks. old $IBP^{+/+}$ DO11.10 (white bars) or $IBP^{trap/trap}$ DO11.10 (black bars) mice were cultured for 3 days as described above and the mRNA expression of RORγt (left panel) and IL-22 (right panel) genes was measured by real-time RT-PCR. Experiment shown is representative of 3 independent experiments. **E.** Serum levels of IL-17 and IL-21 in $IBP^{+/+}$ DO11.10 and $IBP^{trap/trap}$ DO11.10 mice. Sera from $IBP^{+/+}$ DO11.10 and $IBP^{trap/trap}$ DO11.10 mice (12–25 weeks old, male and female, n=4) were collected and levels of IL-17 and IL-21

analyzed by ELISA. *$p<0.05$. **F.** Expression of IL-17 and IL-21 in the joints of arthritic $IBP^{trap/trap}$ DO11.10 mice. Joints from 3 pairs of $IBP^{+/+}$ DO11.10 and $IBP^{trap/trap}$ DO11.10 mice (12–25 weeks old, male and female) were collected and IL-17 and IL-21 gene expression was analyzed by real-time RT-PCR. White bars represent $IBP^{+/+}$ DO11.10 mice and black bars represent $IBP^{trap/trap}$ DO11.10 mice. **G.** Blimp-1 and CD3 staining of spleens of $IBP^{trap/trap}$ DO11.10 mice. Anti-Blimp-1 (blue) and anti-CD3 (red) staining was performed on spleens from $IBP^{+/+}$ DO11.10 (left panel) and $IBP^{trap/trap}$ DO11.10 (right panel) mice. Light microscopy images (magnification of 4×, upper panels, and 40×, lower panels) are shown.

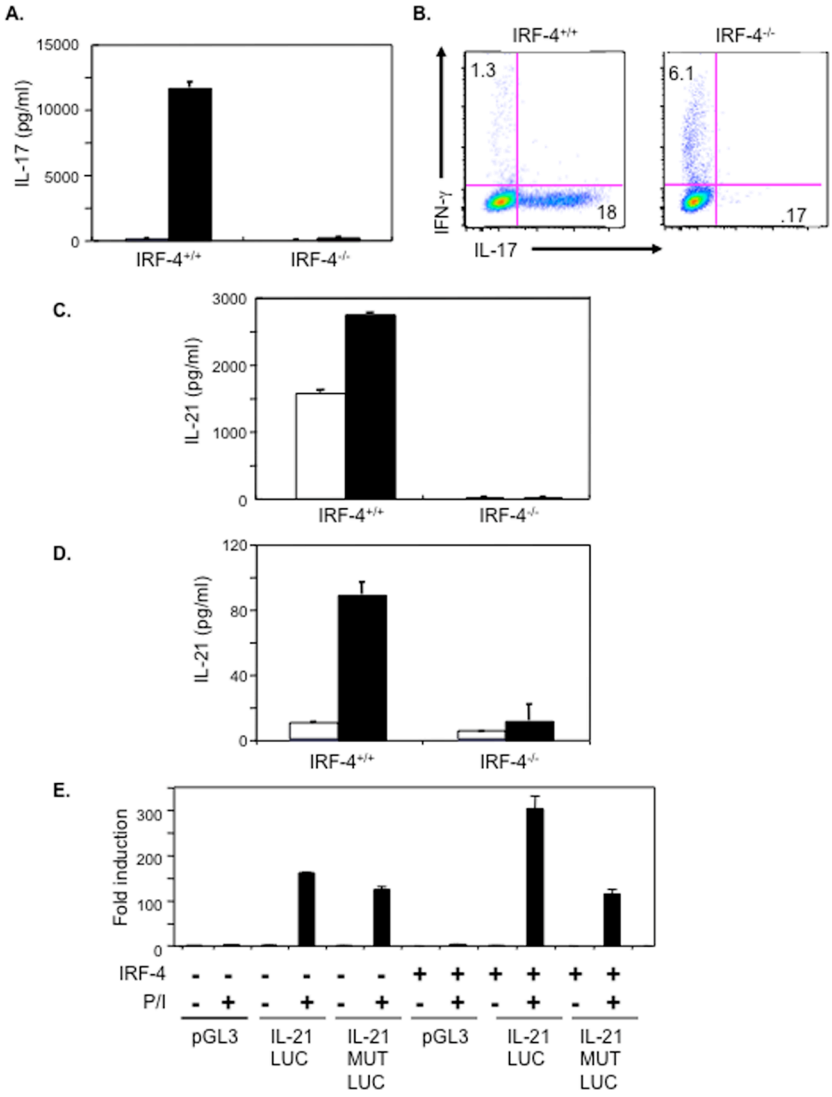


Figure 4.

IRF-4 is a critical regulator of both IL-17 and IL-21 production. **A.** IL-17 production by wt and IRF-4$^{-/-}$ CD4^{+} T cells. Purified naïve CD4^{+} T cells were stimulated under either TH0 (white bars) or TH17 (black bars) conditions for 4 days. IL-17 levels in culture supernatants were determined by ELISA. The experiment is representative of four independent experiments. **B.** Intracellular IL-17 and IFN-γ production by wt and IRF-4$^{-/-}$ CD4^{+} T cells. Cells were cultured under TH17 conditions as above and then stimulated with PMA and ionomycin for 5 hours. Intracellular IFN-γ and IL-17 production was measured by FACS. The experiment is representative of three independent experiments. **C.** IL-21 production by wt and IRF-4$^{-/-}$ CD4^{+} T cells cultured under TH17 conditions. Purified naïve CD4^{+} T cells were stimulated under TH17 conditions for 4 days and then restimulated for either 24 hrs (white bars) or 48 hrs (black bars). IL-21 levels in culture supernatants were determined by ELISA. The experiment is representative of four independent experiments. **D.** IL-21 production by wt and IRF-4$^{-/-}$CD4^{+} T cells cultured under TH0 and TH2 conditions. Purified naïve CD4^{+} T cells were stimulated under TH0 (white bars) or TH2 (black bars) conditions for 7 days and then restimulated for 48 hrs. IL-21 levels in culture supernatants were determined by ELISA. The experiment is representative of four independent experiments. **E.** Effects of IRF-4 on the

transactivation of IL-21. Jurkat cells that express an empty vector or Jurkat cells expressing IRF-4 were transfected with a luciferase reporter construct driven either by the murine IL-21 promoter (IL-21 LUC), by a murine IL-21 promoter containing mutations within the putative IRF-4 binding site (IL-21 MUT LUC) or with a control luciferase reporter construct (pGL3) in the presence or absence of PMA and ionomycin as indicated. Cotransfection with a renilla-luciferase construct was performed to normalize the transfection efficiency of the different samples. Data are representative of 3 independent experiments.

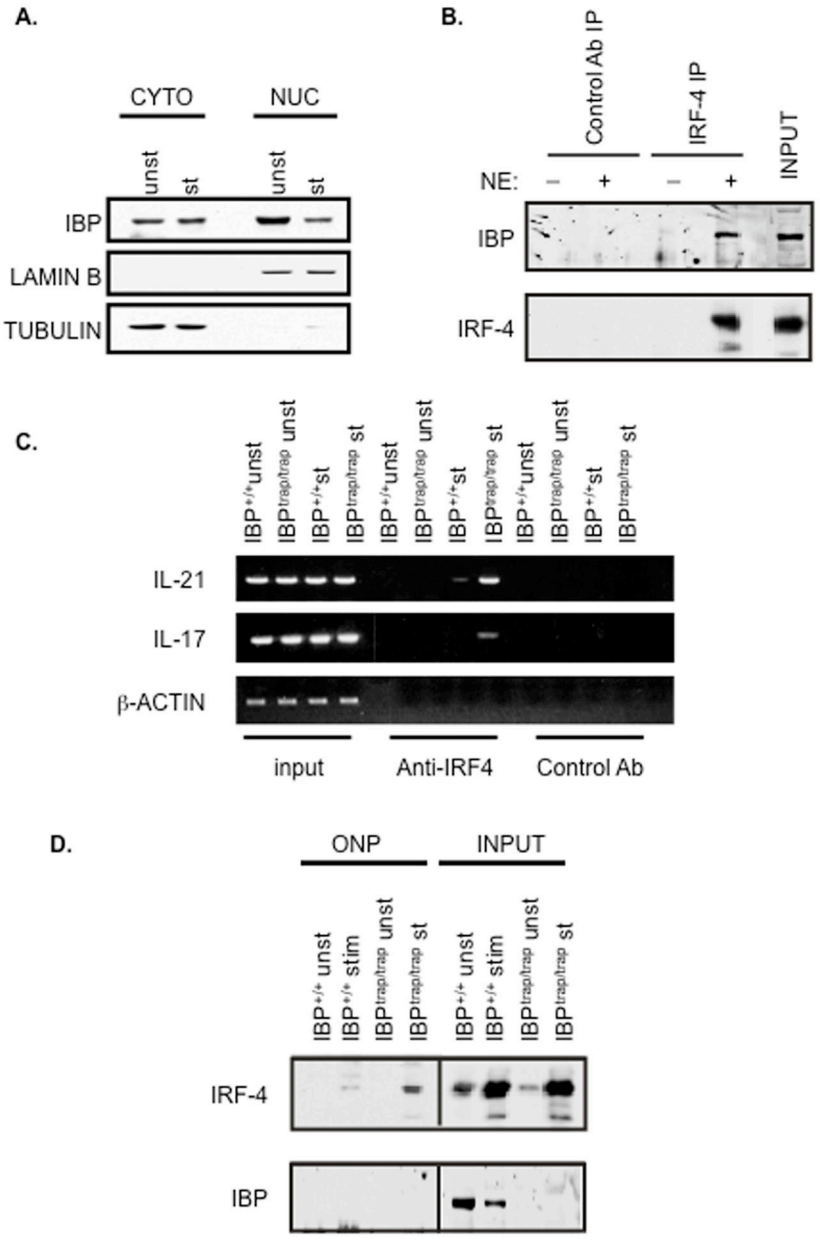
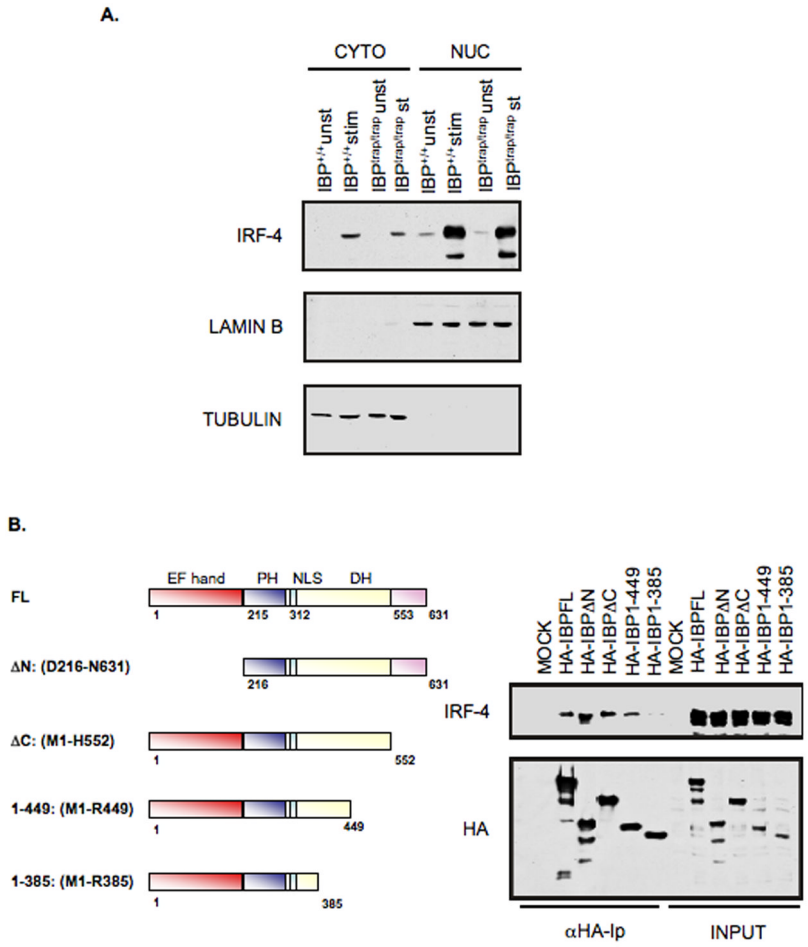


Figure 5.

The absence of IBP leads to enhanced targeting of the IL-17 and IL-21 regulatory regions**.** **A.** IBP can be found within the nuclear compartment. Purified $CD4^+$ T cells were either left unstimulated or stimulated with anti-CD3 and anti-CD28 Abs for 48 hrs. Nuclear and cytoplasmic extracts were then prepared, resolved by 8% SDS-PAGE, and then analyzed by Western blotting using an anti-IBP antiserum (upper panel). The blot was later stripped and reprobed with an anti-Lamin B (middle panel) or an anti-β-tubulin (lower panel) antibody to assess the purity of the different fractions. **B.** Nuclear IBP interacts with IRF-4. Purified $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 Abs for 48 hrs. Nuclear extracts were then prepared and immunoprecipitated with an anti-IRF-4 antibody (IRF-4 IP) or with a control antibody (Control Ab IP). The immunoprecipitates were resolved by 8% SDS-PAGE, and then analyzed by Western blotting using an anti-IBP antiserum (upper panel). The blot was later stripped and reprobed with an anti-IRF-4 antibody (lower panel). **C.** Lack of IBP

leads to enhanced targeting of the IL-17 and IL-21 promoter by IRF-4 *in vivo*. $CD4^+$ T cells were purified from either $IBP^{+/+}$ (wt) or $IBP^{trap/trap}$ mice, cultured under TH0 conditions for 7 days, and then either left unstimulated (unst) or restimulated for 24 hrs (st) with anti-CD3 and anti-CD28 Abs. Chromatin immunoprecipitation assays were then carried out on these cells with either an anti-IRF-4 antibody or a control Ab, and PCR for the IL-21 and IL-17 promoters was performed as indicated. The total input control is shown on the left. Actin was used as a negative control. **D.** Lack of IBP leads to enhanced binding of IRF-4 to the IRF-4 binding site within the IL-21 promoter. $CD4^+$ T cells were purified from either $IBP^{+/+}$ (wt) or $IBP^{trap/trap}$ mice, cultured as described in C. and subjected to an oligonucleotide precipitation assay (ONP) with a biotin-labelled oligonucleotide corresponding to the IRF-4 binding site within the IL-21 promoter. Precipitates were separated by 8% SDS-PAGE and analyzed by Western blotting with an IRF-4 antibody (top panel). The levels of IRF-4 in the input samples are shown on the right (due to the high levels of IRF-4 in the input extracts a shorter exposure time of the IRF-4 input is shown). The blot was then stripped and reprobed with an anti-IBP antibody (lower panel). The levels of IBP in the input samples are shown on the right.



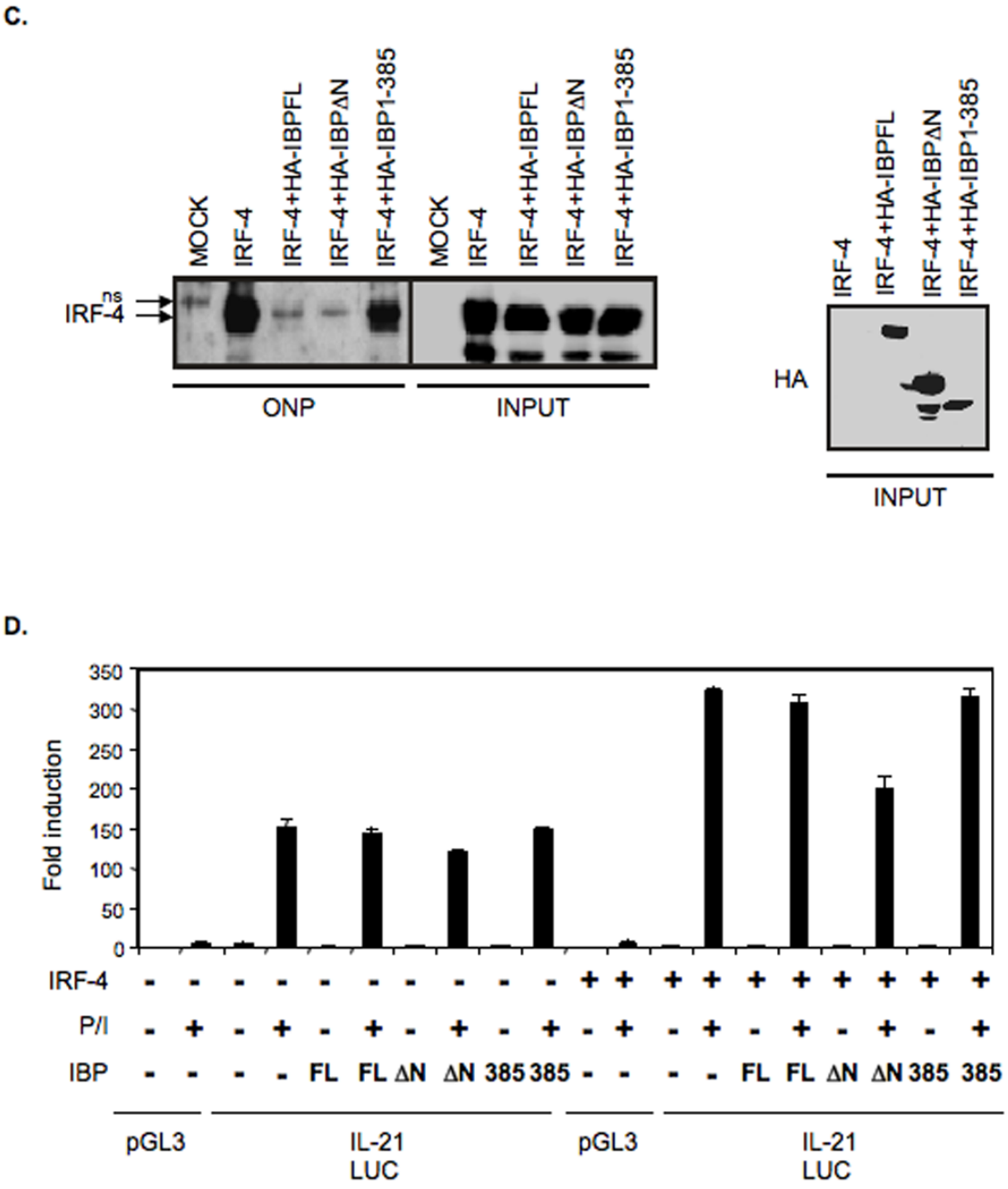


Figure 6.

IBP inhibits the DNA binding and transactivating activity of IRF-4. **A.** The absence of IBP does not alter the expression or localization of IRF-4. $CD4^+$ T cells were purified from either $IBP^{+/+}$ (wt) or $IBP^{trap/trap}$ mice, cultured under TH0 conditions for 7 days, and then either left unstimulated (unst) or restimulated for 24 hrs (st) with anti-CD3 and anti-CD28 Abs. Nuclear and cytoplasmic extracts were then prepared, resolved by 8% SDS-PAGE, and then analyzed by Western blotting using an anti-IRF-4 antiserum (upper panel). The blot was later stripped and reprobed with an anti-Lamin B (middle panel) or an anti-β-tubulin (lower panel) antibody to assess the purity of the different fractions. **B.** Mapping of the IBP domain responsible for the interaction with IRF-4. 293T cells were cotransfected with an IRF-4 expression construct

together with either HA-tagged full length IBP or the panel of HA-tagged IBP mutants described in the diagram. Extracts were immunoprecipitated with an anti-HA antibody, precipitates were separated by 8% SDS-PAGE and analyzed by Western blotting with an IRF-4 antibody (top panel). The blot was later stripped and reprobed with an anti-HA antibody (bottom panel). **C.** IBP can block the binding of IRF-4 to an oligonucleotide containing the IRF-4 binding site within the IL-21 promoter. An oligonucleotide precipitation assay (ONP) was performed on 293T cells that were either mock transfected or transfected with IRF-4 in the presence/absence of HA-tagged FL IBP, HA-tagged IBPΔN, or HA-tagged IBP 1-385. Precipitates were separated by 8% SDS-PAGE and analyzed by Western blotting with an IRF-4 antibody (left panel). The levels of IRF-4 in the input samples are also shown (due to the high levels of IRF-4 in the extracts a shorter exposure time of the IRF-4 input is shown). A nonspecific band detected in the ONP from mock transfected 293 T cells is indicated as ns. The blot was later stripped and reprobed with an anti-HA antibody to assess the levels of the different IBP constructs in the input samples (right panel). **D.** IBP can inhibit the transactivating activity of IRF-4. Jurkat cells that express an empty vector or Jurkat cells expressing IRF-4 were transfected with a luciferase reporter construct driven by the murine IL-21 promoter (IL-21 LUC) together with an empty vector, HA-tagged FL IBP, HA-tagged IBPΔN, or HA-tagged IBP 1-385 as indicated. Cells were either left unstimulated or stimulated with PMA and ionomycin as indicated. Transfections with a control luciferase reporter construct (pGL3) in the presence/absence of PMA and ionomycin were also carried out. Cotransfection with a renilla-luciferase construct was performed to normalize the transfection efficiency of the different samples. Data are representative of 3 independent experiments.

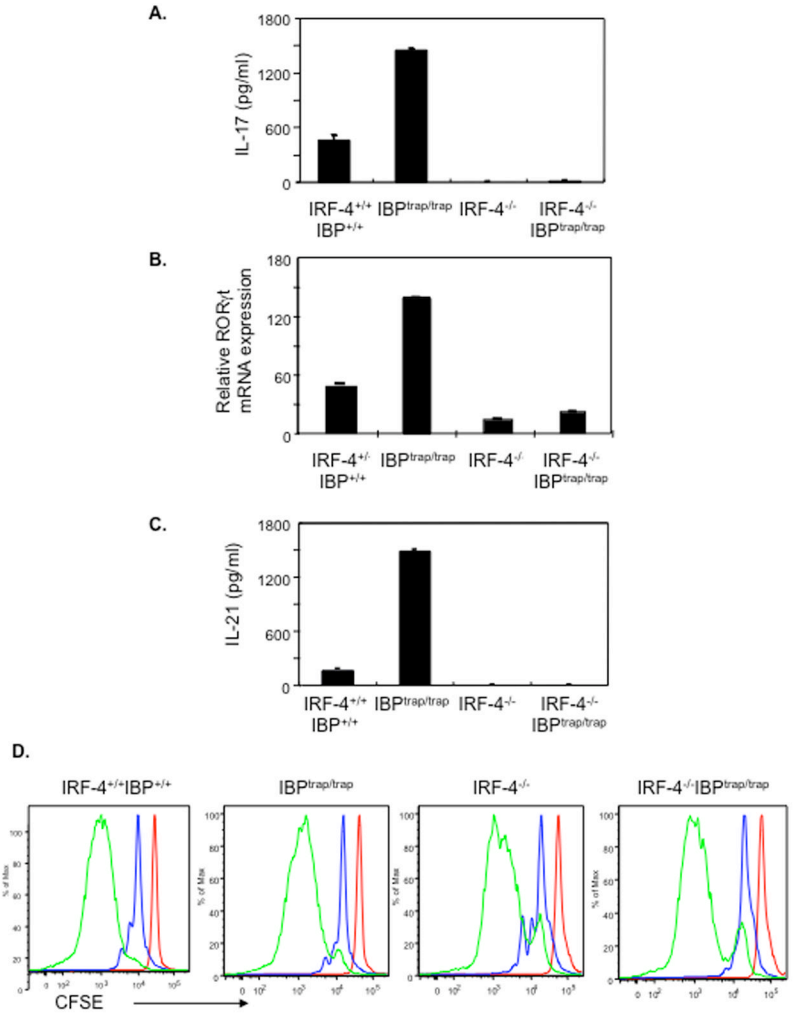


Figure 7.

The deregulated IL-17 and IL-21 production observed in the absence of IBP is dependent on IRF-4**. A.** IL-17 production by wt, IBP$^{trap/trap}$, IRF-4$^{-/-}$, and IRF-4$^{-/-}$IBP$^{trap/trap}$ CD4^{+} T cells. Purified naïve CD4^{+} T cells were stimulated under TH0 conditions for 4 days. IL-17 levels in culture supernatants were determined by ELISA. **B.** RORγt expression by wt, IBP$^{trap/trap}$, IRF-4$^{-/-}$, and IRF-4$^{-/-}$IBP$^{trap/trap}$ CD4^{+} T cells. Purified naïve CD4^{+} T cells were stimulated as above and RORγt gene expression was determined by real-time RT-PCR. **C.** IL-21 production by wt, IBP$^{trap/trap}$, IRF-4$^{-/-}$, and IRF-4$^{-/-}$IBP$^{trap/trap}$ CD4^{+} T cells. Purified naïve CD4^{+} T cells were stimulated under TH2 conditions for 7 days and then restimulated for 48 hrs. IL-21 levels in culture supernatants were determined by ELISA. **D.** CFSE profiles of wt, IBP$^{trap/trap}$, IRF-4$^{-/-}$, and IRF-4$^{-/-}$IBP$^{trap/trap}$ CD4^{+} T cells that were either left unstimulated (red) or stimulated under TH0 conditions for 2 days (blue) or for 4 days (green). Experiments shown are representative of 3 independent experiments.

Table 1

Survival of syngeneic nude mice transferred with $CD4^+$ T cells derived from either $IBP^{+/+}$DO11.10 or $IBP^{trap/trap}$DO11.10 mice.

Donor cells	$IBP^{+/+}$ Do CD4+T cells	$IBP^{trap/trap}$ Do KJ^{low} CD4+T cells	$IBP^{trap/trap}$ Do KJ^{high} CD4+ T cells
Dead mice/Total mice	0/6	0/6	3/7