Dual regulation of IRF4 function in T and B cells is required for the coordination of T–B cell interactions and the prevention of autoimmunity

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Effective humoral responses to protein antigens require the precise execution of carefully timed differentiation programs in both T and B cell compartments. Disturbances in this process underlie the pathogenesis of many autoimmune disorders, including systemic lupus erythematosus (SLE). Interferon regulatory factor 4 (IRF4) is induced upon the activation of T and B cells and serves critical functions. In CD4$^+$ T helper cells, IRF4 plays an essential role in the regulation of IL-21 production, whereas in B cells it controls class switch recombination and plasma cell differentiation. IRF4 function in T helper cells can be modulated by its interaction with regulatory protein DEF6, a molecule that shares a high degree of homology with only one other protein, SWAP-70. Here, we demonstrate that on a C57BL/6 background the absence of both DEF6 and SWAP-70 leads to the development of a lupus-like disease in female mice, marked by simultaneous deregulation of CD4$^+$ T cell IL-21 production and increased IL-21 B cell responsiveness. We furthermore show that DEF6 and SWAP-70 are differentially used at distinct stages of B cell differentiation to selectively control the ability of IRF4 to regulate IL-21 responsiveness in a stage-specific manner. Collectively, these data provide novel insights into the mechanisms that normally couple and coordinately regulate T and B cell responses to ensure tight control of productive T–B cell interactions.

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Abbreviations used: AID, activation-induced cytidine deaminase; ChIP, chromatin immunoprecipitation; DKO, double KO; GC, germinal center; IRF4, interferon regulatory factor 4; SLE, systemic lupus erythematosus; SWAP-70, SWAP switching B-cell complex 70 kD subunit; Tfh, follicular T helper cell.

Effective collaboration between T and B cells is essential for the production of high-affinity antibodies, which confer long-lasting immunity against offending pathogens (McHeyzer-Williams et al., 2009; Elgueta et al., 2010). T cell help for B cells requires the precisely orchestrated antigen-driven repositioning of T and B cells within secondary lymphoid organs (Cyster, 2010; Goodnow et al., 2010). After activation by dendritic cells, T cells migrate to the boundary between the T cell zone and B cell follicles, where the earliest encounter with antigen-bearing B cells occurs. After interaction with T cells, B cells can migrate to extrafollicular areas, where they become short-lived plasmablasts, or they can remain in the follicle and form germinal centers (GCs), the crucial anatomical sites where somatic hypermutation occurs. Upon further productive interactions with specialized T helper cells within the GCs, appropriately

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selected GC B cells will then differentiate into high-affinity plasma cells or memory cells. Disturbances in these tightly regulated processes can have profound pathogenic consequences, and dysregulation of follicular and extrafollicular antibody production is commonly encountered in autoimmune disorders, particularly in systemic lupus erythematosus (SLE; Wardemann and Nussenzweig, 2007; Shlomchik, 2008; Vinuesa et al., 2009).

Among the signals provided to B cells by T helper cells to drive humoral responses, the production of IL-21 has recently emerged as a critical element in this process (Ettinger et al., 2008; Spolski and Leonard, 2008). Production of high-levels of IL-21 is the hallmark of a novel class of effector T helper cells termed follicular helper T cells (Tfh), which are specialized in providing help to B cells in GCs (Crotty, 2011). Synthesis of IL-21 is, however, not exclusive to Tfh cells, as IL-21 can also be produced by other T helper subsets including extrafollicular T helper cells and Th17 cells (Korn et al., 2007; Nurieva et al., 2007; Wei et al., 2007; Zhou et al., 2007; Odegard et al., 2008). IL-21 plays a multifaceted role in T cell–dependent humoral responses. In addition to helping support the maintenance of Tfh cells (Nurieva et al., 2008; Vogelzang et al., 2008), IL-21 acts directly on B cells to promote GC formation, somatic hypermutation, follicular and extrafollicular plasma cell differentiation, and memory B cell responses (Linterman et al., 2010; Zotos et al., 2010; Rankin et al., 2011). The critical effects of IL-21 on B cell responses are related to its capacity to drive the expression of major regulators of the B cell differentiation program including activation-induced cytidine deaminase (AID; also known as AICDA), Bcl-6, and Blimp-1 (Ozaki et al., 2004; Pène et al., 2004; Kobayashi et al., 2009). Given that the presence of these factors marks distinct stages of B cell differentiation, the ability of IL-21 to induce the expression of these molecules must be selectively controlled as B cells proceed along their differentiation program. The mechanisms by which exposure to IL-21 leads to different functional outcomes in B cells as they proceed through different stages of differentiation are, however, unknown.

The molecular pathways regulating the production of IL-21 have recently been investigated. Interferon regulatory factor 4 (IRF4), a transcription factor induced upon stimulation of T and B cells, has emerged as an essential controller of IL-21 production because its absence prevents IL-21 production by multiple T helper subsets (Chen et al., 2008; Huber et al., 2008). The role of IRF4 in T cell activation is not restricted to the control of IL-21 production, as the lack of IRF4 also results in defects in the function of Th0, Th2, Th9, and Th17 cells (Rengarajan et al., 2002; Brüstle et al., 2007; Chen et al., 2008; Honma et al., 2008; Staudt et al., 2010). IRF4 also exerts a fundamental role in B cell differentiation. IRF4 regulates class-switch recombination via its ability to control AID expression and is absolutely required for the generation of plasma cells (Klein et al., 2006; Sciammas et al., 2006). This latter effect has been ascribed to the capacity of IRF4 to control the expression of Blimp-1 by directly targeting the *Prdm1* regulatory regions (Sciammas et al., 2006). Interestingly, recent studies have demonstrated that IRF4 regulates the responsiveness of both T and B cells to IL-21 and that the expression of IL-21–regulated genes is broadly controlled by the functional cooperation of IRF4 with STAT3 (Kwon et al., 2009). Given the essential and complex role of IRF4 in T and B cell responses (Nutt and Tarlinton, 2011), its activity in both of these compartments needs to be tightly regulated to ensure that the execution of T and B cell differentiation programs is strictly controlled in time and space.

During a search for regulatory proteins interacting with IRF4, our laboratory previously isolated a protein termed DEF6 (also known as IBP or SLAT; Hotfilder et al., 1999; Gupta et al., 2003b; Tanaka et al., 2003). DEF6 exerts an important immunoregulatory role in vivo, as demonstrated by the finding that DEF6-deficient (*Def6*$^{tr/tr}$) mice can spontaneously develop either a lupus-like syndrome (on a 129/B6 background) or RA-like arthritis (when crossed to a TCR transgenic mouse, DO11.10, on a BALB/c background; Fanzo et al., 2006; Chen et al., 2008). The autoimmune disorders that develop in the absence of DEF6 are characterized by deregulated production of IL-17 and IL-21, which is caused by the unrestrained ability of IRF4 to target the regulatory regions of IL-17 and IL-21 (Chen et al., 2008; Biswas et al., 2010). DEF6 shares a high degree of homology with only one other protein, SWAP-70 (Hotfilder et al., 1999; Gupta et al., 2003b; Tanaka et al., 2003). Unlike DEF6, which is highly expressed in naive T helper cells and B cells, SWAP-70 is expressed in B cells but not in naive T helper cells (Borggrefe et al., 1999). Consistent with this expression pattern, the lack of SWAP-70 results in defects within the B cell compartment. SWAP-70–deficient B cells are less efficiently recruited into the secondary lymphoid organs during the initial phases of an inflammatory response (Pearce et al., 2006). Furthermore, SWAP-70 deficiency uncouples GC formation from long-lived plasma cell production leading to the extrafollicular generation of high-affinity plasma cells after T cell–dependent immunization (Quemeneur et al., 2008). The molecular mechanisms by which SWAP-70 regulates T cell–driven plasma cell differentiation are, however, unknown. Given the emerging role of this unique family of proteins in the control of immune responses, in this study we investigated the effects of the simultaneous lack of DEF6 and SWAP-70 on the immune system.

RESULTS

Spontaneous development of a lymphoproliferative disorder in double KO (DKO) female mice

DEF6 and SWAP-70 are the only two members of a unique family of proteins (Borggrefe et al., 1998; Hotfilder et al., 1999; Gupta et al., 2003b; Tanaka et al., 2003). To elucidate the broad role of these two molecules in the immune system, *Def6*$^{tr/tr}$ mice on a C57BL/6 background were bred with *Swap-70*$^{-/-}$ mice (also on a C57BL/6 background) to generate *Def6*$^{tr/tr}$*Swap-70*$^{-/-}$ (DKO) mice. No significant differences were observed in the cellularity and surface phenotypic markers of the lymphoid organs in 6–8-wk-old DKO mice except for a slight decrease in the number of splenic CD8^{+}

T cells (unpublished data). By 24 wk of age, however, the DKO female mice, but not the DKO male mice, were found to develop splenomegaly and lymphadenopathy (Fig. 1, A and B). None of the WT, *Def6*$^{tr/tr}$, or *Swap-70*$^{-/-}$ female or male mice developed similar findings (Fig. 1, A and B). Thus, the concomitant absence of DEF6 and SWAP-70 results in the spontaneous development of a lymphoproliferative disorder that selectively affects the female gender.

To better characterize the lymphoproliferative disease exhibited by the DKO female mice, a detailed flow cytometric analysis was conducted. Spleens of 24-wk-old DKO female mice demonstrated a significant expansion of effector

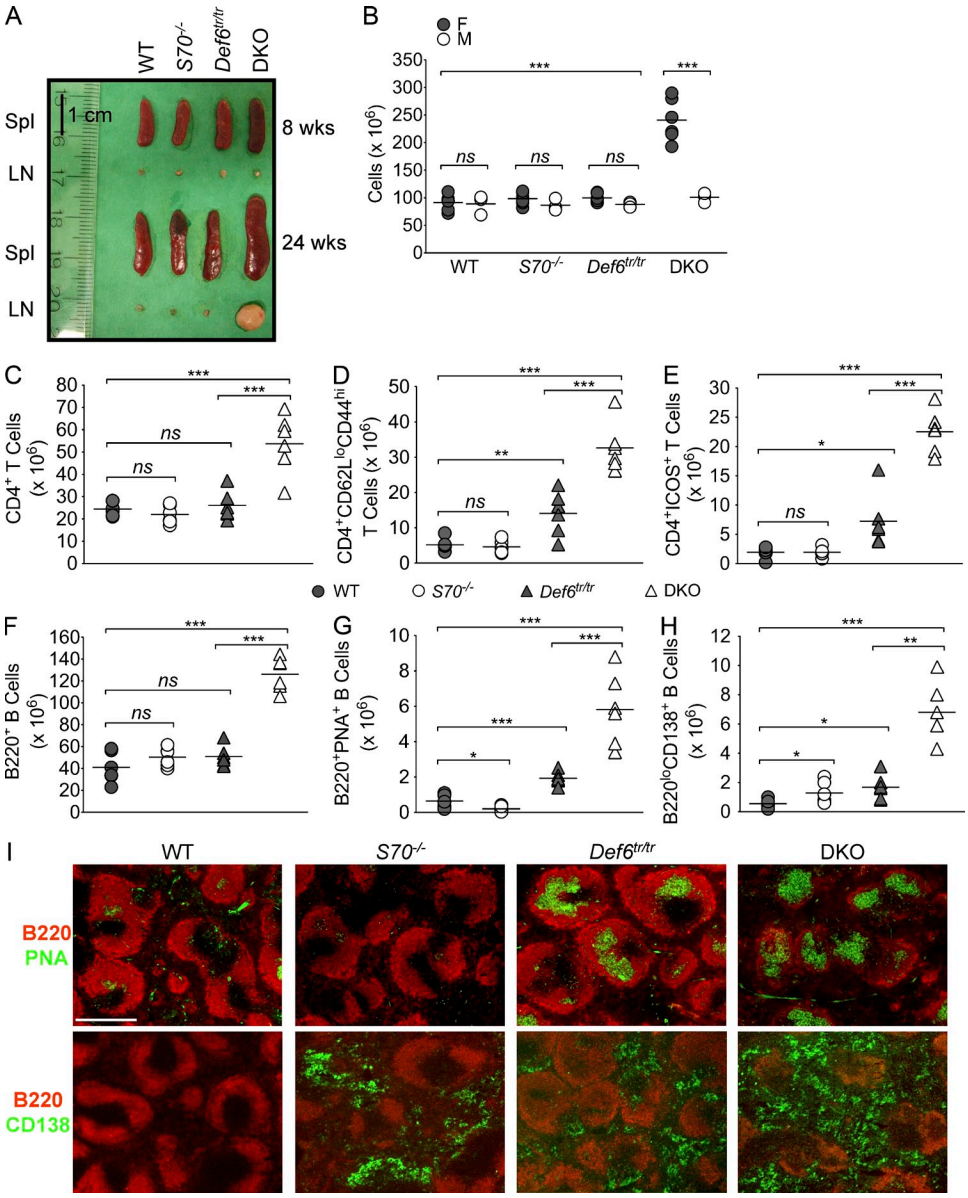

Figure 1. Spontaneous development of lymphoproliferation in DKO female mice. (A) Representative spleen and lymph nodes from 8- and 24-wk-old C57BL/6 (WT), *Swap-70*$^{-/-}$ (*S70*$^{-/-}$), *Def6*$^{tr/tr}$, and *Def6*$^{tr/tr}$*Swap-70*$^{-/-}$ (DKO) female mice. Data are representative of six independent experiments with one mouse/group. (B) Total number of splenic cells in 24-wk-old female (F) and male (M) mice. For female mice, data represent six independent experiments with one mouse/group. For male mice, data represent three independent experiments with one mouse/group. (C–H) Total number of splenic CD4$^+$ T cells (C), CD4$^+$CD62L^{lo}CD44^{hi} T cells (D), CD4$^+$ICOS$^+$ T cells (E), B220$^+$ B cells (F), B220$^+$PNA$^+$ B cells (G), and B220^{lo}CD138$^+$ B cells (H) in 24-wk-old female mice as assessed by flow cytometry. Each dot represents an individual mouse. Horizontal bars indicate mean for each group. Data are representative of six independent experiments with one mouse/group. (I) Immunofluorescence analysis of GCs and plasma cells in the indicated mice. Spleens were stained for GCs with B220 (red) and PNA (green) in the top panels or for plasma cells with B220 (red) and CD138 (green) in the bottom panels. Magnification is 16×. Bar, 500 µm. Data are representative of three independent experiments with one mouse/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ns, not statistically significant.

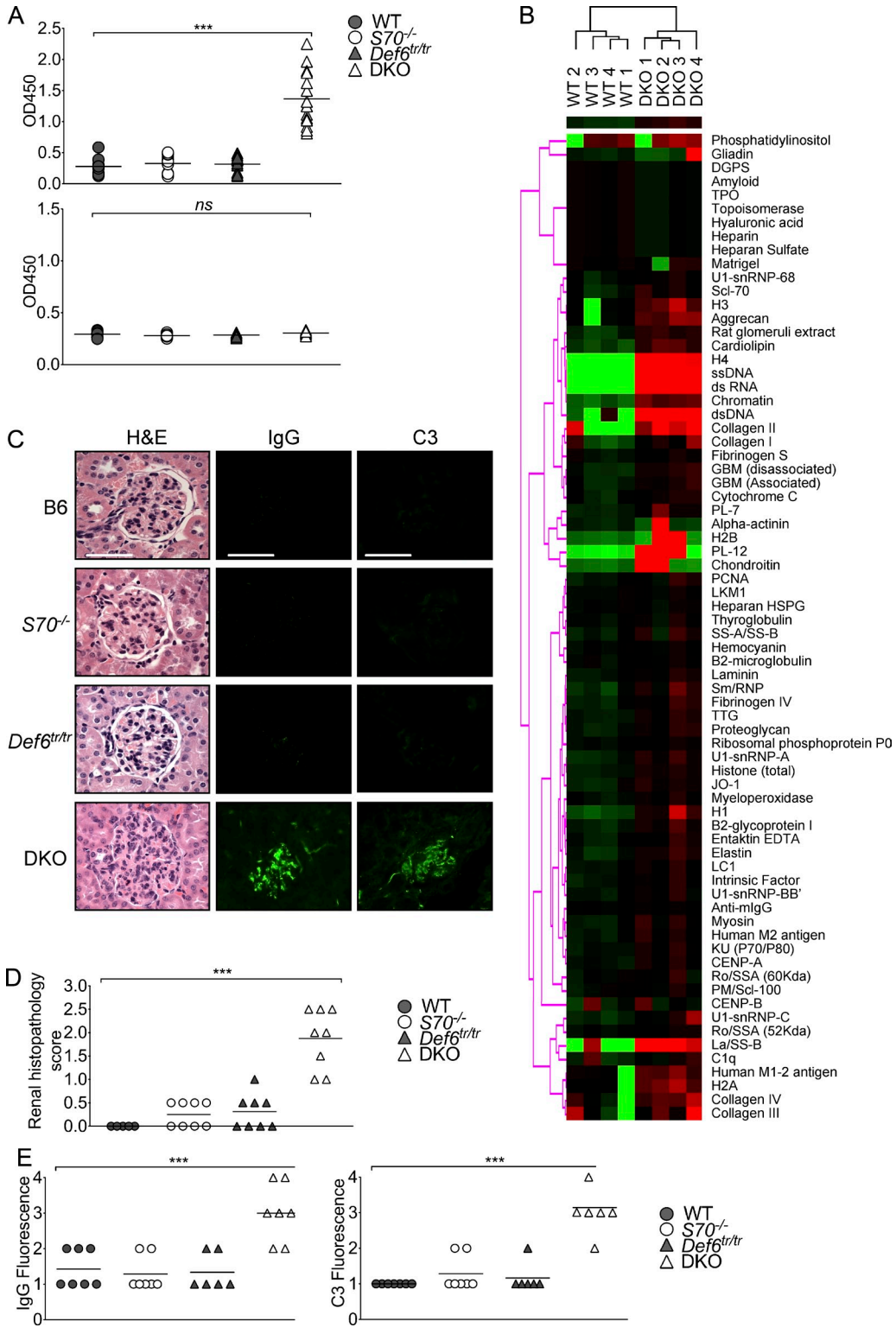

Figure 2. Autoantibody production and renal pathology in DKO female mice. (A) Serum anti-dsDNA antibody levels of 1-yr-old female (top) and male (bottom) mice (n = 13–17/group). Each dot represents individual mice per group, and horizontal bars indicate mean for each group. Data are representative of two independent experiments. ***, $P < 0.005$; ns, not statistically significant. (B) Sera from 4 WT and 4 DKO 1-yr-old female mice were subjected to an autoantigen microarray. The data are presented as a heat map, which shows the IgG seroreactivities of each sample toward the individual antigens on the array. Reactivities above the array mean are shown in red, reactivities below the mean are shown in green, and reactivities close to the mean are shown in black. A clustering algorithm was used to cluster together sera that display similar reactivities (top dendrogram) and group together antigens that were targeted in a

$CD4^+$ T cells ($CD4^+CD62L^{lo}CD44^{hi}$) and $CD4^+ICOS^+$ cells (Fig. 1, C–E; and not depicted). A significant accumulation of $CXCR5^+PD\text{-}1^{hi}Bcl\text{-}6^{hi}CD4^+$ T cells and of a distinct population of $CXCR5^{-/lo}PD\text{-}1^{hi}$ $CD4^+$ T cells expressing intermediate levels of Bcl-6 could also be observed (unpublished data). The number of regulatory T cells ($CD4^+CD25^+Foxp3^+$) was also increased in the DKO female mice (unpublished data). Female mice deficient in DEF6 alone exhibited increased numbers of effector $CD4^+$ T cells and $CD4^+ICOS^+$ cells, but the increase was more modest than that observed in the DKO female mice (Fig. 1, D and E). No T cell aberrancies were detected in *Swap-70*$^{-/-}$ female mice (Fig. 1, D and E). Thus, the absence of both DEF6 and SWAP-70 results in the aberrant accumulation of classical Tfh cells ($CXCR5^+PD\text{-}1^{hi}Bcl\text{-}6^{hi}$), and of a population that may represent extrafollicular T helper cells ($CXCR5^{-/lo}PD\text{-}1^{hi}Bcl\text{-}6^{int}$).

Female DKO mice also exhibited marked aberrancies within the B cell compartment, which were characterized by an increased number of B cells (Fig. 1 F), an expansion of GC B cells ($B220^+PNA^+$), an increased number of switched $IgG1^+$ B cells and an accumulation of plasma cells ($B220^{lo}CD138^+$) in the spleen but not in the bone marrow (Fig. 1, F–H; and not depicted). Slight increases in GC B cells and plasma cells were observed in mice deficient in DEF6 alone. Consistent with previous results (Quemeneur et al., 2008), in comparison to WT mice, mice lacking only SWAP-70 demonstrated a slightly increased frequency of plasma cells but decreased numbers of GC B cells (Fig. 1, G and H). Immunofluorescence studies confirmed the striking expansion of GCs and plasma cells in the spleens of the DKO female mice (Fig. 1 I). Collectively these studies indicate that the simultaneous absence of DEF6 and SWAP-70 results in the spontaneous accumulation of GC B cells and plasma cells.

Lymphoproliferation is often associated with the development of autoantibodies and lupus-like disease. An extensive serologic and histopathologic analysis of the different groups of mice at one year of age was thus performed. Consistent with the finding that only DKO female mice developed lymphoproliferation, anti–double-stranded DNA (dsDNA) autoantibodies were detected exclusively in DKO female mice (Fig. 2 A). Autoantibody production in DKO female mice was associated with an increase in total serum IgG1 levels, but not in other IgG isotypes or in IgM and IgA production (unpublished data). Evaluation of sera from four WT and four DKO female mice with an autoantigen microarray (Fig. 2 B; and not depicted) confirmed these findings and demonstrated that the sera from DKO female mice reacted strongly against several nuclear antigens. 1-yr-old DKO female mice were also found to exhibit glomerular deposition of IgG and C3 and to develop glomerulonephritis (Fig. 2, C–E). Collectively, these results indicate that the concomitant absence of DEF6 and SWAP-70 results in the development of a lupus-like syndrome, which selectively affects the female gender. Remarkably, this occurs on a C57BL/6 background, a strain relatively resistant to the development of lupus.

Young DKO female mice exhibit abnormal responses to T cell–dependent antigens

Given the profound deregulation in humoral responses observed in the older DKO female mice, we sought to assess the responses of young DKO female mice to immunization with a T-dependent antigen, NP-KLH. Immunization with NP-KLH elicited a similar number of $B220^+NP^+$ cells in all four sets of mice (Fig. 3 A). As previously observed (Quemeneur et al., 2008), the lack of SWAP-70 resulted in a decreased number of NP^+PNA^+ B cells when compared with WT mice, whereas the absence of DEF6 led to a higher number of NP^+PNA^+ B cells (Fig. 3 B; and not depicted). The concomitant absence of DEF6 and SWAP-70 resulted in an increase in NP^+PNA^+ B cells comparable in magnitude to what was detected in the absence of DEF6 alone (Fig. 3 B; and not depicted). ELISPOT assays for total and high affinity NP-specific antibody forming B cells (AFCs) demonstrated that, as previously observed (Quemeneur et al., 2008), the lack of SWAP-70 resulted in an increased number of plasma cells when compared with WT mice, an effect that was associated with higher serum titers of anti–NP-specific IgG1 and IgG2a (Fig. 3, C–F). Interestingly, the lack of both DEF6 and SWAP-70 resulted in a more dramatic increase in plasma cells compared with mice lacking either SWAP-70 or DEF6 alone (Fig. 3, C–F). Similar results were also obtained upon immunization with SRBCs (unpublished data). Collectively, these data suggest that the lack of DEF6 may be primarily responsible for the enhanced GC formation observed in the DKO mice, whereas the absence of both DEF6 and SWAP-70 contributes to the aberrant plasma cell formation detected in these mice.

Aberrant production of IL-21 by DEF6-deficient T cells plays a key role in the humoral abnormalities observed in mice lacking DEF6

To further dissect the contribution of DEF6 and SWAP-70 to the aberrant humoral responses observed in the DKO female mice, we set out to separately evaluate the effects of the lack of DEF6 and SWAP-70 in T and B cells. Because we had previously observed that the lack of DEF6 leads to the aberrant production of IL-17 and IL-21 when $CD4^+$ T cells are cultured under neutral conditions (Chen et al., 2008;

similar manner by the different sera (left dendrogram). (C, left) Representative H&E-stained sections from the kidneys of 1-yr-old female mice. Photomicrographs were taken at a magnification of 400×. Deposition of immunoglobulin complexes in the glomeruli was detected by immunofluorescence with anti-IgG (middle) and anti-C3 (right) staining. Magnification of 400× is shown. Results are representative of 5–7 mice/group. Bar, 250 µm. (D) The graph shows the renal histopathologic score of the indicated mice (n = 5–8/group). Data are representative of two independent experiments. (E) The graph on the left represents the IgG deposition scores as detected by immunofluorescence, and the graph on the right represents the C3 deposition scores in the glomeruli of the indicated mice (n = 3–7/group). Data are representative of two independent experiments. ***, $P < 0.005$.

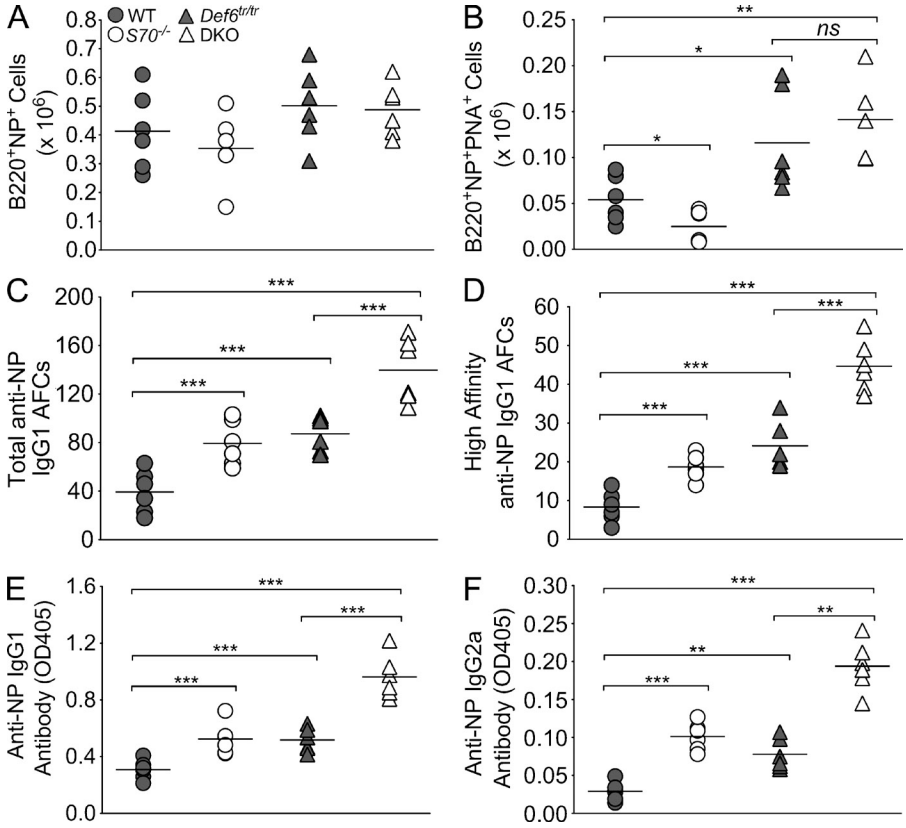

Figure 3. Abnormal responses to immunization with a TD antigen in young DKO female mice. (A and B) 8–10-wk-old female mice (n = 6–7/group) were immunized with NP-KLH emulsified in CFA. Unimmunized control mice received CFA only (not depicted). At day 7 after immunization, spleens were analyzed for the numbers of $B220^+NP^+$ cells (A) and of $B220^+NP^+PNA^+$ cells (B) by flow cytometry. (C and D) ELISPOT analysis of total (C) and high-affinity (D) IgG1 secretion by splenocytes from NP-KLH–immunized mice at day 14 after immunization. (E and F) Serum responses to NP-KLH immunization in the indicated mice. 14 d after immunization, serum samples were collected and total NP-specific anti-IgG1 (E) and IgG2a (F) antibody titers were determined by ELISA with NP14-BSA as capture antigen. Each dot represents individual mice, and horizontal bar represents mean for each group. Data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Biswas et al., 2010), we first explored the effects of the simultaneous lack of DEF6 and SWAP-70 on the production of these cytokines. When cultured under neutral conditions, DKO T cells from young female mice aberrantly produced IL-17 and IL-21 to an extent similar to that exhibited by *Def6*$^{tr/tr}$ T cells (Fig. 4 A). In contrast, *Swap-70*$^{-/-}$ $CD4^+$ T cells behaved similarly to WT $CD4^+$ T cells (Fig. 4 A). Moreover, the lack of SWAP-70 either by itself or in combination with the absence of DEF6 did not affect the production of IFN-γ or IL-4 (Fig. 4 A; and not depicted). Consistent with these findings, intracellular staining for IL-17 and IL-21 demonstrated that, unlike WT and *Swap-70*$^{-/-}$ mice, 24-wk-old *Def6*$^{tr/tr}$ and DKO female mice contained comparable populations of T cells that aberrantly produced both IL-17 and IL-21 (Fig. 4 B).These data suggest that the lack of SWAP-70 does not affect the aberrant cytokine production observed in DEF6-deficient $CD4^+$ T helper cells.

In view of the enhanced ability of *Def6*$^{tr/tr}$ $CD4^+$ T cells to produce IL-21 and the key role that IL-21 plays in driving B cell responses, we also explored the effects of IL-21 deficiency on the humoral abnormalities observed in the absence of DEF6. For these experiments, we crossed *IL-21*$^{-/-}$ mice with *Def6*$^{tr/tr}$ mice on a mixed background where DEF6 deficiency alone is sufficient to mediate exaggerated T and B cells responses (Fanzo et al., 2006). Control mice, *Def6*$^{tr/tr}$, *IL-21*$^{-/-}$, and *Def6*$^{tr/tr}$*IL-21*$^{-/-}$ mice were then immunized with SRBCs and splenic B cell responses were quantified 7 d after immunization (Fig. 4, C and D). As expected, in response to immunization, *Def6*$^{tr/tr}$ mice exhibited higher numbers of GC B cells and plasma cells as compared with control mice. These effects were clearly inhibited by the absence of IL-21. IL-21 deficiency did not exert any effects on the expansion of effector ($CD62L^{lo}CD44^{hi}$) $CD4^+$ T cell and $ICOS^+$ $CD4^+$ T cells, which is detected in *Def6*$^{tr/tr}$ mice (Fig. 4, E and F). These results thus support the notion that aberrant IL-21 production by DEF6-deficient $CD4^+$ T cells plays a key role in the exaggerated B cell responses observed in mice that lack DEF6.

Differential requirements for DEF6 and SWAP-70 in B cells during T cell–dependent humoral responses

Given that the lack of DEF6 in $CD4^+$ T helper cells appeared to be primarily responsible for the cytokine abnormalities detected within the T cell compartment, we next used WT or *Def6*$^{tr/tr}$ $CD4^+$ T cells in a series of transfer experiments together with B cells purified from each of the four genotypes. Different combinations of WT and *Def6*$^{tr/tr}$ $CD4^+$ T cells and resting B cells from WT, *Def6*$^{tr/tr}$, *Swap-70*$^{-/-}$, or DKO mice were transferred into *Rag1*$^{-/-}$ mice, and, after appropriate reconstitution, the recipient mice were immunized with SRBCs. 7 d after immunization, the frequencies of PNA^+ GC B cells and plasma cells were assessed by FACS. Transfer of either WT T cells or DEF6-deficient T cells with WT B cells led to comparable frequencies of GC B cells and plasma cells (Tables 1 and 2). Consistent with previous results (Quemeneur et al., 2008), transfer of *Swap-70*$^{-/-}$ B cells with WT T cells resulted in lower numbers of GC B cell, but increased numbers of plasma cells, as compared with WT B cells. Interestingly, transferring *Swap-70*$^{-/-}$ B cells with

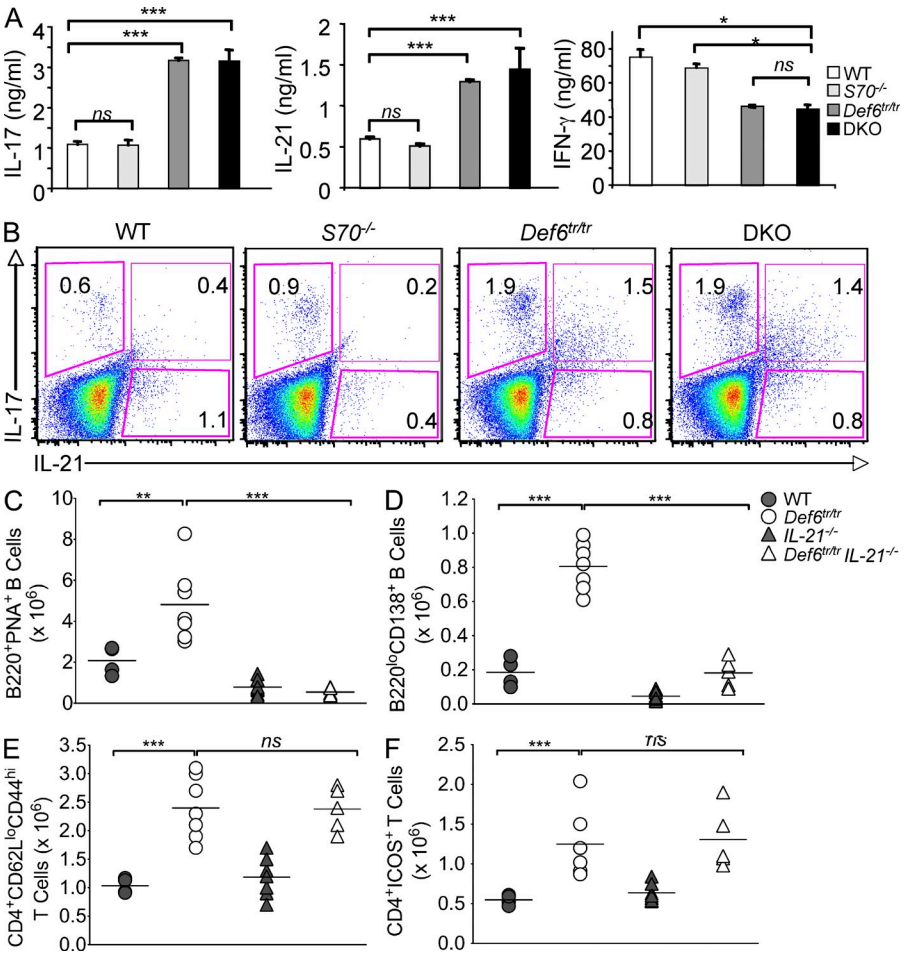

Figure 4. Aberrant production of IL-21 by DEF6-deficient T cells plays a key role in the humoral abnormalities observed in mice lacking DEF6. (A) Purified CD4+ T cells from 8–10-wk-old WT, *S70*$^{-/-}$, *Def6*$^{tr/tr}$, and DKO female mice were subjected to a primary stimulation, and then restimulated with αCD3 and αCD28 for 48 h. Supernatants were then collected and assayed for IL-17 (left), IL-21 (middle), and IFN-γ (right) production by ELISA. The data are representative of three independent experiments. The error bars represent mean ± SD. (B) Splenocytes from 24-wk-old WT, *S70*$^{-/-}$, *Def6*$^{tr/tr}$, and DKO female mice were stimulated with PMA (50 µg/ml) and Ionomycin (1 µM) for 5 h. After stimulation, cells were subjected to intracellular staining for IL-17 and IL-21, and then analyzed for cytokine production by flow cytometry. The data are representative of three independent experiments. (C–F) 8–10-wk-old female WT, *Def6*$^{tr/tr}$, *IL-21*$^{-/-}$, and *Def6*$^{tr/tr}$ *IL-21*$^{-/-}$ mice were immunized with SRBC (n = 4–7/group). Unimmunized control mice (not depicted) received PBS only. At day 7 after immunization, splenocytes were analyzed for numbers of B220+PNA+ cells (C), B220loCD138+ cells (D), CD4+CD62LloCD44hi cells (E), and CD4+ICOS+ cells (F). Each dot represents individual mice and horizontal bars indicate mean for each group. The data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Def6$^{tr/tr}$ T cells rather than WT T cells further increased their ability to differentiate into plasma cells. In contrast to the findings with *Swap-70*$^{-/-}$ B cells, *Def6*$^{tr/tr}$ B cells exhibited an enhanced ability to differentiate into GC B cells when co-transferred with *Def6*$^{tr/tr}$ T cells, whereas the differentiation of *Def6*$^{tr/tr}$ B cells into plasma cells was similar to that observed in WT B cells. Strikingly, DKO B cells exhibited an increased capacity to form both GC B cells and plasma cells, which was particularly evident when these cells were co-transferred with *Def6*$^{tr/tr}$ T cells rather than WT T cells. Collectively, these studies suggest that the absence of DEF6 in B cells plays a key role in driving the aberrant GC formation observed in DKO mice, whereas the major contribution of SWAP-70 deficiency to the B cell abnormalities that occur in the DKO mice occurs during the terminal differentiation of B cells into plasma cells, and that both of these abnormalities are markedly augmented by the lack of DEF6 in T cells.

DEF6 regulates IL-21 responsiveness of antigen-engaged B cells by controlling IRF4 function

The transfer experiments suggested that the absence of DEF6 in B cells leads to an enhanced capacity of these cells to respond to help provided by T cells during their initial encounter at the T–B cell border. One of the critical features of such productive interactions is the ability of B cells to up-regulate the expression of AID (Ramiro et al., 2007; Delker et al., 2009). To investigate whether the lack of DEF6 in B cells results in aberrant AID induction upon exposure to T cell signals, we used a recently developed in vitro culture system in which a single-round IgM signal is provided to B cells (Damdinsuren et al., 2010). Such a system is believed to more closely resemble the short-term physiological exposure to antigen encountered in vivo by B cells that have migrated to the T–B border. Accordingly, purified resting B cells (CD43^{-}) from 8–10-wk-old WT, *Swap-70*$^{-/-}$, *Def6*$^{tr/tr}$, or DKO female mice were stimulated with a single round of anti-IgM treatment, followed by anti-CD40 stimulation (Fig. 5 A). Given that increased production of IL-21 by *Def6*$^{tr/tr}$ T cells plays a crucial role in this system, these stimulations were conducted in the presence or absence of IL-21. Under these culture conditions, B cells from *Def6*$^{tr/tr}$ mice expressed higher levels of *Aicda* mRNA as compared with WT B cells, an effect that was augmented by the presence of IL-21. Consistent with the transfer experiments, DKO B cells expressed a pattern of abnormalities similar to those observed in *Def6*$^{tr/tr}$ B cells, whereas *Swap-70*$^{-/-}$ B cells behaved similarly to WT B cells. Western blotting experiments with an anti-AID antibody confirmed the aberrant induction of AID observed in the absence of DEF6, which was

again particularly evident in the presence of IL-21 (Fig. 5 B). These data suggest that DEF6, but not SWAP-70, regulates AID induction during the early phases of the T–B interaction.

We had previously found that nuclear DEF6 can physically interact with IRF4 and inhibit IRF4 function in $CD4^+$ T cells (Chen et al., 2008). Given that IRF4 regulates the expression of AID in B cells (Klein et al., 2006; Sciammas et al., 2006), we explored the possibility that DEF6 could also interact with IRF4 in B cells and regulate its function in this compartment. Coimmunoprecipitation experiments, indeed, confirmed that nuclear DEF6 physically interacts with IRF4 in B cells (Fig. 5 C). Because the *Aicda* promoter contains potential IRF-binding sites (Lee et al., 2006), chromatin immunoprecipitation (ChIP) assays with an anti-IRF4 antibody were conducted next. When compared with WT B cells, stimulated *Def6*$^{tr/tr}$ B cells exhibited increased binding of IRF4 to the *Aicda* promoter, although WT and *Def6*$^{tr/tr}$ B cells expressed similar levels of IRF4 (Fig. 5 D; and not depicted). Intriguingly, the enhanced binding of IRF4 to the *Aicda* promoter was only minimally affected by the presence of IL-21 (Fig. 5 D). Given the known ability of IL-21 to activate STAT3 and the broad cooperativity between IRF4 and STAT3 (Kwon et al., 2009), we reasoned that the aberrant binding of IRF4 observed in the absence of DEF6 may result in increased STAT3 recruitment to the *AICDA* promoter upon exposure to IL-21. Although WT and DEF6-deficient B cells activated STAT3 at similar levels in response to IL-21 stimulation (Fig. 5 E), ChIP assays with a STAT3 antibody demonstrated that *Def6*$^{tr/tr}$ B cells exhibited enhanced binding of STAT3 to the *Aicda* promoter upon stimulation with IL-21 (Fig. 5 F). These data therefore suggest that the hyperresponsiveness of DEF6-deficient B cells to IL-21 is caused not only by deregulated binding of IRF4 but also by the subsequent ability of the bound IRF4 to recruit STAT3.

To further confirm the role of IRF4 in the abnormal activation of DEF6-deficient B cells, we used B cells from mice doubly deficient in DEF6 and IRF4, which we had previously generated (Chen et al., 2008). As shown in Fig. 5 G, the concomitant lack of IRF4 abolished the aberrant induction of *Aicda* mRNA observed in *Def6*$^{tr/tr}$ B cells. Consistent with a mechanism whereby DEF6 can sequester IRF4 and prevent it from binding to the *Aicda* promoter, retroviral transduction experiments furthermore revealed that reintroduction of full-length DEF6 (DEF6 FL), but not of a DEF6 mutant (DEF6 1–385) that is unable to interact with IRF4 (Chen et al., 2008), could rescue the abnormal induction of AID detected in *Def6*$^{tr/tr}$ B cells (Fig. 5 H), although both constructs were expressed at similar levels (unpublished data). Collectively, these data support the notion that nuclear DEF6 can sequester IRF4 in activated B cells and inhibit its function.

SWAP-70 regulates IL-21–induced plasma cell differentiation by controlling the activity of IRF4

In line with previous findings (Quemeneur et al., 2008), the transfer experiments had indicated that SWAP-70 plays a key role in regulating plasma cell formation and that its absence in B cells was a major contributor to the plasma cell accumulation observed in the DKO female mice. To explore the molecular mechanisms by which SWAP-70 regulates T cell–driven plasma cell differentiation, we employed a standard in vitro culture system where resting B cells are stimulated in the presence of continuous IgM signaling (cIgM) and CD40 stimulation. In light of the importance of IL-21 in the abnormalities that we had observed, these stimulations were conducted in the presence or absence of this cytokine. Under these stimulatory conditions, no significant differences in the proliferation of WT, *Swap-70*$^{-/-}$, *Def6*$^{tr/tr}$, or DKO B cells were

Table 1. Percentages of $B220^+PNA^+$ cells after transfer into *Rag1*$^{-/-}$ mice

T cells	B cells	$B220^+$ PNA^+
		%
WT	WT	1.2 ± 0.6
Def6$^{tr/tr}$	WT	1.4 ± 0.4
WT	*S70*$^{-/-}$	0.5 ± 0.2
Def6$^{tr/tr}$	*S70*$^{-/-}$	1.2 ± 0.2
WT	*Def6*$^{tr/tr}$	2.0 ± 0.6
Def6$^{tr/tr}$	*Def6*$^{tr/tr}$	3.9 ± 1.8
WT	DKO	3.8 ± 1.5
Def6$^{tr/tr}$	DKO	5.8 ± 1.0

Percentage of $B220^+PNA^+$ cells after transfer of indicated combinations of T and B cells into *Rag1*$^{-/-}$ mice followed by immunization with SRBCs. $B220^+PNA^+$ cells were evaluated 7 d after immunization by flow cytometry. P values are as follows: WT T+WT B vs. *Def6*$^{tr/tr}$ T+WT B, NS; WT T + *S70*$^{-/-}$ B vs. *Def6*$^{tr/tr}$ T + *S70*$^{-/-}$ B, $P < 0.005$; WT T + *Def6*$^{tr/tr}$ B vs. *Def6*$^{tr/tr}$ T + *Def6*$^{tr/tr}$ B, $P < 0.05$; WT T + DKO B vs. *Def6*$^{tr/tr}$ T + DKO B, $P < 0.01$; WT T+WT B vs. WT T + *S70*$^{-/-}$ B cells, $P < 0.005$; WT T + WT B vs. WT T + *Def6*$^{tr/tr}$ B cells, $P < 0.05$; WT T + WT B vs. WT T + DKO B cells, $P < 0.005$; *Def6*$^{tr/tr}$ T + WT B vs. *Def6*$^{tr/tr}$ T + *S70*$^{-/-}$ B cells, NS; *Def6*$^{tr/tr}$ T + WT B vs. *Def6*$^{tr/tr}$ T + Def6tr/tr B cells, $P < 0.01$; *Def6*$^{tr/tr}$ T + WT B vs. *Def6*$^{tr/tr}$ T + DKO B cells, $P < 0.005$.

Table 2. Percentages of $B220^{lo}CD138^+$ cells after transfer into *Rag1*$^{-/-}$ mice

T cells	B cells	%$B220^{lo}CD138^+$
		%
WT	WT	0.3 ± 0.2
Def6$^{tr/tr}$	WT	0.3 ± 0.3
WT	*S70*$^{-/-}$	0.8 ± 0.3
Def6$^{tr/tr}$	*S70*$^{-/-}$	1.5 ± 0.7
WT	*Def6*$^{tr/tr}$	0.2 ± 0.4
Def6$^{tr/tr}$	*Def6*$^{tr/tr}$	0.3 ± 0.8
WT	DKO	0.7 ± 0.2
Def6$^{tr/tr}$	DKO	1.5 ± 0.7

Percentage of $B220^{lo}CD138^+$ cells after transfer of indicated combinations of T and B cells into *Rag1*$^{-/-}$ mice followed by immunization with SRBCs. $B220^{lo}CD138^+$ cells were evaluated 7 d after immunization by flow cytometry. P values are as follows, WT T+WT B vs. *Def6*$^{tr/tr}$ T+WT B, NS; WT T + *S70*$^{-/-}$ B vs. *Def6*$^{tr/tr}$ T + *S70*$^{-/-}$ B, $P < 0.05$; WT T + *Def6*$^{tr/tr}$ B vs. *Def6*$^{tr/tr}$ T + *Def6*$^{tr/tr}$ B, NS; WT T+DKO B vs. *Def6*$^{tr/tr}$ T + DKO B, $P < 0.01$; WT T+WT B vs. WT T + *S70*$^{-/-}$ B cells, $P < 0.01$; WT T + WT B vs. WT T + *Def6*$^{tr/tr}$ B cells, NS; WT T + WT B vs. WT T+ DKO B cells, $P < 0.005$; *Def6*$^{tr/tr}$ T + WT B vs. *Def6*$^{tr/tr}$ T + *S70*$^{-/-}$ B cells, $P < 0.05$; *Def6*$^{tr/tr}$ T + WT B vs. *Def6*$^{tr/tr}$ T + *Def6*$^{tr/tr}$ B cells, NS; *Def6*$^{tr/tr}$ T + WT B vs. *Def6*$^{tr/tr}$ T + DKO B cells, $P < 0.005$.

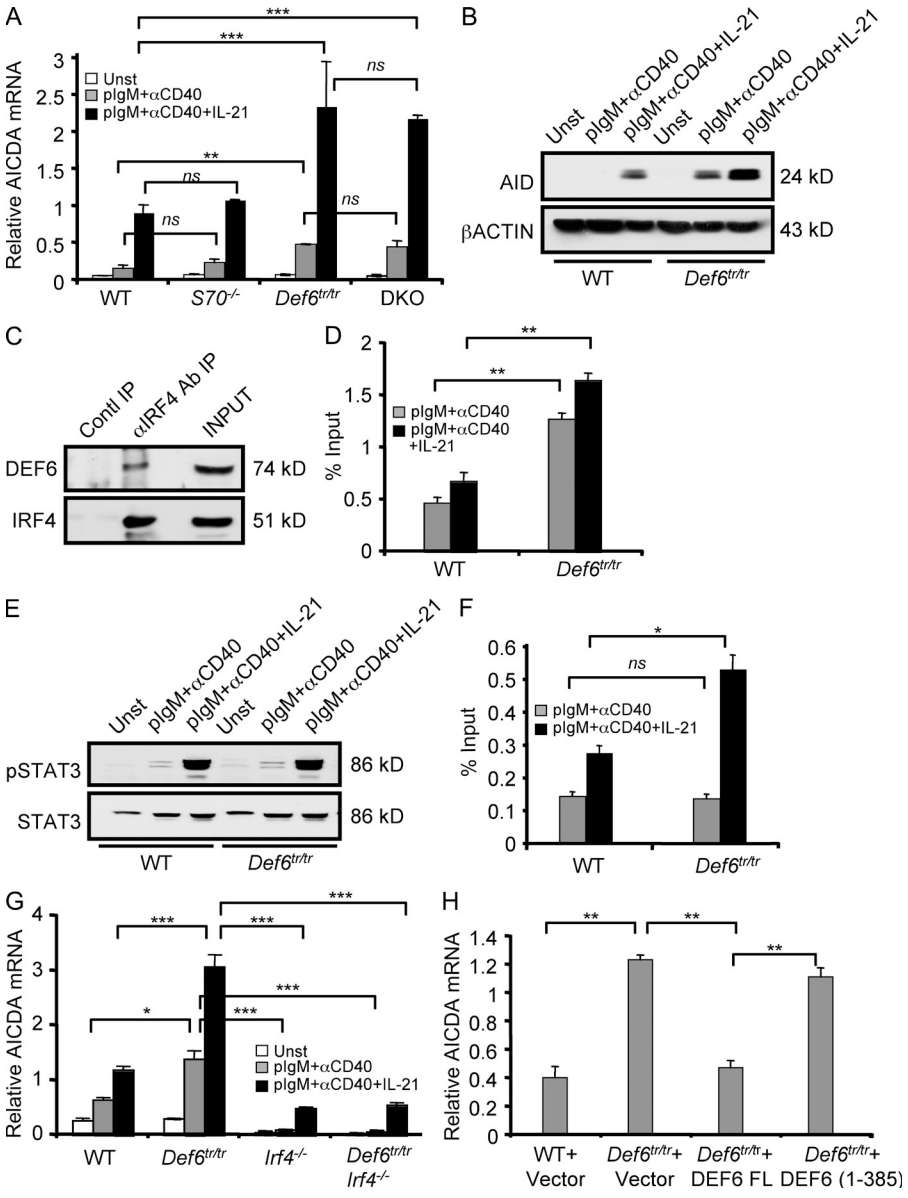

Figure 5. DEF6 regulates the expression of AID in antigen-engaged B cells by controlling IRF4 function. (A) Purified B cells from indicated 8–10-wk-old female mice were stimulated with single-round pulse αIgM (plgM) followed by stimulation with an αCD40 antibody in the presence or absence of 50 ng/ml IL-21. 48 h after stimulation, *Aicda* mRNA expression was measured by real-time RT-PCR. The data are representative of three independent experiments. (B) Purified B cells from female WT and *Def6*$^{tr/tr}$ mice were stimulated as in A. 48 h later, extracts were obtained and analyzed by Western blot using an anti-AID antibody (top). The blot was reprobed with an antibody against β-actin as a loading control (bottom). The data are representative of three independent experiments. (C) Purified B cells from female WT mice were stimulated as in A. Nuclear extracts were then prepared and immunoprecipitated with anti-IRF4 (αIRF4 Ab IP) or with a control antibody (Contrl IP). The immunoprecipitates were then analyzed by immunoblotting using an antiserum against DEF6 (top). The blot was later stripped and reprobed with an IRF4 antibody (bottom). The data are representative of four independent experiments. (D) ChIP assays were performed on purified B cells from female WT and *Def6*$^{tr/tr}$ mice stimulated as in A with either an anti-IRF4 antibody or a control antibody, and real-time PCR for the *Aicda* promoter was performed as indicated. The data are representative of two independent experiments. (E) Purified B cells from female WT and *Def6*$^{tr/tr}$ mice were stimulated as in A. 48 h later, extracts were obtained and analyzed by Western blot using an antibody recognizing phospho-STAT3 (Y705; top). The blot was reprobed with an antibody recognizing total STAT3 (bottom). The data are representative of two independent experiments. (F) ChIP assays were performed on purified B cells from female WT and *Def6*$^{tr/tr}$ mice stimulated as in A with either an anti-STAT3 antibody or a control antibody, and real-time PCR for the *Aicda* promoter was performed as indicated. The data are representative of two independent experiments. (G) Purified B cells from 8–10-wk-old WT, *Def6*$^{tr/tr}$, *Irf4*$^{-/-}$, and *Def6*$^{tr/tr}$*Irf4*$^{-/-}$ female mice were stimulated as described in A. 48 h after stimulation, *Aicda* mRNA expression was measured by real-time PCR. The data are representative of three independent experiments. (H) Purified B cells from female WT and *Def6*$^{tr/tr}$ mice were retrovirally transduced with a control vector or with vectors expressing either a full-length form of DEF6 (DEF6 FL) or DEF6 (1–385) in presence of stimulation with plgM+αCD40. 5 d after stimulation, cells were harvested and sorted for YFP$^+$ cells. Sorted YFP$^+$ cells were then subjected to real-time RT-PCR for *Aicda* mRNA expression. The data are representative of two independent experiments. Throughout the figure white bars represent unstimulated cells, gray bars represent cells stimulated with plgM+αCD40 and black bars represent cells stimulated with plgM+αCD40+IL-21. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ns, not statistically significant.

observed (Fig. 6 A). Interestingly, although no differences in IL-21R expression could be observed in B cells from the 4 genotypes (unpublished data), B cells lacking SWAP-70 were found to generate a greater number of plasma cells than WT B cells especially when the cultures were supplemented with IL-21 (Fig. 6 B). This effect could be observed even at early time points in the culture (unpublished data). In line with the transfer experiments, B cells from DKO female mice behaved similarly to *Swap-70*$^{-/-}$ B cells, whereas *Def6*$^{tr/tr}$ B cells generated an equivalent number of plasma cells as WT B cells. Consistent with the known role of Blimp-1 as the master regulator of plasma cell differentiation, *Swap-70*$^{-/-}$ and DKO B cells expressed higher levels of *Prdm1* mRNA than WT or *Def6*$^{tr/tr}$ B cells (Fig. 6, C and D). Thus, these data indicate that SWAP-70 inhibits IL-21–driven plasma cell differentiation.

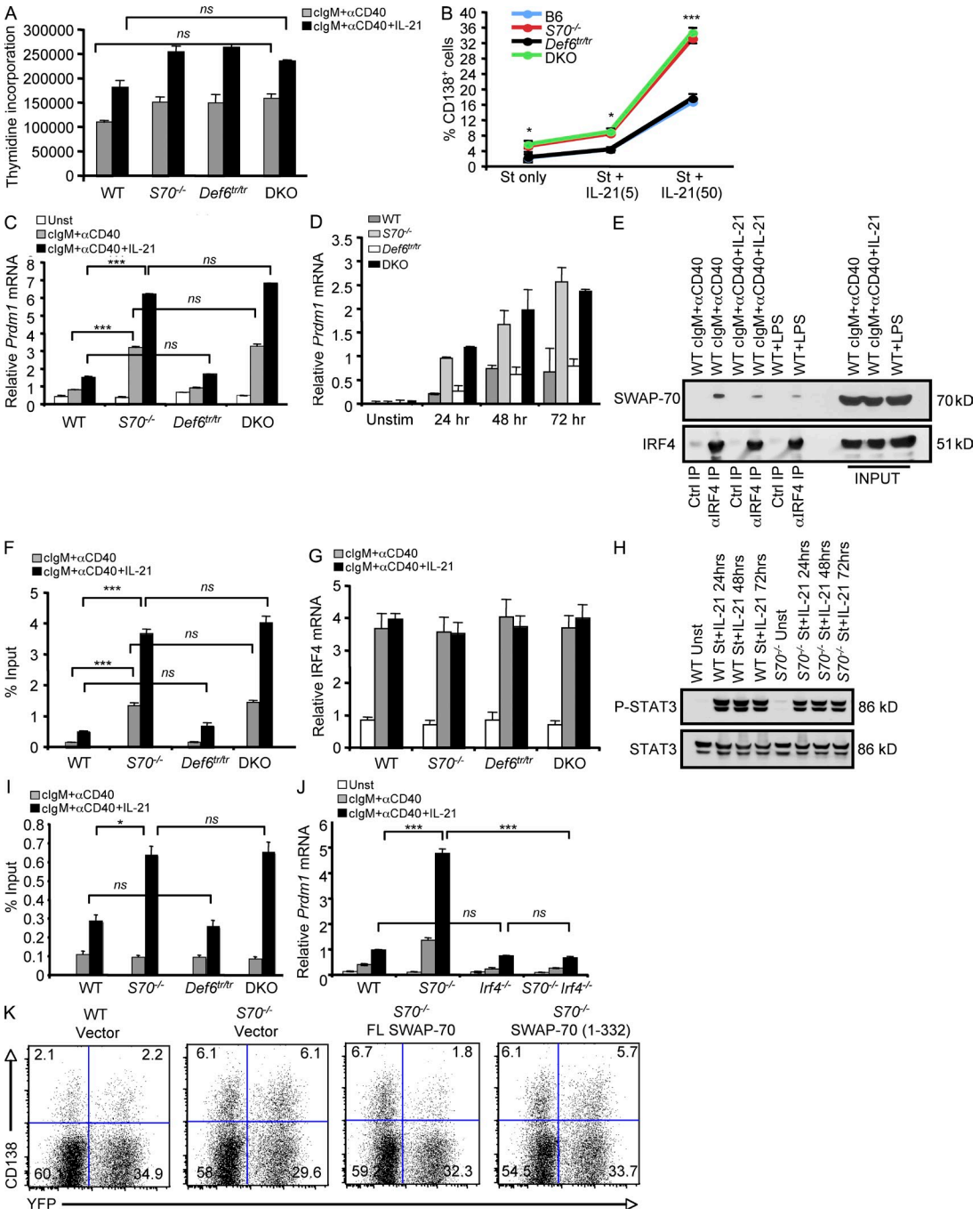

Figure 6. SWAP-70 regulates IRF4 function during IL-21–induced plasma cell differentiation. (A) Purified B cells from indicated 8–10-wk-old female mice were stimulated with continuous αIgM (cIgM) and αCD40 in the presence or absence of 50 ng/ml IL-21. After 96 h of stimulation, proliferation of B cells was measured by thymidine incorporation. The data are representative of three independent experiments. (B) Purified B cells were stimulated with cIgM and αCD40 in presence or absence of IL-21 (5 or 50 ng/ml, as indicated). After 4 d of culture, in vitro plasma cell differentiation was measured by quantifying the percentage of $CD138^+$ cells by FACS. The data are representative of three independent experiments. (C) Purified B cells were stimulated as in A. 72 h after culture, cells were harvested, and mRNA levels of *Prdm1* were measured by real-time PCR. The data are representative of three independent experiments. (D) Purified B cells were stimulated as in A. At the times indicated, cells were harvested and mRNA levels of *Prdm1* were measured by real-time PCR. The data are representative of two independent experiments. (E) Purified B cells from WT or $S70^{-/-}$ female mice were stimulated as indicated. 48 h after stimulation, nuclear extracts were prepared and immunoprecipitated with an anti-IRF4 antibody (αIRF4 IP) or with a control antibody (Ctrl IP). The immunoprecipitates were resolved by 8% SDS-PAGE, and then analyzed by Western blot using an anti–SWAP-70 antiserum (top). The blot was reprobed with an anti-IRF4 antibody (bottom). The data are representative of two independent experiments. (F) Purified B cells were stimulated for 48 h, as indicated in A. ChIP assays were performed with either an anti-IRF4 antibody or a control antibody. Real-time PCR for the IL-21 re-

Because IRF4 can control plasma cell generation by regulating the expression of Blimp-1 (Kwon et al., 2009), we explored the possibility that SWAP-70, given its high degree of structural homology to DEF6 (Gupta et al., 2003b; Tanaka et al., 2003), could also interact with IRF4 and thereby regulate its capacity to promote plasma cell differentiation in response to T cell help. We first performed coimmunoprecipitation experiments to assess whether SWAP-70 and IRF4 could interact in B cells. These experiments demonstrated that nuclear SWAP-70 can indeed physically interact with IRF4 in B cells stimulated with cIgM and αCD40 (Fig. 6 E). Interestingly, the interaction between SWAP-70 and IRF4 was weaker when these experiments were conducted in the presence of IL-21 or in LPS-stimulated B cells, suggesting that stimuli known to strongly drive plasma cell differentiation may be able to modulate this interaction.

ChIP assays were subsequently conducted to assess the effects of the absence of SWAP-70 on the recruitment of IRF4 to the IL-21 response element, a crucial functional element in the *Prdm1* gene that is cooperatively targeted by IRF4 and STAT3 (Kwon et al., 2009). Stimulated *Swap70*$^{-/-}$ B cells exhibited increased binding of IRF4 to this crucial regulatory element when compared with WT B cells, despite expressing similar IRF4 levels (Fig. 6, F and G). Presence of IL-21 further augmented this effect. In contrast, the absence of DEF6 alone did not alter the binding of IRF4 to the *Prdm1* IL-21 response element. B cells from the DKO mice exhibited a pattern similar to the one displayed by B cells lacking SWAP-70 alone. Given the known ability of STAT3 to bind to this element together with IRF4 (Kwon et al., 2009), we also examined the recruitment of STAT3 to this region. No STAT3 phosphorylation was observed in the absence of IL-21 stimulation in either WT or *Swap-70*$^{-/-}$ B cells (unpublished data). Although, in response to IL-21, STAT3 was phosphorylated to a similar extent in WT and *Swap-70*$^{-/-}$ B cells (Fig. 6 H), *Swap-70*$^{-/-}$ B cells exhibited enhanced binding of STAT3 to the *Prdm1* gene in the presence of IL-21 (Fig. 6 I). The abnormalities displayed by the DKO B cells mirrored those of B cells lacking SWAP-70 alone, whereas DEF6-deficient B cells behaved similarly to WT B cells. Thus, SWAP-70 restricts the recruitment of IRF4 and STAT3 to the *Prdm1* IL-21 response element.

To gain additional support for the role of IRF4 in mediating the enhanced IL-21–driven plasma cell differentiation observed in *Swap-70*$^{-/-}$ B cells, we explored the effects of concomitantly deleting IRF4 in *Swap-70*$^{-/-}$ B cells (Fig. 6 J). Absence of IRF4 in *Swap-70*$^{-/-}$ B cells prevented the aberrant induction of Blimp-1 observed in these cells upon exposure to T cell–dependent stimuli. Because a deletional analysis revealed that the C-terminal portion of SWAP-70 is critical for its interaction with IRF4 (unpublished data), we next used a mutant of SWAP-70 that is unable to interact with IRF4 (SWAP-70 1–332) in retroviral transduction experiments (Fig. 6 K). These experiments demonstrated that the increased generation of plasma cells observed in *Swap-70*$^{-/-}$ B cells could be corrected by full-length SWAP-70, but not by SWAP-70 (1–332). Altogether, these data support the notion that SWAP-70 regulates the ability of IRF4 to regulate T cell–induced plasma cell differentiation.

DISCUSSION

The molecular mechanisms that ensure the precise orchestration of T and B cell interactions and the factors that perturb such regulatory mechanisms are not fully understood. Here we demonstrate that mice deficient in both DEF6 and SWAP-70, the only two members of a unique family of molecules, develop aberrant T-dependent humoral responses and a lupus-like disorder that, similarly to human SLE, preferentially affects the female gender. One of the key mechanisms by which DEF6 controls T-dependent humoral responses is via its ability to tightly regulate the production of IL-21 by CD4$^+$ T cells, an effect that we have previously shown to be dependent on the ability of DEF6 to control IRF4 function (Biswas et al., 2010; Chen et al., 2008). Consistent with studies in other autoimmune models (Bubier et al., 2009; Jang et al., 2009), deletion of IL-21 corrects the B cell abnormalities observed in our mice supporting the idea that, in this system, deregulated production of IL-21 primarily exerts its effects by driving aberrant B cell responses. Although we have not observed any in vitro abnormalities in the DKO B cells upon exposure to IL-17 (unpublished data) it is possible that the aberrant production of IL-17 by *Def6*$^{tr/tr}$ T cells might also augment the autoimmune responses observed in the DKO mice as previously shown in other autoimmune models (Hsu et al., 2008; Wu et al., 2010).

sponse element in the *Prdm1* gene was performed as indicated. The data are representative of two independent experiments. (G) Purified B cells were stimulated as indicated in A. 48 h after stimulation, *Irf4* mRNA expression was evaluated by real-time PCR. The data are representative of two independent experiments. (H) Purified B cells from WT and *S70*$^{-/-}$ female mice were stimulated as indicated. The phosphorylation of STAT3 at the different time points was evaluated by Western blot with an antibody recognizing phospho-STAT3 (Y705; top). The blot was reprobed with an antibody recognizing total STAT3 (bottom). The data are representative of two independent experiments. (I) Purified B cells were stimulated as in A. 48 h after culture, ChIP assays were performed with either an anti-STAT3 antibody or a control antibody, and real-time PCR for the *Prdm1* IL-21 response element was performed as indicated. The data are representative of two independent experiments. (J) Purified B cells were stimulated as in A. 72 h after culture, cells were harvested, and mRNA levels of *Prdm1* were measured by real-time PCR. The data are representative of three independent experiments. (K) Purified B cells from female WT and *S70*$^{-/-}$ mice were retrovirally transduced with a control vector or with vectors expressing either a full-length form of SWAP-70 (FL SWAP-70) or SWAP-70 (1–332) in presence of stimulation with cIgM+αCD40. 5 d after stimulation, cells were stained for CD138 and then analyzed by FACS for CD138 expression. The data are representative of two independent experiments. Throughout the figure white bars represent unstimulated cells, gray bars represent cells stimulated with cIgM+αCD40, and black bars represent cells stimulated with cIgM+αCD40+IL-21. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ns, not statistically significant.

Although Tfh cells are normally viewed as the key source of IL-21 in the course of T cell–dependent B cell responses (Crotty, 2011), in autoimmune models such as the BXSB-Yaa and the MRL/lpr mice, one can observe the expansion of extrafollicular T helper cells, which express high levels of ICOS and IL-21 but do not express high-levels of CXCR5 (Odegard et al., 2008; Bubier et al., 2009). Because the DKO mice exhibited increased numbers of Tfh cells and an expanded population of $CXCR5^{-/lo}PD\text{-}1^{hi}Bcl\text{-}6^{int}$ T cells that might represent extrafollicular T helper cells, this latter subset may also contribute to the aberrant production of IL-21 in the DKO mice. Interestingly, in some settings, PD-1 on Tfh cells can regulate the survival of GC B cells and the production of long-lived plasma cells (Good-Jacobson et al., 2010; Hams et al., 2011), raising the possibility that abnormal up-regulation of PD-1 by these extrafollicular T helper cells may also participate in promoting the aberrant humoral responses detected in the female DKO mice. Experiments are presently being conducted to gain additional insights into the precise localization of these distinct subsets in the lymphoid organs of DKO female mice.

Although B cells express both DEF6 and SWAP-70, our studies now provide evidence that DEF6 and SWAP-70 do not appear to function in a redundant manner in this compartment. Our in vivo findings support the idea that DEF6 regulates the early phases of activation that are triggered upon the encounter of an antigen-specific B cell with its cognate T helper cell. One of the key outcomes of these productive engagements, which are critically mediated by the CD40L–CD40 interaction and the production of cytokines, is the up-regulation of AID expression (Ramiro et al., 2007; Delker et al., 2009). Consistent with the known role of IRF4 in the control of AID expression (Klein et al., 2006; Sciammas et al., 2006), our studies demonstrate that DEF6 inhibits the expression of AID by restricting the ability of IRF4 to bind to the *Aicda* promoter, suggesting that the DEF6–IRF4 interaction is a new component of the complex regulatory networks, which have recently been implicated in the control of AID expression (Park et al., 2009; Tran et al., 2010; Ise et al., 2011). Interestingly, the expression of IRF4 can be regulated by estrogen (Carreras et al., 2010), suggesting that deregulation of IRF4 activity could exert more profound pathogenic consequences in the female gender. Whether this effect plays a role in the known estrogen-mediated regulation of AID expression (Pauklin et al., 2009; Mai et al., 2010), and whether it contributes to the striking gender bias observed in the development of lupus-like disease in the DKO mice, however, remains to be established. Studies are now in progress to further delineate the role of estrogen in the pathophysiology that develops in DKO mice.

Although DEF6 in B cells appears to primarily control the early phases of B cell differentiation during T cell–dependent responses, the presence of SWAP-70, as shown by these and previous studies (Quemeneur et al., 2008), is required for the proper control of terminal B cell differentiation in response to T cell–dependent antigens. In line with these results, we have found that expression of DEF6 decreases in GC B cells and that plasma cells express lower levels of both DEF6 and SWAP70 (unpublished data). Our findings demonstrate that SWAP-70, similarly to DEF6, can physically interact with IRF4 and that this interaction is critical for the ability of SWAP-70 to regulate T cell–driven plasma cell differentiation because it restricts the ability of IRF4 to access the *Prdm1* regulatory regions, thus limiting the expression of Blimp-1. Thus, in addition to the known requirement for high levels of IRF4 expression (Sciammas et al., 2006), plasma cells may also need to decrease the expression of SWAP-70 to ensure that IRF4 is fully functional. We have recently observed that, in T cells, DEF6 can also regulate the phosphorylation of IRF4 by ROCK2 (Biswas et al., 2010), but addition of ROCK inhibitors did not affect the differentiation abnormalities observed in DEF6-deficient or SWAP-70–deficient B cells in our in vitro systems (unpublished data).

One of the striking features of B cells lacking either DEF6 or SWAP-70 is that these cells become hyperresponsive to IL-21. One of the key mechanisms responsible for this effect is that, in the absence of DEF6 or SWAP-70, deregulated binding of IRF4 to its target genes also results in enhanced recruitment of STAT3 upon exposure to IL-21. Thus, by inhibiting IRF4, DEF6 and SWAP-70 can prevent its known capacity to recruit and/or cooperate with STAT3 (Kwon et al., 2009), and thus regulate the IL-21 responsiveness of B cells. Importantly, the differential ability of DEF6 and SWAP-70 to control IRF4 function at distinct stages of B cell differentiation can help ensure that IRF4, and subsequently STAT3, only access restricted sets of target genes that are appropriate for the specific differentiation step. This, in turn, can enable IL-21 to act in a stage-specific manner, and thus play a multifaceted role in humoral responses.

One of the major benefits of using a complex system of regulators and counter-regulators is that it can help ensure that only appropriately selected B cells proceed along specific differentiation pathways and that this process is tightly controlled in time and space. The remarkable alterations in splenic lymphoid architecture and the spontaneous development of a lupus-like syndrome in mice lacking both DEF6 and SWAP-70 support the idea that these two molecules are key components of the regulatory networks that ensure the precise orchestration of these processes. Intriguingly, although the lack of DEF6 alone can result in the spontaneous development of autoimmune syndromes in other strains (Chen et al., 2008; Fanzo et al., 2006) it fails to do so in C57BL/6 mice, where the concomitant lack of SWAP-70 is required. Given the profound B cell abnormalities observed in the DKO mice, the known ability of B cells to promote T helper responses (Crotty, 2011), and the known differences in thresholds for maintaining B cell tolerance among strains (Putterman and Diamond, 1998), a likely scenario is that, although the lack of DEF6 alone predisposes toward autoimmunity, this predisposition can only convert to full-blown autoimmunity in the context of a "permissive" B cell environment. Interestingly, *Swap-70*$^{-/-}$ mice

on a 129/Sv background developed autoantibodies with age (Borggrefe et al., 2001) suggesting that the absence of SWAP-70 alone can also predispose to autoimmunity in specific genetic settings.

The concomitant absence of DEF6 and SWAP-70 altered some of the B cell phenotypes detected in mice lacking SWAP-70 alone. Indeed, in contrast to *Swap-70*$^{-/-}$ mice, which exhibit decreased GC formation upon TD immunizations (Quemeneur et al., 2008), DKO mice displayed enhanced GC formation. In view of our findings that SWAP-70 inhibits the ability of IRF4 to up-regulate Blimp-1, B cells lacking SWAP-70 alone would be predicted to prematurely up-regulate Blimp-1 upon exposure to T cell help and might thus preferentially commit to an extrafollicular plasma cell fate. Such a commitment would be modified by the simultaneous absence of DEF6 because the lack of DEF6 imparts B cells with an increased capability of becoming a GC B cell. Thus, consistent with the phenotype of the DKO, the simultaneous absence of DEF6 and SWAP-70 would lead to both increased GC formation and plasma cell differentiation upon exposure to TD antigens.

Although our studies have mostly focused on the inhibitory roles of DEF6 and SWAP-70 on lymphocyte function, it is important to note that DEF6 and SWAP-70 do not exclusively function as negative regulators of T and B cell activation. For instance although DEF6 restricts the ability of T cells to produce IL-17 and IL-21 under neutral conditions (Chen et al., 2008; Biswas et al., 2010), it promotes Th17 differentiation upon exposure to a proinflammatory environment (Canonigo-Balancio et al., 2009). A similar complex role is emerging for SWAP-70. Although, as demonstrated here and in a previous study (Quemeneur et al., 2008), it functions as an inhibitor of plasma cell differentiation under T cell–dependent conditions, it can promote the initiation of plasma cell differentiation under T cell–independent conditions (Chopin et al., 2011). Interestingly, this effect appears to be caused by the ability of SWAP-70 to fine-tune IRF4 expression, highlighting the intricate cross-talk between IRF4 and this family of molecules. In addition to regulating gene expression, DEF6 and SWAP-70 can also regulate cytoskeletal reorganization as a result of their ability to control the activation of Rac and Cdc42 (Shinohara et al., 2002; Gupta et al., 2003a; Bécart et al., 2008). The employment of molecules like DEF6 and SWAP-70, which can simultaneously control gene expression programs and cytoskeletal dynamics, might thus provide lymphocytes with a unique molecular system to precisely couple the acquisition of specific differentiation programs with their localization and even with their antigen responsiveness. These molecules thus likely do not act simply as "inhibitors" of lymphocyte activation but rather might function as "sensors," helping ensure that lymphocyte activation is regulated in a dynamic and plastic manner, and thus enabling immune responses to be appropriately tailored to the offending pathogen.

MATERIALS AND METHODS

Mice. DEF6-deficient (*Def6*$^{tr/tr}$) mice were generated by Lexicon Pharmaceuticals (OmniBank) using a gene trapping strategy as previously described (Fanzo et al., 2006). These mice were backcrossed onto a C57BL/6 background for >10 generations. *Swap-70*$^{-/-}$ mice were generated by R. Jessberger (Institute of Physiological Chemistry, Dresden University of Technology, Dresden, Germany; Borggrefe et al., 2001) and were also backcrossed onto a C57BL/6 background for >10 generations. *Irf4*$^{-/-}$ mice on a C57BL/6 background were obtained from T. Mak at University of Toronto (Toronto, Canada; Mittrücker et al., 1997). To generate *Def6*$^{tr/tr}$*Swap-70*$^{-/-}$ (DKO) mice, *Def6*$^{tr/tr}$ mice were crossed with *Swap70*$^{-/-}$ mice that had been backcrossed onto the C57BL/6 background. To generate *Def6*$^{tr/tr}$*Irf4*$^{-/-}$ and *Swap-70*$^{-/-}$*Irf4*$^{-/-}$, *Irf2*$^{-/-}$ mice were crossed with either *Def6*$^{tr/tr}$ or *Swap-70*$^{-/-}$ mice, respectively. *IL21*$^{-/-}$ mice on mixed strain background were obtained from the Mutant Mouse Regional Resource Centers (Lexicon strain ID 011723-UCD), and then crossed with *Def6*$^{tr/tr}$129/SvJ mice to generate *Def6*$^{tr/tr}$*IL21*$^{-/-}$ mice. 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 and The Hospital for Special Surgery.

Flow cytometry. Single-cell suspensions from thymus, spleen, and lymph nodes were isolated, stained with Biotin- or Fluorochrome-conjugated CD3e (eBioscience), CD4 (eBioscience), CD8 (eBioscience), CD25 (eBioscience), CD44 (eBioscience), CD62L (eBioscience), CD69 (eBioscience), ICOS (eBioscience), PD-1 (eBioscience), CXCR5 (eBioscience), Foxp3 (eBioscience), B220 (eBioscience), CD21 (BD), CD23 (BD), IgM (eBioscience), IgD (eBioscience), CD138 (BD), IgG1 (eBioscience), and PNA (Jackson ImmunoResearch Laboratories) and analyzed by FACS. To enumerate the number of NP-specific B cells in spleen, spleen cells were stained with NP-PE (Biosearch Technologies) 7 and 14 d after NP-KLH immunization, as previously described (Quemeneur et al., 2008). Data were analyzed using FlowJo (Tree Star) software, as previously described (Chen et al., 2008). For intracellular staining, splenocytes were stimulated with 50 μg/ml PMA and 1 μM Ionomycin (EMD) in the presence of 1 μM Monensin (eBioscience) for 5 h. After stimulation, cells were subjected to intracellular staining for IL-17 and IL-21 cytokines using anti–mouse IL-17 PE (BD) and anti–human IL-21R Fc (R&D Systems) antibodies. FITC-conjugated anti–human antibody (Jackson ImmunoResearch Laboratories) was used as a secondary antibody.

Immunizations. In SRBC immunizations, 8–10-wk-old female mice were immunized once intraperitoneally with 1×10^5 sheep red blood cells in PBS (Cocalico Biologicals), as per standard protocol (Klein et al., 2006). Control mice received PBS only. 7 d after immunization, control and immunized mice were sacrificed and spleens were harvested for flow cytometric analysis. In NP-KLH immunizations, 8–10-wk-old female mice were immunized once intraperitoneally with NP-KLH (100 μg per mouse; Biosearch Technologies) in complete Freund's adjuvant, as per standard protocol (Klein et al., 2006). 7 and 14 d after immunization, control and immunized mice were sacrificed and spleens and bone marrow were analyzed for anti–NP-KLH response. ELISPOT assays were performed as previously described (Quemeneur et al., 2008).

Histopathology, immunohistochemical, and immunofluorescence 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. The severity of glomerulonephritis was assessed as previously described (Atkinson et al., 2008). Immunofluorescence analysis on frozen kidney sections was performed by staining with FITC-labeled goat anti–mouse IgG or anti-C3 (Jackson ImmunoResearch Laboratories) and specimens were analyzed with a LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc.). The severity of immune complex deposition was determined in a blinded manner according to previously described parameters (Atkinson et al., 2008).

For immunofluorescence staining of the spleens, the spleen tissue was embedded in Tissue-tek (OCT compound; Sakura) and snap-frozen. 8-µm cryostat sections were air dried for an hour before staining. The sections were fixed for 10 min with ice-cold acetone, and then incubated for 1 h at room temperature with the primary antibody (biotinylated peanut agglutinin [Vector Laboratories], rabbit anti–mouse IgG (H+L)-biotin [Southern Biotech], rat anti–mouse IgM-biotin [BD], and PE anti–mouse CD45R/B220 [BD]). Primary antibodies were diluted in PBS/0.5% BSA. Sections were then incubated for 1 h with Alexa Fluor 488–conjugated Streptavidin (Invitrogen) before mounting in Gel/Mount (Biomeda). Samples were analyzed using a microscope (Eclipse E600; Nikon) and images were captured by Q capture software (version 2.68.4). The channels were overlaid using Photoshop software (Adobe).

Serum autoantibodies. Serum levels of anti-dsDNA were determined by ELISA (Alpha Diagnostics) as previously described (Chen et al., 2008; Fanzo et al., 2006). Autoantibody array was performed in UTSW Genomics and Microarray Core Facility as previously described (Li et al., 2005). For intergroup comparisons, the Student's *t* test was used. Heat map diagrams with row-wise and column-wise clustering were generated using Cluster and TreeView software. In these diagrams, fluorescence intensities that were higher than the row mean were colored red, those that fell below the row mean were colored green, and cells with signals close to the mean were left black.

In vitro B cell assays. Resting B cells ($CD43^-$ B cells) were purified from mice by negative selection using CD43 microbeads (Miltenyi Biotec). B cell assays were performed as previously described (Kuchen et al., 2007). In brief, for short stimulation, B cells were incubated with 10 µg/ml goat anti–mouse IgM $F(ab')_2$ (Jackson ImmunoResearch Laboratories) at 4°C for 30 min. Unbound anti-IgM was removed from the medium by washing and centrifuging the cells at 4°C. The cells were resuspended in chilled complete medium and shifted to 37°C by placement in an incubator or in water bath. 4 h later, 5 µg/ml monoclonal anti-CD40 (BD) in the presence or absence of 50 ng/ml mouse IL-21 (R&D Systems) was added, and the cells were further incubated for various time periods depending on the assay. For plasma cell studies, B cells (10^6/well) were stimulated with αIgM (5 µg/ml; Jackson ImmunoResearch Laboratories) and αCD40 (2 µg/ml) for 96 h in the presence or absence of recombinant mouse IL-21 (50 ng/ml). For proliferation assays, cultures were pulsed with $[^3H]$thymidine (1 µCi/well; PerkinElmer) for the last 12 h of culture. In vitro differentiation of plasma cells was determined by FACS after staining with anti-CD138 antibody (BD).

Real-time RT-PCR. Total RNA was isolated from cells or organs using RNeasy Mini kit (QIAGEN). 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 *Prdm1*, *Aicda* were purchased from QuantiTect Primer Assay (QIAGEN). The expression of each gene was normalized to the expression of β-actin.

Cell extracts, Western blotting, and coimmunoprecipitation. Nuclear and cytoplasmic extracts were prepared using 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 (Santa Cruz Biotechnology, Inc.) and β-tubulin (Sigma-Aldrich). For coimmunoprecipitation experiments, cell lysates were immunoprecipitated with an anti-IRF4 antibody, as previously described (Chen et al., 2008). The immunoprecipitates were resolved by 8% SDS-PAGE, transferred to a nitrocellulose membrane, and then immunoblotted with either an anti–SWAP-70 antibody (Santa Cruz Biotechnology, Inc.) or anti-DEF6 antiserum (Chen et al., 2008). For the AID Western blots, the blots were probed with an anti-AID antibody, which was a gift from J. Chaudhuri (Memorial Sloan-Kettering Cancer Center, New York, NY; Chaudhuri et al., 2003).

ChIP assays. ChIP assays were performed as previously described (Chen et al., 2008). In brief, $CD43^-$ B cells were purified and either left unstimulated or stimulated for 48 h. 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 IRF4-specific antibody or a control antibody. After cross-linking was reversed and proteins were digested, the DNA was purified from the immunoprecipitates as well as from input extracts, and then analyzed by quantitative PCR using primers specific for the murine *AICDA* promoter (Lee et al., 2006), and the IL-21 response element in the *Prdm1* gene (Kwon et al., 2009; forward, 5′-AACCGTTGAAAGACGGTGACTGA-3′; reverse, 5′-CACATCATC-TGTGCTGGAGGCA-3′) as previously described (Chen et al., 2008).

Adoptive transfer experiments. For transfer experiments, total $CD4^+$ T cells purified from WT and $Def6^{tr/tr}$ mice were mixed with $CD43^-$ B cells from all the four genotypes (WT, $S70^{-/-}$, $Def6^{tr/tr}$, and DKO) at 1:2 ratio and transferred in $Rag1^{-/-}$ recipient mice. 21 d after transfer, recipient mice were checked for successful reconstitution followed by immunization with 2% SRBC. 7 d after immunization, spleens of recipient mice were analyzed for B cell response to SRBC immunization.

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