IL-27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells

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Maturation and selection of high-affinity B cell clones in the germinal center (GC) relies on support from T follicular helper (TFLH) cells. TFLH cells are characterized by their localization to the B cell follicle and their high expression of the costimulatory molecules ICOS and PD1 and the cytokine IL-21, which promotes immunoglobulin (Ig) class switching and production by B cells. We show that the heterodimeric cytokine IL-27 is critical for the function of TFLH cells and for normal and pathogenic GC responses. IL-27 signaling to T cells results in the production of IL-21, a known autocrine factor for the maintenance of TFLH cells, in a STAT3-dependent manner. IL-27 also enhances the survival of activated CD4+ T cells and the expression of TFLH cell phenotypic markers. In vivo, expression of the IL-27Rα chain is required to support IL-21 production and TFLH cell survival in a T cell–intrinsic manner. The production of high-affinity antibodies is reduced, and pristane-elicited autoantibodies and glomerulonephritis are significantly diminished, in il27ra−/− mice. Together, our data show a nonredundant role for IL-27 in the development of T cell–dependent antibody responses.

IL-27 is a heterodimeric cytokine consisting of the protein subunits IL-27p28 and Epstein Barr virus–induced protein 3 (Pflanz et al., 2002). It signals through a heterodimeric receptor consisting of the ligand-specific IL-27Rα chain and gp130 (Pflanz et al., 2004), which is shared with several other cytokines, including the structurally related cytokine IL-6. Like IL-6, IL-27 signaling involves the activation of Jak1, STAT1, and STAT3 (Batten and Ghilardi, 2007). Despite shared use of the gp130 chain, the contribution of the IL-27Rα subunit makes IL-27 functionally distinct from IL-6 in that it promotes early aspects of TFLH differentiation, such as upregulation of the transcription factor T-bet and the IL-12 receptor β2 chain while suppressing IL-6–driven T cell proliferation and TFLH17 differentiation (Batten and Ghilardi, 2007).

In vivo, IL-27 acts to constrain inflammatory under most circumstances that have been studied to date (Batten and Ghilardi, 2007). Several possible mechanisms for this immunosuppressive activity have been identified; IL-27 is known to antagonize TFLH17 development (Batten et al., 2006; Stumhofer et al., 2006), induce IL-10 production (Awasthi et al., 2007; Fitzgerald et al., 2007; Stumhofer et al., 2007; Batten et al., 2008), and suppress IL-6–induced T cell proliferation (Batten et al., 2006). Nevertheless, IL-27 apparently plays a proinflammatory role in some situations. For example,
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**RESULTS**

**IL-27 induces IL-21 expression in vitro and in vivo**

We tested the effect of IL-27 on FACS-purified naive T cells during anti-CD3/anti-CD28 stimulation and found that the addition of IL-27 resulted in greatly elevated IL-21 mRNA expression (Fig. 1 A), peaking at ~24–48 h. This was a specific response to ligand–receptor interaction because no effect was observed on IL-27RA-deficient (Il27ra<sup>−/−</sup>) T cells (Fig. 1 A). IL-27 was unable to significantly enhance IL-21 mRNA expression in the absence of IL-21 induction. Analysis of the culture supernatants confirmed that IL-27 induces IL-21 protein production by >10-fold compared with untreated cells by 48 h (Fig. 1 B).

We also found that the addition of rhIL-27 during stimulation induced IL-21 expression by naive CD4<sup>+</sup> T cells purified from human tonsil (Fig. 1 C) and peripheral blood (Fig. 1 D). These data suggest that IL-27 has a similar role in human and mouse IL-21 induction. In comparison to IL-27, IL-12 was a more potent IL-21 stimulant in cultures of human CD4<sup>+</sup> T cells (Fig. 1 C). Because IL-27 stimulation up-regulated the expression of IL-12R<beta>2 (Fig. S1 B), thereby sensitizing the cells to the effects of IL-12, these two cytokines may act in an additive manner to enhance IL-21 expression.

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**Figure 1.** IL-27 induces IL-21 expression. (A) FACS-purified CD4<sup>+</sup>CD25<sup>−</sup> T cells isolated from either Il27ra<sup>−/−</sup> or Il27ra<sup>−/−</sup> mice were stimulated with plate-bound anti-CD3 and soluble anti-CD28 under Th0 polarizing conditions and in the presence or absence of 20 ng/ml rmIL-27 for the times indicated. IL-21 mRNA was determined by real-time RT-PCR and is presented relative to rpl19 mRNA. Data represent the mean of triplicate experimental samples and error bars indicate standard deviation. (B) CD4<sup>+</sup> T cells isolated from C57BL6 spleens were stimulated as in A (n.t., no treatment) and IL-21 protein was measured in the culture supernatant by ELISA. Error bars indicate standard deviation. (C) FACS-purified CD4<sup>+</sup>CD45RA<sup>+</sup>CCR5<sup>−</sup> cells from human tonsil were labeled with CFSE and stimulated with anti-CD3 + anti-CD28 + anti-CD2 beads (2:1 ratio of beads to cells) in the presence of no additional cytokine, 20 ng/ml rhIL-12 or IL-23, or 50 ng/ml rhIL-27 for 5 d. IL-21 expression was assessed by intracellular staining and flow cytometry and is plotted against CFSE division. The percentage of IL-21–expressing cells is indicated. Similar data were obtained from three individuals. (D) Naive CD4<sup>+</sup> T cells were FACS purified from three healthy controls and three AD-HIES patients and stimulated as in C. The mean percentage of IL-21<sup>+</sup> cells ± SEM is given. (E) Groups of Il27ra<sup>−/−</sup> (filled circles) and Il27ra<sup>−/−</sup> (open squares) mice were immunized with OVA (30 µg/mouse) in CFA. 4 and 8 d after immunization, CD4<sup>+</sup> T cells were isolated from the spleens and IL-21 mRNA was determined by real time RT-PCR (relative to rpl19). Data from individual animals are shown. Bars indicate the mean of five animals ± SEM.

* P < 0.05; ***, P < 0.001 (unpaired Student’s t test). One of three (A and B) or two (E) independent experiments is shown. C shows a single donor representative of six. D shows data combined from three independent donors.
work together to enhance IL-21 production. Indeed, IL-12 and IL-27 had an additive effect on IL-21 secretion by murine CD4+ T cells (Fig. S1 C).

A prominent feature of IL-27 signaling is the activation of STAT1; however, induction of IL-21 protein expression was not dependent on activation of this transcription factor because IL-27 was able to induce IL-21 expression by STAT1−/− CD4+ T cells (Fig. S1 D). To investigate the importance of STAT3 activation for IL-27–induced IL-21 expression, we used naive CD4+ T cells from autosomal dominant hyper IgE syndrome (AD-HIES) as a result of dominant-negative mutations in STAT3 (Minegishi et al., 2007). The addition of rhIL-27 during the stimulation of AD-HIES CD4+ T cells could no longer induce IL-21 expression (Fig. 1 D), indicating that the induction of IL-21 by IL-27 is STAT3 dependent.

Having established that IL-27 is sufficient to induce IL-21 production in vitro, we next sought to determine whether IL-27 signaling is required for IL-21 induction in vivo. To this end, we immunized WT and Il27ra−/− mice with OVA/CFA and measured Il21 mRNA expression in splenic CD4+ T cells 4 and 8 d after immunization. CD4+ T cells from Il27ra−/− mice contained significantly diminished levels of Il21 mRNA, demonstrating that IL-27 signals are nonredundant for IL-21 expression in vivo (Fig. 1 E). High levels of IL-21 mRNA expression by WT cells at day 4 compared with day 8 may reflect IL-21 expression by recently activated CD4+ T cells in addition to the fully differentiated T FH cells (King, 2009).

**T FH cells are reduced in the absence of IL-27ra**

Because Il27ra−/− mice had reduced IL-21 expression, and because IL-21 is both a differentiation factor and for hallmark cytokine of T FH cells (Nurieva et al., 2008; Vogelzang et al., 2008; Eddahri et al., 2009; Yu et al., 2009a; Linterman et al., 2010), we investigated whether these mice had a reduced T FH cell population. To this end, we immunized mice twice with TNP-OVA in Freund’s adjuvant and analyzed CD4+ T cell phenotypes in spleen and LNs 7 d after the second immunization. A statistically significant reduction in both the percentage and absolute number of PD1+CXCR5+CD4+ T cells was observed in the spleens and draining LN (DLN) of Il27ra−/− mice (Fig. 2). To ensure proper discrimination between T FH and activated T cells, we stained with additional markers and found that Il27ra−/− mice have a reduction in CD4+ T cells with a CXCR5+, PD1+, ICOS+, CCR7hi, CD62Llo, and CD127lo cell phenotype (Fig. S2 A), a population matching the published phenotype of T FH cells. Interestingly, more ICOS+ cells were observed in the LN compared with spleen, both in the CXCR5- and -negative gates, suggesting that there may be differences in the phenotype or activation level of CD4+ cells in the DLN (Fig. S2 A). Although the percentage of T FH cells was not significantly different between WT and Il27ra−/− mice before immunization (Fig. 2 B), we used a double immunization strategy to ensure that the response to immunization overcome any small baseline differences in T FH cell numbers and to provide sufficient opportunity for the development of high-affinity antibody. However, a similar requirement for Il27ra was observed 7 d after a single immunization (Fig. S2, C and D; and not depicted)

We reproducibly noted a significantly reduced population of T FH cells in both the spleen and DLN of Il27ra−/− mice; however, we consistently saw that the magnitude of the reduction was greatest in the spleen (Fig. 2 and Fig. S2). Inhibition of lymphocyte migration using FTY720 administration did not prevent the tissue-specific effect (Fig. S2 C), suggesting that IL-27–dependent lymphocyte migration is not responsible for these tissue-specific differences. Furthermore, although blockade of IL-21 using a soluble IL21R–Fc molecule diminished the percentage of CXCR5+PD1+ cells in WT mice, particularly in the LN, it was unable to further suppress T FH cell numbers in Il27ra−/− mice (Fig. S2 D), suggesting that these two cytokines work in the same pathway.

**Reduced GC activity in the absence of IL-27ra**

Mouse strains that have a deficiency of IL-21 or T FH cells also display abortive GC reactions (de Vinuea et al., 2000; Nurieva et al., 2008; Vogelzang et al., 2008). Thus, to confirm that T FH cell function was diminished, we examined the formation of GC in the spleens of immunized Il27ra−/− mice. Flow cytometric analysis showed that significantly fewer Fas+GL7+ GC B cells were present in the absence of Il27ra signaling.
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(Harris et al., 1999). In general, antibody derived from GC-independent responses appears to be unaffected by the loss of IL-27 signaling. Previous studies showed that Il<sub>27</sub>ra<sup>−/−</sup> mice displayed normal levels of total serum Ig, with the exception of IgG2a (Chen et al., 2000; Miyazaki et al., 2005). In line with these observations, we found that the early IgM response to the T cell–independent Ag, TNP-Ficoll, was similar in Il<sub>27</sub>ra<sup>+/+</sup> and Il<sub>27</sub>ra<sup>−/−</sup> mice (Fig. 3 H). Collectively, global absence of IL-27 receptor results in a reduction in T<sub>FH</sub> cells, along with diminished GC formation and selective reduction of high-affinity antibodies, whereas T cell–independent and low-affinity antibodies remain largely unchanged.

Defects in T<sub>FH</sub> and GC function in Il<sub>27</sub>ra<sup>−/−</sup> mice are T cell intrinsic

The observation that IL-27 directly promotes IL-21 expression by CD4<sup>+</sup> T cells suggested that the defect in T<sub>FH</sub> cell number and GC function in Il<sub>27</sub>ra<sup>−/−</sup> mice could result from a T cell–intrinsic defect. However, other cells, including B cells, express the IL-27 receptor, and thus it remained formally possible that the T<sub>FH</sub> defect in Il<sub>27</sub>ra<sup>−/−</sup> mice was indirect. To discriminate these two possibilities, we generated...
mixed BM chimeras using Il27ra−/− (CD45.2+ and Thy1.2+) BM mixed at a 1:1 ratio with congenic Il27ra+/+ (CD45.1+ and Thy1.2+) BM and transferred into lethally irradiated Il27ra−/−CD45.1+Thy1.1+ hosts such that Il27ra−/− donors, WT donors, and remnant WT host T cells could each be discriminated in the reconstituted chimeric mice. In such mice, WT and Il27ra−/− T cells have the same exposure to the mixed population of WT and KO Ag-presenting cells and B cells. FACS analysis of the blood of reconstituted chimeric mice revealed a similar contribution of both genotypes to T and B cell compartments before immunization (Fig. S3, A and B), confirming that absence of the Il27ra does not impair repopulation. The chimeric mice were immunized twice, and 7 d after the second immunization the contributions of the WT and Il27ra−/− cells to the total CD4+ and CD4+CXCR5+PD1+ TFH cell populations were analyzed by flow cytometry. In the total CD4+ gate, WT cells contributed with somewhat increased frequency after immunization, with the WT/KO ratio being 1.644 ± 0.14 (mean ± SEM) in the spleen and 1.366 ± 0.088 in the DLN (Fig. 4, A and B).

However, WT T cells contributed highly disproportionately to the PD1+CXCR5+ TFH gate, with a WT/KO ratio of 3.08 ± 0.3 in the spleen and 2.689 ± 0.26 in the DLN (Fig. 4, A and B). These data suggest that Il27ra−/− T cells have an intrinsic defect in their follicular helper capacity, and this defect cannot be compensated for by the presence of WT APC and B cells. Because the T FH compartment cell population of the mixed chimeric mice was comprised mainly of WT cells, GC function was restored and the levels of high-affinity class-switched Ig were comparable with mice reconstituted with 100% WT cells (Fig. S3 C).

To conclusively rule out direct effects of IL-27 on B cells in this model, we constructed BM chimeras using B cell–deficient μMT BM plus either WT or Il27ra−/− BM (at a 80:20 ratio) such that all B cells in the resultant chimeras were derived from either the WT (control) or the Il27ra−/− BM (a B cell–specific Il27ra deletion), whereas the majority of non–B cells were derived from WT BM. The absence of IL-27 signaling in the B cell compartment alone did not affect the development of T FH cells in the spleen or DLN (Fig. 4, C and D). Although the μMT chimeras generally produced lower amounts of high-affinity IgG compared with mice reconstituted with only WT BM, we noted no difference based on the Il27ra genotype of the B cells (Fig. 4 E). Thus, absence of IL-27 signaling in the B cell compartment alone affected neither the development of T FH cells nor the production of high-affinity antibody.

An adoptive transfer system, in which OVA-specific Il27ra+/+ or Il27ra−/− DO11.10 transgenic (Tg) CD4+ T cells were injected into either Il27ra+/+ or Il27ra−/− hosts before immunization, further reinforced the T cell–intrinsic nature of the GC defect. Although complete removal of IL-27Ra from the system recapitulated the Il27ra−/− phenotype, as expected, the percentage of T cells that expressed PD1 and CXCR5 was not significantly changed based on donor Il27ra genotype (Fig. S3 D). These data suggest that the differentiation of T FH cells can occur in the absence of IL-27 signaling. However, lack of Il27ra on the transferred CD4+ T cells significantly reduced their recovery rates (Fig. S3 E), with a corresponding reduction in Fas+GL7+ GC B cells (Fig. S3 F), suggesting that IL-27 supports the survival and/or proliferation of the DO11.10+ cells in a T cell–dependent manner. The effect was most striking when DO11.10+ Il27ra+/+ and DO11.10+ Il27ra−/− cells were transferred into Il27ra−/− recipients (Fig. S3 E), which is consistent with the notion that the transferred DO11.10+.Il27ra+/+ T cells have a greater
IL-27 signaling is required for T\textsubscript{FH} cell function.

Our data suggest that IL-27 is important for the differentiation and/or maintenance of T\textsubscript{FH} cells. To test whether IL-27 stimulation of CD4\textsuperscript{+} T cells directly induces differentiation of T\textsubscript{FH} cells, we performed in vitro experiments using OT-II Tg T cells. The cells were activated by OVA peptide in the presence of irradiated splenocytes and various concentrations of rmIL-27 or rmIL-21 for 5 d and then restimulated with anti-CD3 for 4 h (RNA expression) or 24 h (protein expression), as described by Nurieva et al. (2008). Under these conditions, the addition of rmIL-27 resulted in a concentration-dependent enhancement of the expression of CXCR5, PD1, and ICOS mRNA, and proteins on the cell surface (Fig. 5 and Fig. S4 A). In our hands, this enhancement was superior to rmIL-21. However, when purified CD4\textsuperscript{+} T cells were stimulated in the absence of accessory cells, ICOS, but not PD1 or CXCR5, was induced (not depicted; Pot et al., 2009). Moreover, IL-27 had little effect on Bcl-6 mRNA expression (Fig. S4 A), either under the conditions described in this paragraph or after a short-term primary stimulation (4 or 24 h; not depicted), suggesting that IL-27 did not induce full T\textsubscript{FH} differentiation. In vitro stimulation of OTII TCR Tg T cells in the presence of rmIL-21 resulted in enhanced development of GC in adoptive hosts (Nurieva et al., 2008); however, stimulation with rmIL-27 was not able to enhance B helper activity under these conditions (Fig. S4 B). Although IL-21 is highly up-regulated by IL-27 stimulation, maximal expression appears to reach around 1 ng/ml (Fig. 1 B), which is markedly less than the 50 ng/ml IL-21 used in those in vitro T\textsubscript{FH} differentiation systems. Moreover, other consequences of IL-27 signaling that diverge from that of IL-21 may also contribute to the differences observed. These data suggest that although IL-27 enhances the expression of T\textsubscript{FH} effector molecules and cytokines, it is not able to do so in the absence of additional signals and is not sufficient to produce fully differentiated T\textsubscript{FH} cells.

In adoptive transfer experiments, IL-27 signaling influenced the amount of Ag-specific CD4\textsuperscript{+} T cells that were present after immunization (Fig. S3, D–F). In vitro culture systems, we noted that the addition of rmIL-27 to Ag-stimulated CD4\textsuperscript{+} T cells enhanced the survival of these strongly stimulated T cells (Fig. 5 B and Fig. S4 C). The survival advantage conferred to cells by the addition of rmIL-27 was not dependent on IL-21R signaling (Fig. S4 D). To investigate whether the altered cell survival observed in vitro was reflected by changes in T\textsubscript{FH} cell survival in vivo, we examined the percentage of apoptotic (annexin V\textsuperscript{+}) total CD4\textsuperscript{+} and T\textsubscript{FH} cells in immunized Il27\textsuperscript{+/-} and Il27\textsuperscript{-/-} mice. We chose a time point of 4 d after immunization to examine early changes in viability and examined the percentage of annexin V\textsuperscript{+} apoptotic cells in the live (7-aminoactinomycin D [7AAD\textsuperscript{-}]) gate. Although we found that the percentage of annexin V\textsuperscript{+} cell in competitive advantage when the endogenous recipient T cells, which also participate in the response, are unable to respond to IL-27. Alternatively, the increased recovery of DO11.10\textsuperscript{+/-} Il27ra\textsuperscript{+/-} T cells from KO hosts might be the result of increased IL-27 levels in mice that lack expression of the receptor, as is observed in other receptor-deficient mice (Cho et al., 2007). Differences between WT and Il27ra\textsuperscript{-/-} cells were not evident in the LN in the adoptive transfer system (unpublished data). Together, the BM chimera and adoptive transfer data described strongly support a T cell–intrinsic defect in GC responses in Il27ra\textsuperscript{-/-} mice.

IL-27 enhances the expression of T\textsubscript{FH} cell markers and supports CD4\textsuperscript{+} T cell survival after Ag activation

In our hands, this enhancement was superior to rmIL27 for 72 h, and viability was assessed by flow cytometry using annexin V and 7AAD staining. The CD4-B220\textsuperscript{-} gate is shown. (C) Viability of cells in groups of Il27ra\textsuperscript{+/-} and Il27ra\textsuperscript{-/-} mice was assessed by flow cytometry 4 d after immunization with TNP-OVA in CFA. (C) Representative plots for annexin V staining in the CD4-B220– 7AAD– gate (Il27ra\textsuperscript{+/-}, black fill; Il27ra\textsuperscript{-/-}, gray fill) or CXCR5–PD1–CD4–B220– 7AAD– cell gate (Il27ra\textsuperscript{+/-}, dotted line; Il27ra\textsuperscript{-/-}, solid line) in the spleen or DLN. (D) The mean percentage of annexin V\textsuperscript{+} cells in the CD4–CXCR5–PD1–7AAD– gate. Error bars indicate SEM with n = 5 mice per group. Each set of data are indicative of two individual experiments. **, P < 0.01 (unpaired Student’s t test).
polyclonal hypergammaglobulinemia, and glomerulonephritis (Satoh and Reeves, 1994; Reeves et al., 2009). The IgG hypergammaglobulinemia and the development of IgG1 and IgG2a anti-nuclear antibodies appear to be dependent on T cell help (Nacionales et al., 2009). Because genetic factors influence susceptibility to TMPD-induced lupus, we used Il27ra−/− mice on the BALB/c background to study the importance of IL-27 in the development of disease in this model. 42 wk after TMPD injection, the Il27ra−/− displayed significantly reduced populations of T FH and GC B cells (Fig. 6, A and B), along with significantly lower levels of serum anti-nuclear (Fig. 6, C) and anti-dsDNA IgG (Fig. 6, D) antibodies compared with WT controls. Although a global reduction in IgG2a production in Il27ra−/− mice has been noted previously (Chen et al., 2000; Miyazaki et al., 2005), the reduction in anti-dsDNA antibodies was not confined to this isotype because IgG1 anti-dsDNA antibody was also significantly lower in Il27ra−/− serum (Fig. 6, D). Consistent with the reduction in autoantibody production, histological analysis revealed a lower mean renal histopathology score in the Il27ra−/− mice (Fig. 6, E and F) and a reduction in histological evidence of IgG immune complex deposition in the kidney (Fig. 6, G). In both groups, renal lesions consistent with SLE are mild, although there was greater individual variability with occurrence of more severe lesions in the WT group. These data demonstrate that the defects observed in GC function in the absence of IL-27 signaling are relevant in the disease setting.

Loss of IL-27 signaling ameliorates disease in a murine lupus model

Our data suggest that IL-27 might play a pathogenic role in diseases that are characterized by affinity-matured autoantibody production such as systemic lupus erythematosus (SLE; Wellmann et al., 2005; Mietzner et al., 2008). To test this hypothesis, we used a model in which a single i.p. injection of tetrathymethylpentadecane (TMPD; commonly known as pristane) results in an SLE-like syndrome characterized by the development of a variety of autoantibodies, the CD4+ T cell pool was similar between WT and Il27ra−/− mice (Fig. 5, C), the percentage of annexin V+ cells in the PD1+CXCR5+ T FH cell gate was significantly higher in Il27ra−/− spleen and LN compared with WT (Fig. 5, C and D). Together, these data suggest that IL-27 is important for the survival of T FH cells as well as enhancing the expression of T FH cell effector proteins.
DISCUSSION

The development of T<sub>FH</sub> cells is not fully understood and represents a topic of intense current interest. Our data position IL-27 as a new factor that is critical for T<sub>FH</sub> cell function. Stimulation of T cells with IL-27 promoted IL-21 production and expression of the T<sub>FH</sub> hallmark proteins PD-1, CXCR5, and ICOS. In I<sup>27</sup>a<sup>−/−</sup> mice, we observed a reduction in IL-21 production, fewer T<sub>FH</sub> cells, reduced GC formation, and a reduction in high-affinity immunoglobulin production, indicating that IL-27 signaling is required for efficient T cell–dependent humoral immune responses. A functional consequence of IL-27Ra ablation was partial protection in the pristane-induced lupus model, which is characterized by active GC development, production of multiple autoantibodies, and renal pathology. Collectively, these data strongly suggest that IL-27 is required for normal and pathogenic GC responses in vivo.

IL-27 also induced IL-21 expression by human CD4<sup>+</sup> T cells, suggesting that the IL-27–IL-21 axis is conserved across species. However, our results differ from recent observations by Schmitt et al. (2009), who observed that rhIL-12, but not rhIL-27, induced IL-21 expression by human PBMC. The reason for this discrepancy remains unresolved at this time but may result from differences in the experimental setup and the strength of TCR stimulation, which we observed to be proportional to IL-21 production (unpublished data). Our data also suggest that although IL-12 and IL-27 can independently induce IL-21 (Fig. 1 C), they can also act in concert to induce maximal production of this cytokine (Fig. S1, B and C). Mechanistically, IL-21 induction was independent of STAT1 in murine T cells (Fig. S1 D) but failed to occur in STAT3-deficient T cells from human AD-HIES patients (Fig. 1 D).

IL-27 was unable to induce the functional T<sub>FH</sub> phenotype in an in vitro system. This was surprising because IL-27 induced IL-21 production, which by itself is sufficient to induce functional T<sub>FH</sub> cells (Nurieva et al., 2008). However, IL-21 levels peak only at 48 h and ~1 ng/ml when induced by IL-27, whereas direct stimulation with recombinant IL-21 was conducted at 50 ng/ml and affected cells from the outset (Nurieva et al., 2008). Consequently, these two in vitro stimulation experiments cannot be directly compared. Moreover, isolated stimulation with a single recombinant cytokine in vitro falls short of mimicking the much more complex stimulation environment in vivo, where IL-27 is clearly required for a functional T<sub>FH</sub> response.

In addition to inducing hallmark T<sub>FH</sub> genes, our data also suggest that IL-27 promotes the survival of T<sub>FH</sub> cells. T<sub>FH</sub> cells represent a highly activated population, which express high levels of Fas and low levels of IL-7R (Ma et al., 2009) and are exquisitely sensitive to activation-induced cell death (Marinova et al., 2006). In T cell transfer experiments, DO11.10<sup>+</sup>rag<sup>2/−/−</sup> T cells assumed the T<sub>FH</sub> phenotype readily and independently of IL-27 signaling (Fig. S3 D). This is perhaps not surprising because the DO11.10<sup>+</sup> cells uniformly express a high-affinity Ag-specific TCR and because Ag affinity controls T<sub>FH</sub> differentiation (Fazilleau et al., 2009). However, DO11.10<sup>+</sup>rag<sup>2/−/−</sup> T cells that lacked I<sup>27</sup>a were recovered at diminished numbers (Fig. S3 E), suggesting that IL-27 is important for the maintenance of T<sub>FH</sub> cells. This hypothesis is supported by our observations that recombinant IL-27 enhances the survival of newly activated CD4<sup>+</sup> T cells in vitro and that T<sub>FH</sub> cells undergo increased apoptosis in vivo when I<sup>27</sup>a is genetically ablated. Although the cells studied in vitro were not fully differentiated T<sub>FH</sub> cells, the death of recently activated CD4<sup>+</sup> T cells would likely influence the number of cells that subsequently become T<sub>FH</sub> cells. IL-27 also induced ICOS, and ICOS signals have been shown to contribute to the survival and expansion of Ag-specific T cells in an immunization system similar to the one used in this paper (Burmeister et al., 2008). Thus, although the relative contributions remain to be determined, ICOS induction, IL-21 production (Nurieva et al., 2008), and other IL-27–elicited effects may all contribute to the enhanced survival of T<sub>FH</sub> cells.

Somewhat surprisingly, the reduction in T<sub>FH</sub> cells was more pronounced in spleen compared with the DLN (Fig. 2 and Fig. S2). The transfer of TCR Tg cells confirmed that Ag-specific responses occur in the spleen after immunization in the flank (Fig. S4, C and D). Because inhibition of lymphocyte migration using FTY720 administration did not prevent the tissue-specific effect (Fig. S2 C), we favor a hypothesis in which the proximity of the LN to the site of injection results in increased local exposure to Ag and inflammatory cytokines (Fu et al., 2006). Strong Ag signals promote T<sub>FH</sub> differentiation (Vogelzang et al., 2008; Fazilleau et al., 2009; Deenick et al., 2010), thus, the dependence on IL-27 signals may be reduced. Despite these possible compensatory effects close to the site of immunization, it is clear that IL-27 signaling is not redundant because significant reductions in T<sub>FH</sub> cells in the DLN and reduced serum high-affinity antibodies remain.

Our data suggest that the T<sub>FH</sub>-promoting effect of IL-27 is mediated through direct action on T cells. First, ICOS and IL-21 production were induced in T cell–only systems in vitro (Fig. 1; Pot et al., 2009). Furthermore, in chimeric animals that harbor both WT and I<sup>27</sup>a<sup>−/−</sup> T cells, the I<sup>27</sup>a<sup>−/−</sup> T cells are selectively underrepresented in the T<sub>FH</sub> Pool (Fig. 4, A and B). In contrast, no differences in the T<sub>FH</sub> response or high-affinity Ig levels were noted when I<sup>27</sup>a was selectively ablated on B cells (Fig. 4, C and D), which are known to be functionally responsive to IL-27 in vitro (Yoshimoto et al., 2004; Larrouserie et al., 2006). These data do not preclude the possibility that IL-27 influences certain minor aspects of the T cell–dependent humoral response, such as IgG2a class switching (Yoshimoto et al., 2004), through direct activities on B cells. Although the IL-27R is also expressed on myeloid cells, transfer of I<sup>27</sup>a<sup>−/−</sup>DO11.10<sup>+</sup>rag<sup>2/−/−</sup> T cells into I<sup>27</sup>a<sup>−/−</sup> recipients did not result in a significant decrease of T<sub>FH</sub> differentiation (Fig. S3, D–F), suggesting that IL-27 signaling into myeloid cells plays no major role in this context.
IL-27 is induced by type I IFN (Pirhonen et al., 2007; Remoli et al., 2007), and Ifnar1−/− mice display a similar phenotype to B27α−/− mice with respect to both loss of TFH function (Cucak et al., 2009) and protection from pristane-induced lupus (Nacionales et al., 2007), suggesting that IL-27 is an important downstream effector of type 1 IFN. In human SLE patients, a type 1 IFN signature is readily detectable, and antagonists are currently in clinical trials for the treatment of this disease (Yao et al., 2009; Niewold et al., 2010). Our data suggests that IL-27 might mediate the pathogenic effect of type I IFN and that therapeutic targeting of IL-27 may also be efficacious in SLE and other disorders characterized by excessive GC formation and high-affinity autoantibody production.

MATERIALS AND METHODS

Mouse, cells, and reagents. B27α−/− and B27α−/− (Chen et al., 2000) mice (C57BL/6 background, n > 33 unless otherwise stated or, where indicated, BALB/c background, n > 10), OT-II TCR Tg (C57BL/6), and B27α−/− DO11.10 TCR Tg/rag2-deficient mice (B27α−/− DO11.10tg.rag2−/− or B27α−/− DO11.10tg.ngt2−/− on the BALB/c background) were bred in a specific pathogen-free facility at The Garvan Institute or Genentech. Stat1−/− mice (129Sv/Ev background) and 129Sv/Ev control mice were purchased from Taconic. The TCM (triple congenic) mice, Igh−/−B6, CD45.1_Cross-B6.SJL, which express congenic markers at the CD45.1, Igh and Thy1.1 loci on the C57BL6 background, and µMT mice (Igh−/− B6, 129, or Stat1−/− targeted mutation; C57BL/6 background) were bred in a specific pathogen-free facility at Genentech. The rag2−/− animals were purchased from The Jackson Laboratory. All live animal experiments were approved by the Genentech Institutional Animal Care and Use Committee or The Garvan Institute/St. Vincent’s Animal Experimentation Ethics Committee. Human PBMC buffy coats were obtained from the Australian Red Cross Blood Service, and patients with the clinical diagnosis of AD-HIES as a result of mutations in STAT3 were recruited from Immunology Clinics in Canberra and Sydney, Australia. All human experiments were approved by jurisdictional ethics committees in Canberra and Sydney. Unless otherwise indicated, all cytokines were purchased from R&D Systems and all antibodies were from BD. Cycloheximide was purchased from Sigma-Aldrich. FTY720 (Caymen Chemical) was reconstituted in DMSO and injected i.p. in H2O. IL-21R-Fc was produced at the Garvan Institute as previously described (Herber et al., 2007).

Isolation of lymphocyte subsets. Unless otherwise indicated, primary mouse CD4+ T cells were isolated by magnetic depletion using MACS kits (Miltenyi Biotech) according to the manufacturer’s instructions. The purity ranged from 90 to 95%. Where indicated, FACS sorting was performed using a FACSAnia instrument (BD) to obtain specific cell populations of >99% purity. After immunization, splenic leukocyte populations were isolated as follows: CD4+ (CD4+ B220−), Tfh (CD4+CXCR5+PD1+), non–GC B (B220+CD38−), GC B (B220+CD38+), FDC (B220+CD35−CD32−), CD11b+ (CD11b+CD11c−B220+), and CD11c+ (CD11c+CD11b−B220+). For cell culture systems that required Ag-presenting cells, splenocyte samples were magnetically depleted of T cells with anti-CD90 (Thy-1.2) MACS MicroBeads (Miltenyi Biotech) and irradiated with a dose of 2,600 rads.

In vitro T cell stimulation. Primary mouse cells were cultivated in Isevo’s modified Dulbecco’s medium (Invitrogen) supplemented with 10% (volume/volume) heat-inactivated FBS (HyClone PerBio), 1 mM L-glutamine, 1% (volume/volume) penicillin and streptomycin (Invitrogen), and 55 mM 2-mercaptoethanol (MP Biomedicals). Splenic CD4+ T cells from C57BL/6, B27α−/−, 129, or Stat1−/− mice were activated for the indicated times in plates coated with 5 µg/ml of anti-CD3 and in the presence of 1 µg/ml anti-CD28. Unfractionated DO11.10tg.ngt2−/− or OTIlg spleenocytes were stimulated with OVA25–33 peptide at the indicated concentrations. TH0 conditions consist of 5 µg/ml each of hamster anti-mouse IFN-γ (H22), rat anti-mouse IL-4 (BVD4-1D11), and rat anti-mouse IL-12 (C15.6) and 100 ng/ml TGFβRII-Fc (R&D Systems). Where indicated, rmIL-27 was added at a concentration of 20 ng/ml.

Human naive CD4+ T cells were freshly purified from tonsil cell or PBMC preparations based on the CD4+CD45RA+CXCR5+ phenotype. Cells were labeled with CFSE and stimulated with T cell activation and expansion beads (Miltenyi Biotec) at a bead/cell ratio of 2:1 in the presence of no additional cytokine, 20 ng/ml rhIL-12, 20 ng/ml rhIL-23, or 50 ng/ml rhIL-27 for 5 d. Where irradiated splenocytes were used as APC, total splenocytes were irradiated with 2,000 rad x-ray irradiation.

Immunization. For T cell–dependent immunization, groups of age- and sex-matched mice were immunized with TNP−/−OVA (30 µg/mouse) or OVA (100 µg/mouse), as indicated, and emulsified in 100 µl CFA (Sigma-Aldrich) by subcutaneous injection into the flank. Where a second immunization was required, this was performed 21 d after the initial injection using the same dose of Ag emulsified in IFA (Sigma-Aldrich) to a volume of 100 µl/mouse and injected subcutaneously into the alternate flank.

For T cell–independent immunization, groups of six mice per genotype were immunized i.p. with 100 µg TNP-aminoethylcarboxymethyl-Ficoll in PBS. Serum was harvested 5 d later.

Flow cytometric analysis. Cells were treated with Fc blocking Abs (anti-CD16/32/2.4G2) and then surface stained with the specified antibodies. To measure cell viability, cells were stained with FITC-conjugated annexin V and 7-AAD according to the manufacturer’s instructions (BD). Viable cells exclude both stains. IL-21 expression in human T cells was assessed by intracellular staining using Alexa Fluor 647–labeled anti–human IL-21 (eBioscience; clone 3A3-N2). Samples were analyzed using a FACSCanto II or LSR II (BD) and data analyzed using FlowJo software (Tree Star, Inc.). Contour profiles are presented as 5% probability contours with outliers.

ELISA. IL-21 was detected in culture supernatants using the mouse IL-21 DuoSet ELISA (R&D Systems) as per the manufacturer’s instructions. To measure the relative amounts of TNP-specific antibodies in mouse serum, plates were coated with 5 µg/ml TNP−/−BSA or TNP−/−BSA (Biosearch Technology) overnight at 4°C. TNP-specific ELISA was performed as previously described (Roets and Rajewsky, 1993). To standardize and quantify relative amounts of TNP-specific Ig responses, all experimental samples were compared with a standardized dilution of pooled serum obtained from B27α−/− mice immunized with TNP-OVA. This standard was given the arbitrary concentration of 100. In the case of IgM, an anti-TNP monoclonal (BD; clone G155-228) was used as a standard control.

For serum autoantibody measurement, mouse ANA, anti-Sm, and anti-RNP antibodies were measured using ELISA kits from Alpha Diagnostic International, as per the manufacturer’s instructions. Anti-dsDNA antibodies were measured by coating ELISA plates with 0.01% p-L-Lysine (Sigma-Aldrich) for 1 h at room temperature and then 2.5 µg/ml of calf thymus DNA (Sigma-Aldrich) in PBS at 4°C overnight. After blocking with PBS, 0.5% BSA + 10 ppm Proclin, pH 7.4, 1 h at room temperature, serially diluted mouse serum was added and incubated at room temperature for 2 h. Antibody was detected using 500 ng/ml biotinylated rat anti–mouse IgG, IgG1, or IgG2a (BD) for 1 h at room temperature, followed by HRP-conjugated streptavidin and visualized using TMB substrate. NZB/F1 serum was used as a positive control.

BM chimeras. For mixed BM chimeras, triple congenic mice (CD45.1, Thy1.1, and IgH) were lethally irradiated (137Cs; gamma source, 1,150 rad) and reconstituted with equal numbers of BM cells from B27α−/− (CD45.1 and Thy1.2) and B27α−/− (CD45.2 and Thy1.2) mice by i.v. injection. After 8 wk of reconstitution, the mice were bled to assess reconstitution by flow cytometry and immunized as described in Immunization.
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For µMT chimeras, Rag<sup>2−/−</sup> mice were lethally irradiated (<sup>60</sup>Co; gamma source, 1100 rad) and reconstituted with BM from B cell-deficient µMT mice plus either WT or IL-27ra<sup>−/−</sup> BM (at a 80:20 ratio), such that all B cells in the resultant chimeras were derived from either the WT (control) or the IL-27ra<sup>−/−</sup> BM (a B cell–specific B27ra deletion), with the majority of non–B cells derived from WT BM. After 8 wk of reconstitution, the mice were bled to assess reconstitution by flow cytometry and immunized as described in Immunization.

Adoptive transfer. CD4<sup>+</sup> T cells from either IL-27ra<sup>−/−</sup> or WT donors were isolated by magnetic sorting and 10<sup>6</sup> CD4<sup>+</sup> T cells were transferred to either IL-27ra<sup>−/−</sup> or WT mice on the BALB/c genetic background via tail vein injection. The next day, the mice were immunized with OVA in CFA/IFA as described in Immunization and tissue harvested 7 d after the second immunization. Do11.10 TCR<sup>+</sup> Tg cells were detected using directly fluorescein-labeled clonotypic KJ1-26 antibody (BD).

Statistical analysis. Data were analyzed with Prism software (GraphPad Software, Inc.) to calculate an unpaired two-way Student’s t test.

RT–PCR. Total RNA from FACS-sorted or cultured cells was isolated with the RNaseasy kit using on-column DNAse I digestion (Qiagen). Tagman quantitative RT–PCR was performed according to the manufacturer’s instructions (Applied Biosystems). A LightCycler480 (Roche) instrument was used in the case of human samples. For each sample, triplicate test reactions and a control reaction lacking reverse transcription were analyzed for expression of the gene of interest and results were normalized to those of the housekeeping ribosomal protein L19 mRNA or GAPDH. Arbitrary units given are the fold change relative to RPL19 (mouse) or GAPDH (human) and multiplied by 1,000. Primer sequences for each target are provided in Table S1.

Histochemical analysis. For GC quantification, spleens were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. To detect GC, 5-µm sections were stained with biotin-conjugated PNA followed by visualization with HRP-linked streptavidin and diamino benzidine. The sections were lightly counterstained with hematoxylin. Slides were scanned on a NanoZoomer digital pathology system (Olympus), and Matlab image analysis software (The MathWorks) was used to quantify the percentage of each spleen that was positive for PNA staining.

For kidney glomerulopathy, tissue collected at necropsy was fixed in neutral buffered formalin and subsequently embedded in paraffin. To detect GC, 5-µm sections were stained with biotin-conjugated PNA followed by visualization with HRP-linked streptavidin and diamino benzidine. The sections were lightly counterstained with hematoxylin. Slides were scanned on a NanoZoomer digital pathology system (Olympus), and Matlab image analysis software (The MathWorks) was used to quantify the percentage of each spleen that was positive for PNA staining.

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