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A role for IL-27p28 as an antagonist of gp130-mediated signaling

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Abstract

The heterodimeric cytokine interleukin 27 (IL-27) signals through the IL-27Rα subunit combined with gp130, a common receptor chain utilized by several cytokines, including IL-6. Interestingly, the IL-27 subunits p28 and EBI3 are not always co-expressed, suggesting that they may have

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Author Contributions

J.S.S. and C.A.H. contributed to all studies and wrote the manuscript; E.D.T., W.J.Q. III, N.H., M.P.C. and S.D.L. were involved in the analysis of the p28 transgenic mice; R.G. contributed to the studies on GC formation; M.M.E contributed to the studies with the *Ebi3*$^{-/-}$ mice; A.C.O. contributed to the studies on the intracellular staining for IL-27p28, and B.S., S.R-J. and J.G. performed the p28-gp130 modeling and contributed to its analysis. C.A.F. and S.A.J. performed the biacore assays and contributed to its analysis. M.L.J. provided the recombinant IL-27p28 protein. Y.C. and D.J.C. performed the hydrodynamics-based transfection experiments with the mini-circle DNA and contributed to its analysis.

unique functions. Here, we show IL-27p28, independent of EBI3 antagonized cytokine signaling through gp130 and IL-6 mediated production of IL-17 and IL-10. Similarly, the ability to generate antibody responses was dependent on the activity of gp130-signaling cytokines. IL-27p28 transgenic mice showed a major defect in germinal center formation and antibody production. Thus, IL-27p28, as a natural antagonist of gp130-mediated signaling, may be useful as a therapeutic for managing inflammation mediated by cytokines that signal through gp130.

Introduction

Type I cytokines including interleukin 6 (IL-6 [http://www.signaling-gateway.org/molecule/query?afcsid=A004204]), IL-12, IL-23 and IL-27 are related based on structural motifs, a common four-helix bundle, and shared usage of receptor subunits1. These cytokines have numerous biological activities, but their diverse effect on the development of T_H subsets has received considerable attention. IL-12 promotes T_H1 cells, IL-6 and IL-23 are involved in T_H17 differentiation, and IL-27 antagonizes T_H1, T_H2 and T_H17 responses. These ligands signal through membrane bound receptor complexes that include either gp130 [http://www.signaling-gateway.org/molecule/query?afcsid=A001266] or IL-12Rβ1, which activate STAT pathways1. Given the role of these cytokines in cell-mediated immunity, it is not surprising that they are linked to the development of a number of autoimmune inflammatory conditions2. For instance, IL-6 is implicated in the control of leukocyte recruitment, activation, and apoptotic clearance in inflammatory bowel disease (IBD), peritonitis, rheumatoid arthritis, Castleman's disease and asthma, making IL-6 a viable therapeutic target in these conditions3–5.

The receptor subunit gp130 is utilized by several cytokines including IL-6, IL-11, IL-27, oncostatin M (OSM), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1) and cardiotrophin-like cytokine (CLC). Accordingly, these cytokines display similar functions including induction of acute phase proteins6, stimulation of hematopoiesis7, 8, and promotion of B cell development and antibody production9–12. However, they also exhibit distinct activities, owing to the usage of unique receptor alpha chains that pair with gp130 to form functional receptor complexes. For instance, the single subunit cytokine IL-6 binds gp130 in combination with either a membrane bound or secreted version of the IL-6Rα chain [http://www.signaling-gateway.org/molecule/query?afcsid=A001265] 3, 4. IL-27, is a heterodimeric cytokine composed of p28, a four-helix bundle protein similar to IL-6, and EBI3, which resembles the sIL-6Rα chain13. IL-27 employs a unique receptor subunit IL-27Rα (also known as WSX-1 or TCCR [http://www.signaling-gateway.org/molecule/query?afcsid=A002911]) that pairs with gp130 to initiate signaling13, 14.

For the heterodimeric cytokines in this family (IL-12, IL-23, IL-27) current models dictate that their secretion is dependent on the regulated transcription of the IL-12p35, IL-23p19 and IL-27p28 subunits, while the p40 and EBI3 subunits are constitutively expressed. For IL-12, this transcriptional regulation may explain why IL-12p40 is produced in excess of IL-12p35, resulting in p40 homodimers that can function as IL-12 antagonists15. Whereas a disulfide bond links IL-12p40 with IL-12p35 or IL-23p19, it is unclear how the subunits of

IL-27 interact, suggesting an alternative mechanism of folding and assembly16. Thus, p28 and EBI3 might be secreted independently, allowing for association or pairing of each subunit with other proteins. This idea is supported by cases where EBI3 and p28 are not expressed by the same cells17, 18, differences in the transcriptional regulation of each subunit13, 19, and evidence that EBI3 and IL-12p35 can associate to form IL-35 (refs. 20–22). Nevertheless, based on a number of bioassays13, no role for IL-27p28 has been reported. However, previous work from this laboratory has shown that purified IL-27p28, like heterodimeric IL-27, was capable of suppressing IL-17 production by $CD4^+$ T cells *in vitro*23. The basis for this effect was unclear, but it suggested that IL-27p28 could complex with EBI3 in the cultures to form IL-27, or that it could propagate an inhibitory signal on its own. The studies reported here indicate that IL-27p28, independently of EBI3, can block the ability of IL-6 to promote T_H17 responses, and can function as a natural antagonist of gp130 signaling mediated by IL-6, IL-11 and IL-27. Moreover, an analysis of transgenic mice, which over-express IL-27p28, revealed defective thymus-dependent (TD) B cell responses, associated with an inability to form germinal centers (GCs), and a lack of class switching and affinity maturation. As a number of cytokines that utilize gp130 are linked with GC formation and production of high-affinity antibodies24–27, these results are consistent with a role for IL-27p28 in blocking these events, and suggest that IL-27p28 can act as a natural antagonist of gp130 signaling.

Results

EBI3 is not required for the secretion of IL-27p28

It is unclear whether IL-27p28 can be secreted independently of EBI3 under physiological conditions. Previous studies have reported that over-expression of IL-27p28 by established cell lines led to its secretion independent of EBI3 13, 28. To determine if IL-27p28 can be secreted by primary cells in the absence of EBI3, bone-marrow derived macrophages (BMMϕs) and dendritic cells (BMDCs) from wild-type and *Ebi3*$^{-/-}$ mice were incubated with lipopolysaccharide (LPS), interferon-γ (IFN-γ), or this combination, and the secretion of IL-27p28 was determined. As previously reported29, 30, LPS and IFN-γ induced IL-27p28 secretion by wild-type BMMϕs and BMDCs (Fig. 1a). In addition, these stimuli also resulted in the equivalent production of IL-27p28 by *Ebi3*$^{-/-}$ cells. Similar results were seen with other TLR agonists, including CpG (data not shown). Furthermore, infection of wild-type and *Ebi3*$^{-/-}$ mice induced detectable amounts of IL-27p28 in the serum, with the highest concentrations measured in the *Ebi3*$^{-/-}$ mice (Fig. 1b). Together with reports that IL-27p28 and EBI3 can be differentially regulated and produced by different cell types13, 17–19, the finding that IL-27p28 can be secreted in the absence of EBI3 suggests that this subunit may have additional IL-27 independent functions.

IL-27p28 is biologically active in the absence of EBI3

We previously reported that under conditions where transforming growth factor-β (TGF-β) plus IL-6 was used to induce T_H17 development, IL-27 antagonized IL-17 production and IL-27p28 alone also had a reproducible inhibitory effect23. However, it was unclear whether IL-27p28 was binding to EBI3 present in the cultures to form IL-27, which suppressed T_H17 development. When, splenocytes from *Ebi3*$^{-/-}$ mice were used in this assay. IL-27p28

antagonized the production of IL-17 by wild-type and *Ebi3*$^{-/-}$ CD4^{+} T cells under T_H17 inducing conditions (Fig. 1c). Previous studies have shown that TGF-β in combination with IL-6 or IL-27 can induce CD4^{+} T cells to produce IL-10 (ref. 31, 32); however, IL-27p28 alone or in the presence of TGF-β did not support the development of IL-10-producing T cells (data not shown). Furthermore, while stimulation of T cells with TGF-β plus IL-6 led to the production of IL-10, this was not affected by the addition of IL-27, but IL-27p28 reduced the capacity of wild-type and *Ebi3*$^{-/-}$ CD4^{+} T cells to make IL-10 under these conditions (Fig. 1d). Taken together, these results indicate that the inhibitory effects of IL-27p28 are independent of EBI3 and that it has inhibitory activities distinct from IL-27.

IL-27p28 antagonizes IL-6 and IL-27 STAT signaling

Since IL-27p28 can antagonize the ability of IL-6 to promote T_H17 differentiation or IL-10 production, we initially hypothesized that IL-27p28 alone could act in a manner analogous to IL-27 and induce STAT signaling to mediate these effects. As IL-6 and IL-27 primarily activate STAT1 and STAT3, the ability of IL-27p28 to induce phosphorylation of these proteins in CD4^{+} T cells was assessed. A 15-minute incubation with IL-6 or IL-27 resulted in phosphorylation of STAT1 and STAT3, while IL-27p28 alone did not induce STAT phosphorylation (Fig. 2a). This result was consistent at multiple time points examined over a two-hour period (data not shown). As IL-6 and IL-27 signal through gp130, an alternative explanation for the inhibitory effects of IL-27p28 is competition with IL-6 for binding to gp130. Therefore, the induction of STAT1 and STAT3 phosphorylation by IL-6 in the presence of IL-27p28 was tested. Co-incubation of these two proteins led to a marked reduction in STAT1 and STAT3 phosphorylation in CD4^{+} T cells (Fig. 2a). A similar result was observed when IL-27p28 was incubated with IL-27 (Fig. 2a). This effect was dose dependent and typically required a 5- to 50-fold excess of IL-27p28 (Supplementary Fig. 1a). Furthermore, phosphorylation of STAT3 by IL-11, a cytokine that utilizes gp130, but not IL-6Rα or IL-27Rα for signaling, was also reduced by addition of IL-27p28 (Supplementary Fig. 1b). Moreover, when CD4^{+} T cells were incubated with hyper-IL-6, a fusion protein consisting of human IL-6 and sIL-6Rα that can signal *in trans* through gp130 (ref. 33), phosphorylation of STAT1 and STAT3 occurred and this signaling was antagonized by inclusion of IL-27p28 (Fig. 2b). It should be noted that the ability of IL-12, which does not signal through gp130, to phosphorylate STAT4 was not blocked by IL-27p28 (Supplementary Fig. 1c).

IL-27p28 antagonizes the interaction of IL-6 with gp130

The data described above suggest that IL-27p28 inhibits IL-6 *trans*-signaling by binding to gp130 and not IL-6Rα, thus limiting the availability of this receptor subunit for binding to hyper-IL-6. This result led us to examine the structural basis for this inhibitory effect. Using the available crystal structure of the human IL-6–gp130 complex as a template (see **Methods**), a three-dimensional model was constructed to assess whether IL-27p28 is capable of binding gp130 without interacting with EBI3. This model predicted that similar to IL-6, IL-27p28 would bind the Ig-like domain of gp130 using amino acid residues located in the AB loop of the protein (Fig. 2c). The amino acid residues that are predicted to be critical for gp130 binding are L81 and E85. In this model L81 interacts with L3 in gp130 and would increase the hydrophobicity of IL-27p28 compared to the Ser residue at this

position in IL-6. Moreover, E85 (Leu in IL-6) would form a salt bridge with the amino terminus of gp130 that is lacking when IL-6 interacts with gp130.

While this model does not resolve whether IL-27p28 must associate with IL-27Rα in order to antagonize gp130-mediated signaling, the absence of IL-27Rα on T cells did not prevent IL-27p28 from inhibiting STAT phosphorylation in response to IL-6 (data not shown). What this model does indicate is that due to the increased hydrophobicity of IL-27p28, compared to IL-6, it is capable of interacting with gp130 in the absence of EBI3 and may antagonize the ability of IL-6, IL-11 and IL-27 to signal through the Ig-like domain of gp130. This model also suggests that IL-27p28 would not inhibit the effects of other cytokines such as OSM that utilizes the cytokine-binding domain of gp130 (ref. 34). When mouse embryonic fibroblasts (MEFs) were stimulated with Hyper-IL-6 or OSM STAT3 phosphorylation was observed (Fig. 2d), and while addition of IL-27p28 antagonized this response to Hyper-IL-6, IL-27p28 did not inhibit STAT3 phosphorylation by OSM (Fig. 2d). In order to investigate the capacity of IL-27p28 to block the interaction of IL-6 with gp130 surface plasmon resonance analysis was performed. The combination of IL-6–sIL-6Rα was able to interact with chip bound gp130 and induce a binding curve response (Supplementary Fig. 2). When IL-27p28 was introduced in this system it resulted in a dose dependent inhibition of the ability of IL-6–sIL-6Rα to interact with gp130. When IL-27p28 was used at a concentration that was in 10-fold excess of IL-6–sIL-6Rα, a slightly slower association (ka) and a faster dissociation (kd) as well as an overall lower affinity (lower KA and higher KD) of IL-6–sIL-6Rα for gp130 was observed (Supplementary Fig. 2). This finding along with the structural model suggests that the ability of IL-27p28 to block gp130-mediated signaling is a consequence of a low affinity interaction.

Analysis of IL-27p28 transgenic mice

To examine the functional role of IL-27p28 *in vivo*, the murine *Il-27p28* gene was cloned into an expression vector downstream of a regulatory element in which the immunoglobulin intronic heavy chain enhancer (Eμ) and the *lck* proximal promoter are juxtaposed to drive expression in B and T cells35 (Fig. 3a). The resulting *Not*I digested plasmid DNA was microinjected into female B6C3F1 oocytes fertilized by C57BL/6J males. The founder mice, which age and reproduce normally, were crossed to C57BL/6J mice to form a stable p28 transgenic line. Basal expression of IL-27p28 was detected by intracellular staining in B and T cells from transgenic, but not wild-type mice (data not shown), which subsequently increased following 48 h of BCR or TCR stimulation (Fig. 3b). These results were also reflected in the amount of IL-27p28 detected in supernatant from these cultures (Supplementary Fig. 3), but IL-27p28 was not detected in supernatants from resting BMMφs or BMDCs of p28 transgenic mice (data not shown). The amount of IL-27p28 in the serum of naïve p28 transgenic mice was 10–15 fold higher than that detected in the serum of naïve wild-type mice (Fig. 3c).

An assessment of the lymphocyte compartment in these transgenic mice revealed no differences in the number of mature B cells (Fig. 3d) or any defect in the developmental stages of B2 B cells in the bone marrow or spleen (data not shown). Similarly, the ratio of $CD4^+$ and $CD8^+$ T cells (Fig. 3e) in the spleen of p28 transgenic mice was comparable to

littermate controls. However, an increase in the total number of $CD4^+$ and $CD8^+$ T cells in the spleen of p28 transgenic mice was noted (Fig. 3f), which was associated with an increase in the number of $CD4^+$ and $CD8^+$ T cells that display an activated phenotype ($CD44^{hi}CD62L^{lo}CD69^+$) compared to wild-type mice (Fig 3g and Supplementary Fig. 4a). Further comparison revealed no difference in CD25 expression (Supplementary Fig. 4b), the number of splenic $Foxp3^+$ regulatory T cells (Supplementary Fig. 4c), or IL-2, IFN-γ, IL-4, IL-17 and IL-10 production following activation of splenocytes with anti-CD3 and anti-CD28 for 48 h (data not shown). Nevertheless, no overt signs of autoimmune disease were noted in p28 transgenic mice as old as 1.5 years of age (data not shown).

To determine if the phenotype of p28 transgenic $CD4^+$ T cells complemented that seen with recombinant IL-27p28 *in vitro*, $CD4^+$ T cells from wild-type and p28 transgenic mice were cultured under T_H17-inducing conditions and IL-17 was measured. IL-17 production was limited by transgenic expression of IL-27p28 (Fig. 4a), and p28 transgenic $CD4^+$ T cells produced lower amounts of IL-10 in response to TGF-β plus IL-6, or IL-27 alone (Fig. 4b). As seen with recombinant IL-27p28, over-expression of IL-27p28 by purified $CD4^+$ T cells did not lead to increased phosphorylation of STAT1 or STAT3 over a 2 h period of incubation (Fig. 4c and data not shown), whereas pre-incubation of transgenic $CD4^+$ T cells in media for 2 h prior to addition of IL-6 or IL-27 led to a marked reduction in STAT1 and STAT3 phosphorylation (Fig. 4c and Supplementary Fig. 5a). Similarly, when p28 transgenic $CD4^+$ T cells were stimulated with IL-11 they displayed reduced phosphorylation of STAT3 compared to wild-type $CD4^+$ T cells (Supplementary Fig. 5b). Together, these studies indicate that IL-27p28 produced by transgenic cells was able to efficiently antagonize the activity of IL-6, IL-11 and IL-27 (Fig. 1c, d). Given the ability of IL-27p28 to antagonize development of T_H17 cells *in vitro* the capacity of IL-27p28 to inhibit the development of experimental autoimmune encephalomyelitis (EAE), a mouse model in which IL-17 producing T cells have been implicated as a cause of this inflammatory disease of the CNS, was tested. Five days prior to induction of EAE by immunization with myelin oligodendrocyte glycoprotein ($MOG_{35–55}$), plasmid DNA containing cDNA for IL-27, IL-27p28 or GFP control was administered to C57BL/6 mice. Consistent with *in vitro* findings, expression of IL-27 greatly inhibited the onset and development of signs of disease in this model, while expression of IL-27p28 resulted in a modest delay in the onset and severity of disease compared to mice expressing the GFP control (Supplementary Fig. 6).

Overexpression of IL-27p28 limits B cell responses

Multiple cytokines that utilize gp130 influence the adaptive immune response, including B cell development and antibody production10–12. Examination of antibody production in naïve p28 transgenic mice showed a significant reduction in the total number of IgM and IgG antibody-secreting cells in the spleen and bone marrow compared to wild-type mice (Supplementary Fig. 7a,b), suggesting a role for IL-27p28 in the regulation of B cell responses *in vivo*. Several experimental systems were utilized to evaluate whether over-expression of IL-27p28 could influence antibody production following immunization with thymus-independent (TI) and TD antigens, allowing us to dissect many aspects of the B cell response, including extrafollicular IgM production, GC formation, and antibody class switching and affinity maturation.

To examine IgM production, wild-type and p28 transgenic mice were immunized intraperitoneally with either the TI antigen 2,4 dinitrophenol-conjugated to Ficoll (NP-Ficoll) in saline, or the TD antigen 2,4 dinitrophenol-conjugated to chicken γ-globulin (NP-CGG) in alum, and antigen-specific antibody-secreting cells were enumerated by ELISPOT. There was no significant difference in the number of NP-specific IgM secreting cells detected in the spleen of wild-type and p28 transgenic mice (Fig. 5a,b). Thus, transgenic expression of IL-27p28 does not affect the ability of B cells to mount an IgM-specific antibody response to TI or TD antigens.

On day 14 following NP-CGG immunization, wild-type mice were able to effectively generate a low affinity NP_{33}-specific IgG_1 response, while no antigen-specific IgG_1 secreting cells were detected in the spleen of p28 transgenic mice (Fig. 5c). Moreover, an assessment of affinity maturation at this time point indicated the presence of NP_4-specific IgG_1 secreting cells in the spleen of wild-type mice, but not p28 transgenic mice (Fig. 5d). When the NP_{33}- and NP_4-specific IgG_1 response was measured in bone marrow at day 14 post-immunization, neither low nor high affinity antigen-specific IgG_1 secreting cells were observed in p28 transgenic mice (Supplementary Fig. 8a,b). There was also a significant reduction in the number of antigen-specific IgM secreting cells in the bone marrow (Supplementary Fig. 8c). At day 56 post-immunization, the anti-NP_4-specific IgG_1 response remained deficient in p28 transgenic mice compared to littermates (data not shown). One possible explanation for this phenotype is that there was decreased survival of antibody secreting cells in p28 transgenic mice; however, no difference in B cell death was observed in the spleen between naïve or immunized wild-type and p28 transgenic mice (data not shown). Furthermore, no NP-specific IgG antibodies were detected in the serum of p28 transgenic mice at day 7 or 14 post-immunization (Supplementary Fig. 8d), suggesting that IL-27p28 does not reduce the survival of antibody secreting cells, but rather prevents their development.

To assess the basis for the antibody defect in these models, GCs in the spleen were visualized by peanut agglutinin (PNA) binding, which revealed normal splenic architecture in naïve wild-type and p28 transgenic mice (Fig. 6a). However, while wild-type mice displayed typical GC formation and structure at day 14 following NP-CGG immunization, p28 transgenic mice, based on the absence of PNA^+ B cells inside the follicle, failed to generate GCs (Fig. 6a). As the NP response in C57BL/6 mice is idiotypically restricted36 and characterized by the use of λ1 light chain, λ expression can be used as a surrogate marker for NP specificity to allow for enumeration of $NP^+\lambda^+PNA^+$ GC B cells. Naïve wild-type and p28 transgenic mice showed very few, if any, $NP^+\lambda^+PNA^+$ GC B cells in the spleen. There was an expansion of this population in wild-type mice at day 14 following immunization with NP-CGG (Fig. 6b,c), but immunized p28 transgenic mice displayed minimal expansion of $NP^+\lambda^+PNA^+$ GC B cells (Fig. 6b,c). Furthermore, immunization with another TD antigen, keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant resulted in an expansion in PNA^+ GC B cells in the spleen of wild-type mice that were capable of class switching in an antigen specific manner, but not in p28 transgenic mice (Supplementary Fig. 9). Together, these data indicate that overexpression of IL-27p28 does not affect TI responses, but it has a distinct effect on formation of GCs in response to immunization with TD antigens.

Discussion

The findings reported here indicate a previously unappreciated role for IL-27p28 as a natural antagonist of gp130-mediated signaling in response to IL-6, IL-11 and IL-27, and highlight the increasingly complex biology of the IL-27 subunits. There are numerous endogenously produced antagonists of cytokine receptors, including IL-12p40 homodimers15, the soluble IL-1 receptor antagonist and IL-18 binding protein, which antagonize the activity of IL-12, IL-1 and IL-18 respectively37. In addition, the surface-bound or soluble IL-13Rα2 chain serves as a decoy receptor for IL-13 binding and functions to regulate T_H2 responses38. The data presented here suggests that IL-27p28 can be added to this list of cytokine antagonists.

While IL-27p28 and EBI3 are known to form IL-27, they can display different patterns of transcription in response to some stimuli13, 19. Additionally, the kinetics of their expression differs following activation of monocyte-derived dendritic cells; IL-27p28 expression peaks early following activation, whereas EBI3 expression is sustained and peaks later13. These observations suggest that the individual subunits of IL-27 can have distinct functions. Accordingly, EBI3 binds to IL-12p35 to form IL-35, a cytokine associated with regulatory and effector T cell function20, 21. Also, there are reports that indicate that IL-23p19 binds to EBI3 (ref. 1), and that IL-27p28 binds a receptor-like protein CLF39; however, whether these complexes form *in vivo* is unclear. These findings further emphasize the complex combinatorial biology of this family of cytokines, and raises questions about whether other subunits in this family have additional biological activities.

While it has been recognized that gp130 is a key receptor subunit for multiple cytokines there is still much to learn about the interaction of different ligands with this receptor. Whereas IL-27p28 does not appear to be capable of binding to IL-27Rα alone13, the nature of the interaction between IL-27p28 and gp130 remains unclear. A prior report showed that recombinant sgp130-Fc does not interfere with IL-27 signaling, suggesting that IL-27 must complex with IL-27Rα prior to interacting with gp130 (ref. 40). Similarly, IL-6 has previously been shown to be incapable of solely binding gp130 (refs. 9, 41). However, the model in figure 2 did indicate that IL-27p28, similar to IL-6, is capable of interacting with the Ig-like domain of gp130 and can do so without associating with EBI3. Differences between the association of IL-27p28 and IL-6 with gp130 are due to two amino acid differences in the AB loop between IL-27p28 and IL-6 that increase the hydrophobicity of IL-27p28, and thus its affinity for gp130. Interestingly, it has previously been shown that mutations in the AB loop of human IL-6 contribute to the ability of mutant forms of IL-6 to antagonize wild-type IL-6 activity42. Furthermore, modeling studies incorporating fluorescence-correlation spectroscopy have proposed that a gp130 homodimer first binds one IL-6–IL-6Rα complex, and engages a second IL-6–IL-6Rα complex at higher ligand concentrations43. One possibility for the inhibitory activity of IL-27p28 is that it may act at the transition binding state, which as outlined by Schroer *et al*, would infer that IL-27p28 would block binding of a second IL-6–IL-6Rα complex43. Regardless, a definitive crystal structure will be required to understand how IL-27p28, alone or as part of IL-27, interacts with gp130 in order to further elucidate how this subunit antagonizes signaling through gp130.

Prior studies have indicated that gp130 signaling cytokines are necessary for B cell expansion, differentiation and antibody production9–12. Therefore, based on these findings, we investigated the ability of IL-27p28 to antagonize antibody production in response to immunization with TI and TD antigens. While no defect in IgM production was observed, mice that transgenically over-express p28 had a severe defect in forming GC reactions and IgG class switching in response to immunization with two different TD antigens. Together, these results suggest that IL-27p28 is a natural antagonist of gp130-mediated GC formation and development of antigen-specific antibody production in response to TD antigens. However, these experiments do not provide a clear indication as to which cytokine IL-27p28 blocks i*n vivo*, and there are many potential candidates, including IL-6, CLC and LIF. For instance, the original characterization of *Il-6*$^{-/-}$ mice indicated that upon infection with vesicular stomatitis virus and vaccinia virus, *Il-6*$^{-/-}$ mice produced 5–10-fold lower virus-neutralizing IgG titers while IgM titers were comparable to control mice44. Furthermore, *Il-6*$^{-/-}$ mice immunized with NP_{36}-CGG formed fewer and smaller GCs, and had a reduced capacity to mount an antigen-specific IgG response45. Also, gp130 transgenic mice, which express a dominant negative version of gp130, exhibit a defect in antigen-specific antibody production for most isotypes other than IgM after immunization with NP-OVA24. Moreover, mice that transgenically express CLC (NNT-1/BSF-3), LIF, or IL-6 show B cell hyperplasia and higher levels of serum antibody levels for most isotypes25, 46, 47. Additional studies will be required to address these potential targets of IL-27p28 during GC formation.

The dysregulated production of inflammatory cytokines is associated with many autoimmune diseases. Thus, there has been considerable focus on understanding how cytokines interact with their receptor complexes to develop novel approaches to manage inflammation. Specifically, identification of the important amino acid residues on the ligand surface that mediate binding is key in developing mimics with specific biological properties, such as receptor agonists and antagonists. The observations reported here raise the question of whether IL-27p28 can be used as a therapeutic to treat inflammatory conditions and malignancies involving gp130-signaling cytokines. In support of this idea, a recent study has reported that IL-27p28-expressing myoblasts could suppress an allogenic cytotoxic T cell response and prolong graft survival, which was suggested to be due to the ability of IL-27p28 to block IL-27 activity28. Interestingly, three recent reports have indicated that single nucleotide polymorphisms in human IL-27p28 are associated with susceptibility to asthma and IBD48–50 and, in one study; reduced production of IL-27p28 was associated with increased susceptibility to early-onset IBD49. A lack of functional IL-27 to serve as an anti-inflammatory mediator in the lung and gut is one likely explanation for these findings. However, an alternative explanation for the increased risk of asthma and IBD in these patients is that reduced production of IL-27p28 leads to enhanced gp130 signaling in these settings. Moreover, there is compelling literature regarding intervention strategies that directly target STAT3 (refs. 51, 52) that would be beneficial for treating inflammation-induced gastrointestinal cancers53, and other forms of cancer54. However, STAT3 is a downstream effector of multiple signaling pathways (e.g. IL-6, IL-10, IL-11, IL-21, IL-23, IL-27, OSM, LIF, EGF, PDGF, HGF, Leptin), and its inhibition will likely have broad based effects. Therefore, identification of inhibitors, such as IL-27p28, that specifically antagonize

IL-6 family-mediated STAT3 activation would complement a small molecule approach to prevent or modify ongoing disease.

Methods

Mice

Ebi3$^{-/-}$ mice on a C57BL/6 background were generated by Lexicon Genetics Inc. and provided by M. Elloso (Centocor, Radnor, Pennsylvania). *Il27ra*$^{-/-}$ mice were provided by C. Saris (Amgen). Wild-type C57BL/6 mice were purchased from Jackson laboratories. Mice were housed and bred in specific pathogen–free facilities in the Department of Pathobiology at the University of Pennsylvania in accordance with institutional guidelines.

Generation of the p28 transgenic mice

The open reading frames of mouse *Il27p28* was PCR amplified adding *Fse*I-*Asc*I sites. *Il27p28* cDNA (753bp) was then cloned into the E_{μ} *lck* transgene expression vector, which directs expression primarily to T and B cells as described35. Expression cassettes were excised by *Not*I digestion and microinjected into B6C3f1 murine oocytes fertilized by C57BL/6 males. Microinjection and production of transgenic mice followed procedures as described[64]. Transgenic founders were bred to C57BL/6 mice to generate stable lines of transgenic mice expressing a single allele of *Il27p28*. The p28 transgenic mice were maintained by crossing with wild-type C57BL/6 mice from Jackson laboratories, and age and sex matched wild-type littermates were used as controls in all experiments. Confirmation of IL-27p28 expression by the transgenic mice was determined by measuring IL-27p28 in the sera using an IL-27p28 specific ELISA (R&D Systems). These mice were bred and housed according to institutional guidelines.

Production of recombinant mouse IL-27p28 subunit protein

The murine *Il27p28* gene, Genbank accession number AY099297, was cloned from activated mouse macrophage cDNA via DNA primer extension. The forward DNA primer, 5′-TTCCCAACAGACCCCCTGAGCC-3′, and reverse DNA primer, 5′-TTAGGAATCCCAGGCTGAGCCTG-3′, were used to produce the mature 621 base pair *Il27p28* DNA for expression in a pL promoter system (Invitrogen). The resulting plasmid containing the mature fragment of the *Il27p28* gene was confirmed via nucleotide sequencing and transfected into competent DH5α *E. coli* for fermentation and inclusion body production. Recombinant IL-27p28 inclusion bodies were collected from the bacteria and processed through a refolding platform.

Following folding, the protein was concentrated in a Millipore Cassette concentrator, molecular weight cut off of 3 kDa. The recombinant protein was then centrifuged in a Beckman J2-21centrifuge for 45 min at 7,000 × g to remove any insoluble particulates. It was then carefully titrated to pH 6.0 and loaded onto a 20 ml Pharmacia Ion Exchange column and eluted with a salt gradient from 0 to 1.0 M sodium chloride. Fractions were run on a 4 – 20% SDS-PAGE Tris-Glycine gel and pooled. The pooled sample was dialyzed over night against 10 mM Tris buffer, pH 8.0 at 4°C. The next day the dialyzed sample was loaded onto an 80 micron hydroxylapetite column and a phosphate gradient from 2 mM to

70 mM sodium phosphate, pH 7.5 over 20 column volumes was run to elute the protein. Fractions were pooled based on purity and dialyzed over night at 4°C against 10 mM sodium phosphate, pH 7.5. Protein was quantitated by Bradford assay, sterile filtered through a 0.2 micron filter and lyophilized.

Molecular Modeling

The three-dimensional model structure of the p28–gp130 complex was generated using the IL-6–IL-6R/gp130 structure (pdb accession code: 1p9m) as a template. We used a fold recognition algorithm to prove that the IL-27p28 sequence is compatible with the architecture of the Type I cytokines (ProHit package, ProCeryon Biosciences GmbH). The sequential alignment obtained by the fold recognition algorithm was used to build the IL-27p28 model structure. According to this alignment amino-acid residues were exchanged in the template, and insertions and deletions in IL-27p28 were modeled using a database-search approach included in the software package WHATIF55. For graphical representations the software package RIBBON56 was used.

T cell differentiation

$CD4^+$ T cells were isolated from splenocyte samples and lymph nodes that were depleted of $CD8^+$ and $NK1.1^+$ cells to enrich for $CD4^+$ T cells by magnetic bead separation (Polysciences). Cells were plated in 96-well round-bottom plates (Costar) at a density of 5×10^6 cells per ml. Cells were stimulated with anti-CD3 (1 μg/ml; clone 145-2C11; eBioscience) and anti-CD28 (1 μg/ml; clone 37.51; eBioscience). For the production of $IL\text{-}17^+$ T cells, cultures were supplemented with recombinant mouse IL-6 (10 ng/ml; eBioscience) and human TGF-β1 (1 ng/ml; R&D Systems). In some cases IL-27 (50 ng/ml; Amgen) or IL-27p28 (100 ng/ml; Shenandoah Biotechnology Inc.) were added to the cultures. For the production of $IL\text{-}10^+$ T cells, cultures were supplemented with recombinant mouse IL-27 (50 ng/ml; Amgen). Additionally, IFN-γ and IL-4 were neutralized in all cultures with anti-IFN-γ (10 μg/ml; XMG1.2) and anti-IL-4 (10 μg/ml; 11B11; NCI Preclinical repository). $CD4^+$ T cells were supplemented with fresh medium and reagents on day 3 and were collected on day 4. T cells were restimulated with PMA and ionomycin plus brefeldin A (Sigma) prior to intracellular staining. Cells were stained using the following antibodies: PerCp-anti-CD4 (clone RM4-5), PE-anti-IL-17 (clone TC11-18H10) and APC-anti-IL-10 (clone JES5-16E3) (BD Biosciences). A FACSCalibar (BD Biosciences) or BDFACS CantoII (BD Biosciences) was used for flow cytometry, and the data were analyzed with FlowJo software (Treestar).

Intracellular staining for phosphorylated STAT1, STAT3 and STAT4

T cells were purified from C57BL/6 mice with a mouse T cell enrichment column kit (R&D Systems). Purified T cells (1×10^6) were incubated with IL-6 (10 ng/ml), IL-27 (50 ng/ml), or Hyper-IL-6 (20 ng/ml) for 5, 15, 30, 60 and 120 min. Additionally, T cells were pre-incubated with IL-27p28 (100 ng/ml) for 2 h prior to stimulation with IL-6, IL-27, or Hyper-IL-6. Also, purified T cells were cultured under T_H17 inducing conditions for three days followed by a rest period of 1 h on ice in serum free media before stimulation with IL-11 (10 ng/ml; R&D Systems), or prior to a 2 h pre-incubation with IL-27p28. To assay pSTAT4 activation in response to IL-12 purified T cells were activated for two days with anti-CD3

and anti-CD28, and rested for 1 h on ice in serum free media before stimulation with IL-12 (10 ng/ml; eBioscience) or a 2 h pre-incubation with IL-27p28. Cells were fixed for 10 min at 37°C with 2% (wt/vol) paraformaldehyde. After being fixed, cells were made permeable for 30 min on ice with 90% (vol/vol) methanol, and stained with the appropriate antibodies: AlexaFluor 647-anti-phosphorylated STAT1(pY701), AlexaFluor 488-anti-phosphorylated STAT3(pY705), AlexaFluor 647-anti-phosphorylated STAT4(pY693) and PerCp-anti-CD4 (clone RM4-5;BD Biosciences). Samples were run and analyzed as previously mentioned.

Antibodies and FACS for B cell assays—Splenocytes and bone marrow cells were harvested and stained as previously described57. FACS strategies used the following antibodies: PE-Cy5-anti-CD4 (clone H129.19), anti-CD8 (clone 53-6.7) and anti-Gr-1 (clone RB6-8C5); Alexa700-anti-CD19 (clone eBio1D3(1D3)); Alexa750-anti-B220 (clone RA3-6B2); efluor450-anti-IgD (clone 11-26c(11-26)) (eBioscience). PE-anti-CXCR4 (clone 2B11/CXCR4), PE-Cy5-anti-F4/80 (clone BM8); Biotin-anti-CD3e (clone 145-2C11), anti-GR-1 (clone RB6-8C5), anti-F4/80 (clone BM8) and anti-IgD (clone 11-26) (BD Pharmingen). FITC-anti-Igλ (clone JC5-1); PE-anti-Igλ (clone JC5-1) and anti-Igκ (goat polyclonal) (Southern Biotech). FITC-PNA (Sigma). Q655-anti-Igκ and APC-NP were conjugated in house. Live cells were determined by pre-incubation with AmCyan fixable live/dead stain (Invitrogen). Cells were fixed and permeabilized using solution A and B (Caltag). Flow cytometry was performed on a BD LSRII. Analysis was done using FlowJo software (Tree Star, Inc.).

ELISPOT Assay—Multiscreen HTS plates (Millipore) were coated with either anti-Ig(H+L), NP_{33}BSA, or NP_4BSA in sodium bicarbonate buffer. Plates were blocked with 2% BSA. Cells were incubated in the plate, undisturbed for 4–6 h at 37°C. Biotin-anti-Igλ and/or anti-Igκ (Southern Biotech) were added, followed by SA-alkaline phosphatase (Sigma). Spots were detected using BCIP/NBT (Sigma), scanned and counted on an ImmunoSpot Analyzer (Cellular Technology Ltd.).

NP-Ficoll and NP-CGG immunizations

P28 transgenic mice and wild-type littermates were immunized i.p. with 50–100 μg NP_{16}-CGG in alum or 100 μg NP_{50}-Ficoll in saline as previously described58. Mice were sacrificed on day 5 following NP-Ficoll immunization, while mice immunized with NP-CGG were examined on day 7, 14 and 56 post-immunization.

Immunohistochemistry

Spleens were immersed in O.C.T. (Tissue Tek) and flash frozen using 2-methylbutane and liquid nitrogen, and stored at −20°C. Afterwards, 7 μm sections were sliced in a cryostat (Zeiss HM505E) and fixed with cold acetone and stored at −20°C. Prior to staining, the sections were re-hydrated in PBS and incubated with PBA containing 10% goat serum. The sections were stained with peanut agglutinin (PNA) conjugated to Rhodamine (Vector Labs), anti IgD-AlexaFluor 647 (clone 11-26, eBioscience) and anti-CD3-FITC (clone H57-507, eBioscience). Sections were mounted using Biomedia Gel/Mount (Electron Microscopy Sciences) and visualized on an LSM-510 Meta confocal microscope (Zeiss)

Statistics

Unpaired Student's *t*-test and nonparametric Mann Whitney U *t*-test were used to determine significant differences, and *P* values less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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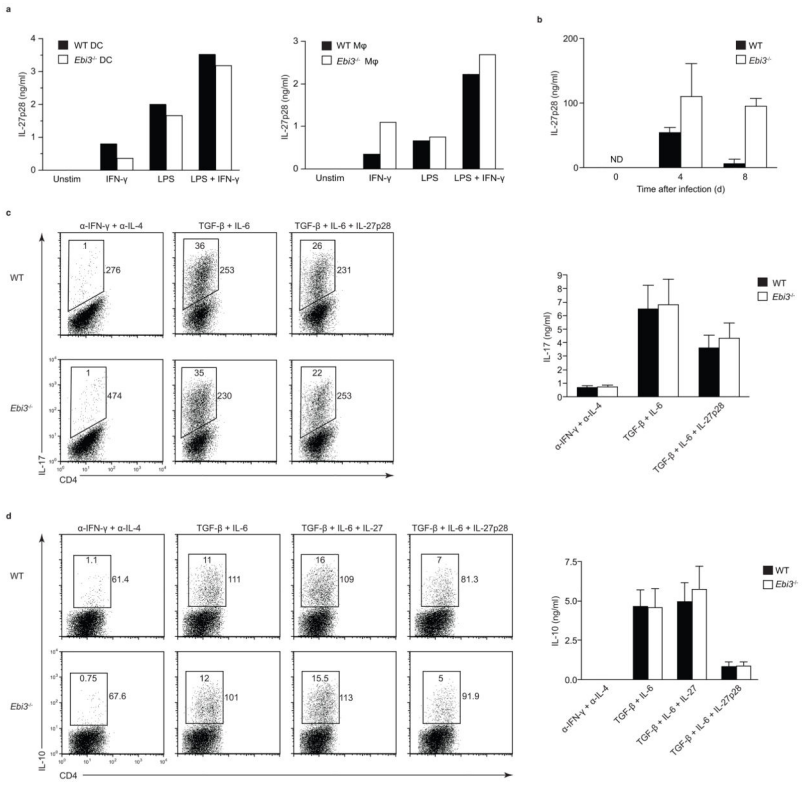

Figure 1.

IL-27p28 has biological activity in the absence of EBI3. (**a**) ELISA of IL-27p28 production by bone-marrow derived dendritic cells and macrophages isolated from wild-type (C57BL/6) or *Ebi3*$^{-/-}$ mice stimulated with IFN-γ, LPS or the combination of LPS and IFN-γ for 24 h. Data are representative of three independent experiments with similar results. (**b)** ELISA of IL-27p28 production in the serum of wild-type and *Ebi3*$^{-/-}$ mice isolated prior to and on day 4, 8 post-infection with *T. gondii*. Data are representative of three independent experiments with groups of three to four mice (error bars, s.d.). (**c, d**) Flow cytometry of intracellular IL-17A and IL-10, or ELISA of IL-17 and IL-10 production in CD4^{+} T cells isolated from the spleen and lymph nodes of wild-type or *Ebi3*$^{-/-}$ mice, activated with anti-CD3 and anti-CD28 in T_H17-polarizing conditions in the presence or absence of IL-27 or IL-27p28 for 4 d. Then the cells were stimulated for 4 h with PMA and ionomycin in the presence of brefeldin A; ELISAs were done after 72 h of stimulation. Numbers in boxes indicate percent IL-17^{+} or IL-10^{+} cells. Numbers outside of boxes represent the MFI of IL-17^{+} or IL-10^{+} cells. Data are representative of three independent experiments with similar results using groups of two to three mice (error bars, s.d.).

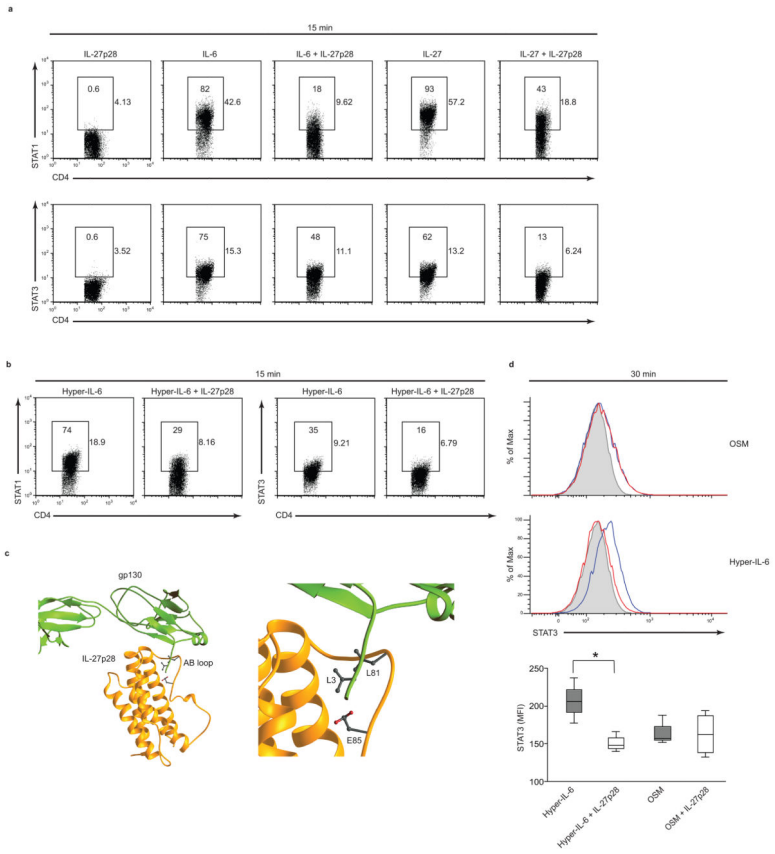

Figure 2.

IL-27p28 antagonizes gp130-mediated STAT phosphorylation. (**a,b**) Flow cytometry of intracellular phosphorylated STAT1 (p-STAT1) or STAT3 (p-STAT3) in $CD4^+$ T cells purified from wild-type mice and stimulated with IL-27p28, IL-6, IL-27 or Hyper-IL-6 for 15 min. Additionally, where indicated IL-27p28 was pre-incubated with T cells for 2 h at 37°C prior to adding IL-6, IL-27 or Hyper-IL-6. Numbers in boxes represent percent $CD4^+$ T cells positive for p-STAT1 or p-STAT3. Numbers outside box represent MFI for p-STAT1 and p-STAT3. Data are representative of four independent experiments with similar results. (**c**) Three-dimensional model illustrating the interaction of IL-27p28 with gp130 highlighting amino acid residues that are key to this interaction, and which differ between IL-27p28 and IL-6. (**d**) Flow cytometry of intracellular p-STAT3 in mouse embryonic fibroblasts (MEFs) stimulated with OSM or Hyper-IL-6 (blue histogram) or without stimulation (gray shaded histogram) following 15 min incubation at 37°C. Additionally, IL-27p28 was incubated with MEFs for 2 h at 37°C prior to stimulation with OSM or Hyper-IL-6 (red histogram). Data are representative of three individual experiments with similar results. Box and whiskers plot represents the change in MFI of p-STAT3 in MEFs pre-incubated with IL-27p28 prior to stimulation with OSM or Hyper-IL-6 for 15 min from the five independent experiments. *, $P = 0.0059$ as determined by an unpaired *t* test (error bars, s.d.).

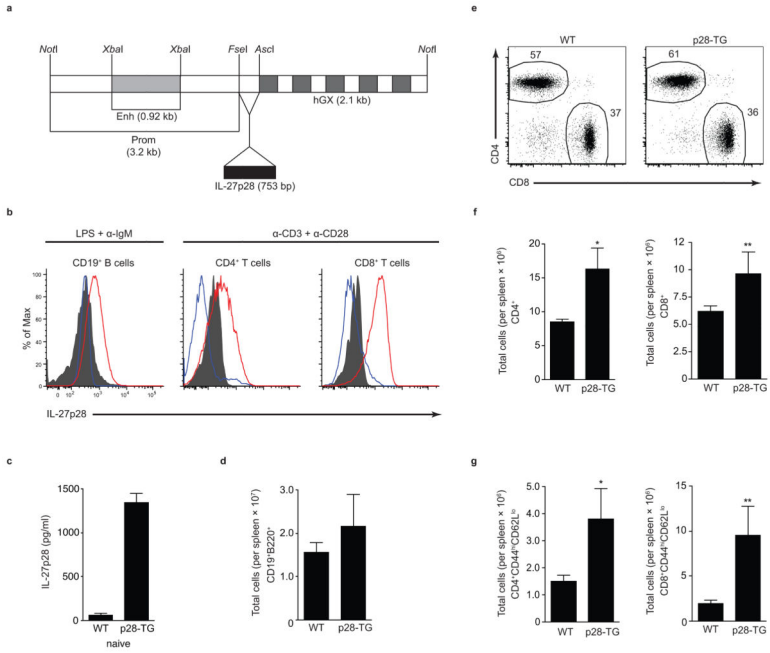

Figure 3.

Phenotypic analysis of the IL-27p28 transgenic mice. (**a**) Schematic of the IL-27p28 transgene construct. Functional elements include the juxtaposed *lck* proximal promoter (Prom) and Eμ enhancer (gray box), the insertion site for IL-27p28 and a mutated (non-translatable) version of the human growth hormone (hGX) gene (alternating black and white boxes represent exons and introns respectively). (**b**) Flow cytometry to detect intracellular IL-27p28 in wild-type (blue histogram) and p28 transgenic (red histogram) $CD19^+$ B cells or $CD4^+$ and $CD8^+$ T cells following stimulation with LPS and anti-IgM or activation with anti-CD3 and anti-CD28 for 48 h. Cells were incubated with Brefeldin A for 4 h prior to staining. Shaded histogram represents fluorescence minus the PE channel (FMO). (**c**) ELISA for IL-27p28 in serum of naïve wild-type littermates and p28 transgenic mice. Results are representative of three individual experiments with groups of three to four mice (error bars, s.d.). **d**) Total $CD19^+B220^+$ B cells in the spleen of naïve wild-type littermates and p28 transgenic mice, calculated from percentages determined by flow cytometry. (**e**) Flow cytometry of splenocytes from naïve wild-type littermates and p28 transgenic mice stained for CD4 and CD8 to determine the ratio of each cell type. Numbers beside outlined areas indicate the percent $CD4^+CD8^-$ (left) and $CD8^+CD4^-$ (right) cells. (**f**) Total $CD4^+$ (left) and $CD8^+$ (right) T cells in the spleen of naïve wild-type littermates and p28 transgenic mice in **e**, calculated from percentages determined by flow cytometry. *, $P = 0.0024$. **, $P = 0.0148$. (**g**) Total $CD4^+CD44^{hi}CD62L^{lo}$ (left) and $CD8^+CD44^{hi}CD62L^{lo}$ T cells in the spleen of naïve wild-type littermates and p28 transgenic mice, calculated from percentages determined by flow cytometry. *, $P = 0.0062$. **, $P = 0.0036$ (**d–g**) Results are representative of three independent experiments with groups of two to four mice (error bars, s.d., **d, f, g**). *P* values determined by an unpaired *t* test.

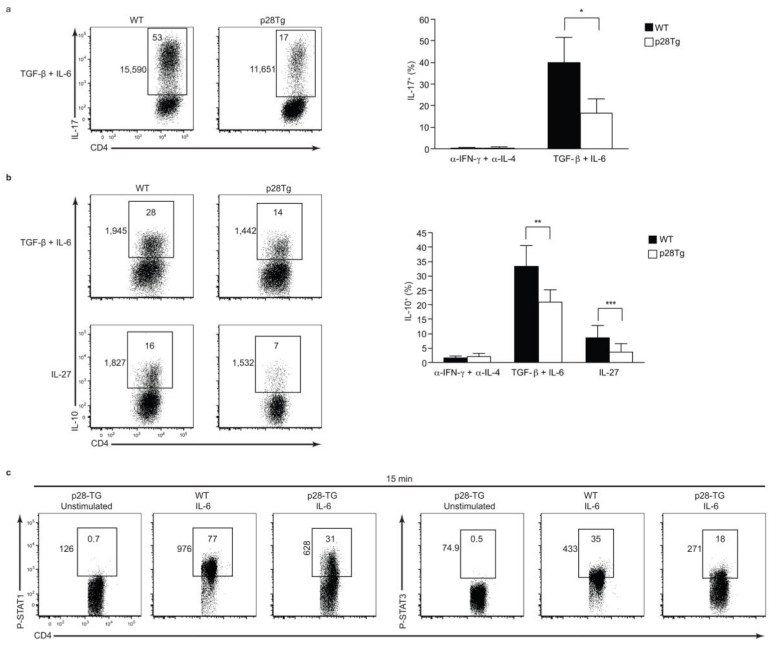

Figure 4.

Transgenic overexpression of IL-27p28 antagonizes the activity of IL-6 and IL-27 on $CD4^+$ T cells. (**a, b**) Flow cytometry (left) for intracellular (**a**) IL-17A and (**b**) IL-10 and graph (right) depicting percentage of (**a**) $IL\text{-}17^+$ and (**b**) $IL\text{-}10^+$ $CD4^+$ T cells isolated from the spleen and lymph nodes of wild-type or p28 transgenic mice, activated with anti-CD3 and anti-CD28 in non-polarizing conditions in the presence of (**a**, **b**) TGF-β plus IL-6 or (**b**) IL-27 for 4 d. Cells were stimulated for 4 h with PMA and ionomycin in the presence of brefeldin A. Numbers in boxes indicate percent $IL\text{-}17A^+$ or $IL\text{-}10^+$ cells, and numbers outside boxes represent the MFI of the $IL\text{-}17^+$ and $IL\text{-}10^+$ cells. Data are representative of four individual experiments with similar results (error bars, s.d.). *, $P = 0.0002$. **, $P = 0.0009$. ***, $P = 0.0158$. (**c**) Flow cytometry of intracellular p-STAT1 or p-STAT3 in $CD4^+$ T cells purified from p28 transgenic mice and left unstimulated or stimulated with IL-6 for 15 min. T cells were pre-incubated at 37°C for 2 h prior to addition of IL-6. Numbers in boxes represent percent $CD4^+$ T cells positive for p-STAT1 or p-STAT3. Numbers outside boxes represent MFI for p-STAT1 or p-STAT3. Data are representative of three independent experiments with similar results. *P* values determined by an unpaired *t* test.

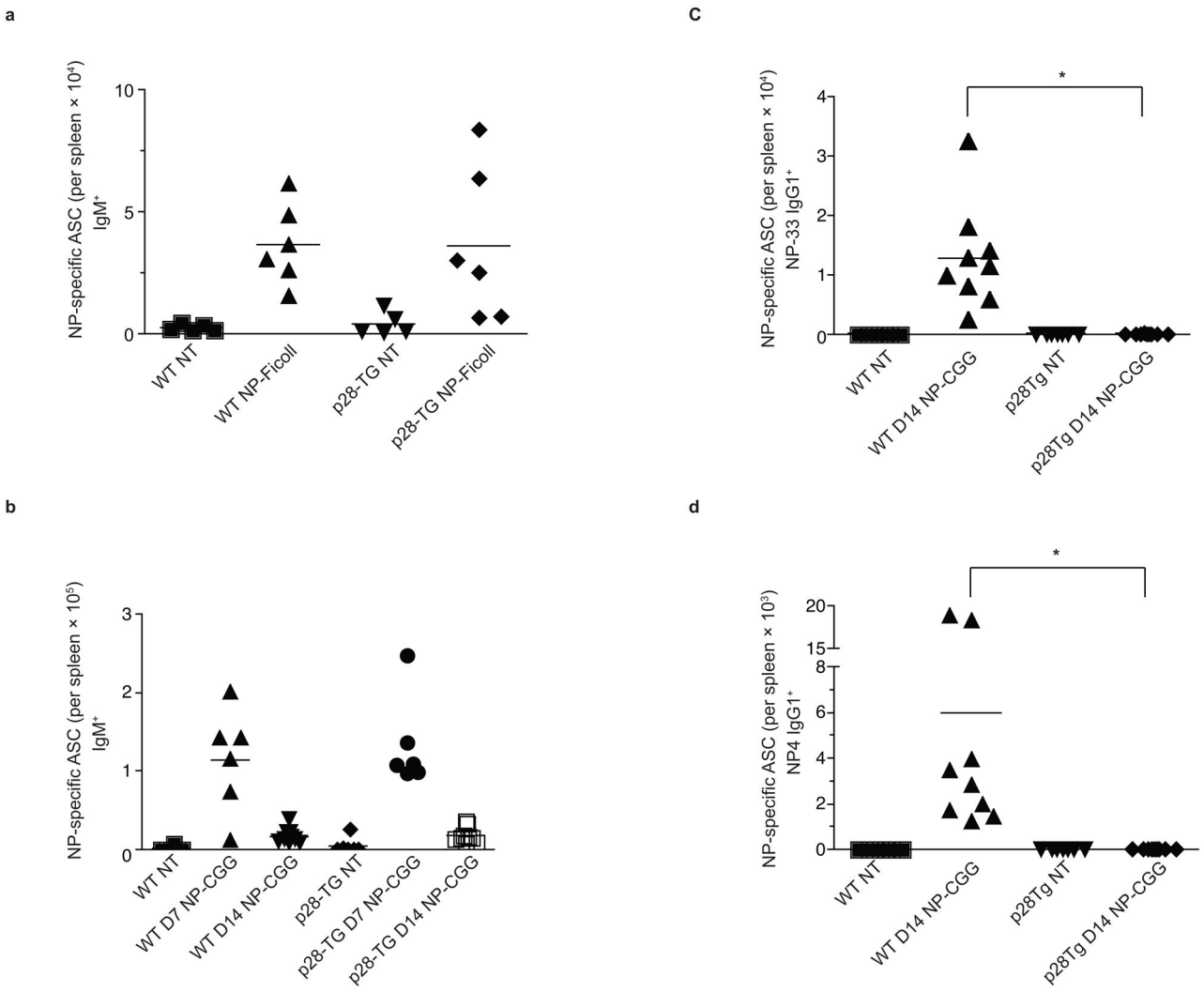

Figure 5.

p28 transgenic mice fail to generate an antigen-specific IgG response following immunization with a TD antigen. (**a**) ELISPOT to measure the number of antibody secreting cells (ASC) able to bind to NP(33)-BSA in the spleen of naïve wild-type littermate controls and p28 transgenic mice or 5 d after immunization with the TI antigen NP-Ficoll in saline. Results are representative of two independent experiments with groups of two to three mice. (**b**–**d**) ELISPOT to measure the number of (**b**) IgM or (**c**) IgG1 ASC able to bind NP(33)-BSA, or (**d**) IgG1 ASC able to bind NP(4)-BSA in the spleen of naïve wild-type littermate controls and p28 transgenic mice or 7 and 14 d after immunization with the TD antigen NP-CCG in alum. Results are representative of two (day 7) or three (day 14) independent experiments with groups of three mice. *, $P < 0.001$. *P* values determined by a non-parametric Mann Whitney U *t* test.

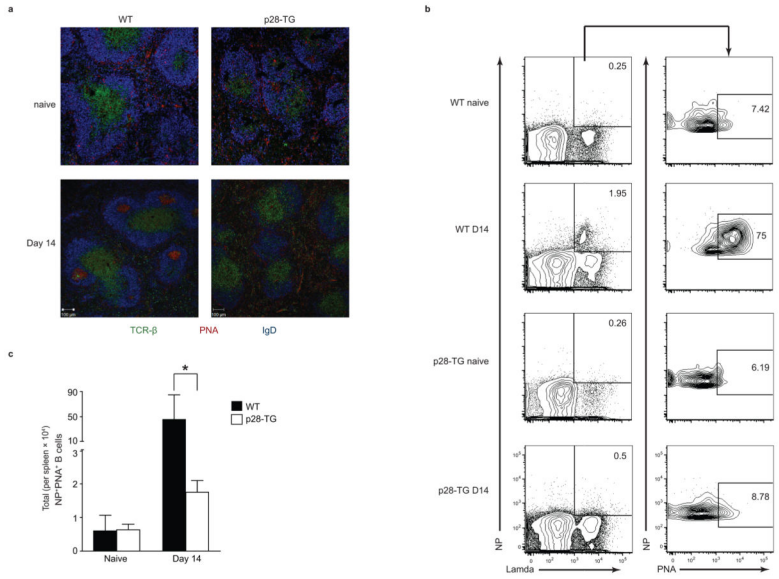

Figure 6.

Transgenic expression of IL-27p28 blocks formation of GC reactions following immunization with a TD antigen. (**a**) Sections of spleen from wild-type littermate control and p28 transgenic mice left unimmunized (top) or immunized with NP-CGG in alum, isolated 14 d after immunization and stained with anti-TCR-β-FITC (T cells), PNA conjugated to Rhodamine (GC B cells) and anti-IgD-AlexaFluor 647 (B cell follicles). Original magnification, ×10. Images are representative of two independent experiments with groups of three mice. (**b**) Flow cytometry to identify PNA^+ B cells (right gate) that possess NP bound to lambda light chain (left gate) in the spleen of naïve wild-type littermate control and p28 transgenic mice or 14 d after NP-CGG immunization. Numbers in boxed areas indicate percent $lambda^+NP^+$ B cells (left) and NP^+PNA^+ GC B cells (right). (**c**) Total number of NP^+PNA^+ B cells found in each spleen from **b**, calculated from percentages determined by flow cytometry. (**b, c**) Results are representative of three independent experiments with groups of three mice. *, $P = 0.0049$ as determined by an unpaired *t* test.