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IL-27p28 inhibits central nervous system autoimmunity by concurrently antagonizing Th1 and Th17 responses

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Abstract

Central nervous system (CNS) autoimmunity such as uveitis and multiple sclerosis is accompanied by Th1 and Th17 responses. In their corresponding animal models, experimental autoimmune uveitis (EAU) and experimental autoimmune encephalomyelitis (EAE), both responses are induced and can drive disease independently. Because immune responses have inherent plasticity, therapeutic targeting of only one pathway could promote the other, without reducing pathology. IL-27p28 antagonizes gp130, required for signaling by IL-27 and IL-6, which respectively promote Th1 and Th17 responses. We therefore examined its ability to protect the CNS by concurrently targeting both effector responses. Overexpression of IL-27p28 *in vivo* ameliorated EAU as well as EAE pathology and reduced tissue infiltration by Th1 and Th17 cells in a disease prevention, as well as in a disease reversal protocol. Mechanistic studies revealed inhibition of Th1 and Th17 commitment *in vitro* and decreased lineage stability of pre-formed effectors *in vivo*, with reduction in expression of gp130-dependent transcription factors and cytokines. Importantly, IL-27p28 inhibited polarization of human T cells to the Th1 and Th17 effector pathways. The ability of IL-27p28 to inhibit generation as well as function of pathogenic Th1 and Th17 effector cells has therapeutic implications for controlling immunologically complex autoimmune diseases.

Keywords

Autoimmunity; gp130; IL-27p28

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.jaut.2013.08.003>.

1. Introduction

Both Th1 and Th17 responses have been connected to debilitating central nervous system diseases such as autoimmune uveitis and multiple sclerosis. Human autoimmune uveitis is a group of intraocular inflammatory diseases that affect the neural retina and are estimated to cause 10–15% of blindness in the western world [1]. Antigen specific CD4⁺ effector T cells have a central role in the pathogenesis and T cell directed therapies ameliorate disease. Published data on cytokine profiles of uveitis patients provide evidence that an elevated Th1 response is associated with some types of human uveitis [2], whereas a role for the Th17 response has been suggested in others [3,4]. Similarly, both Th1 and Th17 responses are reported in patients with multiple sclerosis and different responses determine the efficacy of treatments [5].

Experimental autoimmune uveitis (EAU), induced by immunization with retinal antigen(s) that elicit memory responses in lymphocytes of uveitis patients, serves as a model for clinical autoimmune uveitis. As appears to be the case in the human disease, both Th1 and Th17 responses are generated and both are involved in pathogenesis of EAU. The data supporting this conclusion are: (i) EAU induced by active immunization with interphotoreceptor retinoid binding protein (IRBP) in CFA and EAU induced by infusion of IRBP-pulsed mature dendritic cells (DCs) were found to require, respectively, Th17 and Th1 responses; (ii) EAU could develop in mice deficient in IFN- γ or IL-17; (iii) polarized IL-17-producing Th17 or IFN- γ -producing Th1 uveitogenic T cells could induce full blown disease in recipients lacking the reciprocal signature cytokine [6,7]. Similarly, experimental autoimmune encephalomyelitis (EAE) can also be induced by Th1 polarized and Th17 polarized cells independently [5]. Thus, either Th1 or Th17 effector response is capable of driving CNS autoimmune diseases.

Chronic autoimmune diseases, including uveitis and multiple sclerosis, are believed to involve continuous recruitment and priming of new effector T cells. It has become increasingly clear that immune responses have an inherent plasticity. Th1 and Th17 effectors derive from a common pool of Ag-specific precursors, which can be differentiated along either pathway. Therefore, therapeutic inhibition of one effector pathway might simply shunt the response to the other, equally pathogenic, lineage. This concept is supported by observations in animal models, showing that neutralization or deficiency of the Th1 signature cytokine IFN- γ leads to an elevated Th17 response, whereas deficiency of IL-17 leads to an elevated Th1 response [6,8,9]. The ideal therapy would thus be one that targets both the Th1 and the Th17 responses concurrently.

Type 1 cytokine receptors are transmembrane receptors with a conserved WSXWS motif that recognize and respond to cytokines with 4 α -helical strands such as IL-6, IL-12, IL-23 and IL-27, which are involved in T cell effector choices [10]. Among them, IL-6 signaling is required to mediate STAT3-dependent retinoic acid-related orphan receptor (ROR γ t) expression for Th17 polarization [11]. IL-27 signaling promotes Th1 polarization by inducing T box transcription factor (Tbet) expression through STAT1 and p38 MAPK phosphorylation [12,13]. Both IL-6 and IL-27 receptors share a common β subunit, i.e. gp130, which is also shared with the receptors of other members in the IL-6 cytokine family

[14]. Elimination of IL-27 α signaling inhibits the Th1 pathway, while blockade of IL-6R signaling inhibits the Th17 effector pathway, ameliorating EAU, similarly to direct targeting of IFN- γ or IL-17 [15,16]. However, individual blockade of either the Th1 or the Th17 pathway, while effective in the short term, may be inadequate as a long-term treatment of chronic disease. Clinical evidence to support this notion derives, among others, from limited success of Th17-directed therapy in Behçet's uveitis [17] and Crohn's disease [18], although in both an association with Th17 had been reported, and from varying degrees of success with IFN- β therapy in different forms of multiple sclerosis [5].

IL-27p28, the α subunit of IL-27, is a natural antagonist of gp130, which is required for signaling by IL-27 and IL-6 receptors [19]. IL-27p28 was reported to control B cell responses and to inhibit differentiation of T cells towards the Th17 lineage by blocking gp130 [19]. However, very little is known about its other activities in controlling T cell-mediated autoimmune diseases. In the present study we use the EAU and EAE models of CNS autoimmunity to investigate the ability of IL-27p28 to concurrently regulate auto-pathogenic Th1 and Th17 responses *in vivo*, and we examine the associated mechanisms. We demonstrate that *in vivo* over-expression of IL-27p28 ameliorates actively induced EAU and EAE and reduces development of Th1 and Th17 responses, by interfering with Th1/Th17 lineage commitment through effects on STAT molecules and lineage-specific transcription factors. Importantly, IL-27p28 also ameliorated adoptively transferred EAU induced by already differentiated Th1 or Th17 cells and reduced effector cell numbers, at least in part by impeding lineage stability. Our findings suggest that IL-27p28 effectively suppresses acquisition as well as expression of Th1 and Th17 immunity, providing a potential approach to treatment of CNS and other autoimmune diseases where there is involvement of functionally redundant Th1/Th17 effector responses.

2. Materials and methods

2.1. Mice

p28-TG mice in C57BL/6 background were generated by Zymogenetics, WA. These mice have no difference in the number of mature B cells and CD4⁺T/CD8⁺T cells ratio, but have relatively higher total numbers of CD4⁺ and CD8⁺ T cells in the spleen [19]. C57BL/6 and B10.RIII mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IRBP161-180 T cell receptor transgenic mice (R161H) [60] were produced and bred in-house. All mice were kept in a specific pathogen-free facility and fed standard laboratory chow ad libitum. Animal care and use were in compliance with institutional and ARVO guidelines. The animal study protocol was approved by the Animal Care and Use Committee of the National Eye Institute.

2.2. Human blood samples

Buffy coats from healthy blood donors were obtained from the National Institutes of Health blood bank. Research performed in this study with human samples was in compliance with guidelines of the National Institutes of Health Institutional Review Board.

2.3. Reagents and antibodies

Recombinant mouse IL-6, IL-23 and human IL-1 β , IL-6, IL-12 IL-23 TGF- β 1, antihuman IFN- γ and antihuman IL-4 were obtained from R&D Systems (Minneapolis, MN); recombinant human IL-2 and mouse IL-12 from PeproTech (Rocky Hill, NJ); recombinant mouse and human IL-27 from eBioscience (San Diego, CA); recombinant mouse IL27-p28 from Shenandoah Biotechnology (Warwick, PA); anti-mouse IFN- γ (clone R4-6A2) was made by Bio-XCell (West Lebanon, NH); and anti-mouse IL-4 (11B11) was obtained from National Cancer Institute-Frederick Biological Resources Branch Preclinical Repository (Frederick, MD). Complete Freund's Adjuvant (CFA) and purified *Bordetella pertussis* toxin were purchased from Sigma-Aldrich (St. Louis, MO) and *M. tuberculosis* strain H37RA from Thomas Scientific (Swedesboro, NJ). IRBP was isolated from bovine retinas, as described previously [20]. Human IRBP peptide residues 161–180 (SGIPYIISYLHPGNTILHV, IRBP161-180) and Human IRBP peptide residues 1–20 (GPThLFQPSLVLDMAKVLLD, IRBP1-20) were purchased from AnaSpec (Fremont, CA). Anti-mouse CD3, CD4, CD44, CD90.1, CD90.2, IFN- γ and IL-17A were purchased from Biolegend (San Diego, CA); Anti-pSTAT1 (pY701), pSTAT3 (pY705) and pSTAT4 (pY693) were purchased from BD Biosciences (San Jose, CA).

2.4. Induction of EAU and disease scoring

Induction of EAU by active immunization was described previously [6]. In brief, p28-TG mice and their WT littermates (C57BL/6 background) were immunized subcutaneously with a mixture of 150 μ g native IRBP mixed with 300 μ g IRBP peptide 1-20 emulsified in an equal volume of CFA containing 2.5 mg/ml *M. tuberculosis*. In addition, these mice also received 0.5 μ g of *Bordetella pertussis* toxin intra-peritoneally on the day of immunization. B10.RIII mice were immunized with 7 μ g IRBP peptide 161–180 (1:1 v/v with CFA) subcutaneously without pertussis toxin. In some experiments, immunized mice received IL-27p28 (5 μ g per injection) every other day, starting from day 0.

For induction of EAU by adoptive transfer, lymph nodes from naive R161H mice (B10.RIII background) dispersed into single-cell suspensions were cultured in 12-well plates at 2×10^6 cells/ml (5×10^6 cells/well). Cells were activated with 2 μ g/ml of IRBP161-180 under Th1 or Th17 polarizing conditions in the presence of 10 ng/ml of IL-12 and 10 μ g/ml of anti-IL-4 for Th1, or 25 ng/ml IL-6, 1 ng/ml of TGF- β , 10 μ g/ml of anti-IFN- γ and 10 μ g/ml of anti-IL-4 for Th17 polarization. After 24 h, 10 ng/ml of IL-2 or IL-23 were added to the Th1 and Th17 cultures respectively. After 72 h, cells were purified by centrifugation over Lympholyte M (Cedarlane, Burlington, NC) and washed with 1X PBS. Approximately 4×10^6 cells were injected i.p. into naive B10.RIII mice. In some experiments, recipient mice received recombinant IL-27p28 (5 μ g per injection) twice daily.

Clinical EAU was evaluated by fundus examination on a scale of 0–4 based on the extent of inflammation [21]. Eyes harvested at 21 days after active immunization, or 14 days after adoptive transfer, were processed for histopathology and stained with standard hematoxylin and eosin. Histopathological EAU scores were assigned in a masked fashion on a scale of 0–4, based on the number, type, and size of lesions [21].

2.5. Induction of EAE and disease scoring

EAE was induced in p28-TG mice and their WT littermates by subcutaneous immunization with 200 µg MOG_{35–55} peptide emulsified 1:1 v/v with CFA containing 5 mg/ml *M. tuberculosis*. *Bordetella pertussis* toxin (0.3 µg in 100 µl) was injected i.p at the time of immunization. Disease severity was assessed daily by a masked observer and disease scores were assigned as follows: 0: no clinical signs; 0.5: weakness of the tail; 1: complete tail paralysis; 2: partial hind limb paralysis; 3: complete hind limb paralysis; 4: incontinence and partial paralysis of forelimbs; 5: Complete paralysis of forelimbs or moribund.

2.6. Assay of anti-IRBP antibody titers by ELISA

Serum antibodies were assayed on IRBP-coated plates (2 µg/100 µl/well) using HRP-labeled goat anti-mouse IgG (Zymed Laboratories Inc., San Francisco, California, USA) as developing antibody and 3,3',5,5'-tetramethylbenzidine as substrate (100 µl/well; Endogen Inc., Woburn, Massachusetts, USA).

2.7. Construction and delivery of IL-27p28 expression plasmid

IL-27p28 expression plasmid was provided by Shenandoah Technology, PA. The mouse IL-27p28 gene was PCR amplified and cloned into an expression vector (pcDNA3.1 Directional Topo Expression Kit, Invitrogen, Grand Island, NY) as described previously [22]. The positive clone with IL-27p28 insert is referred to as “pmIL27p28”. pcDNA3.1 vector without insert is referred to as “vector control”. Both DNA constructs were amplified using Qiagen Endotoxin Free Giga kit.

The DNA constructs were delivered to mice by hydrodynamic injection [22]. Briefly, 20 µg of the construct was diluted in 2 ml of Ringer's solution and injected rapidly (<7 s) through the tail vein. Injection of DNA constructs was performed on day 0 and 7 after active immunization, and on day 0 of adoptive transfer-induced EAU.

2.8. Isolation and analysis of eye-infiltrating cells

Eyes were collected from mice with EAU 21 d after active immunization or 4–8 d after adoptive transfer, as specified. After trimming of external tissue, the eyes were carefully dissected along the limbus for lens removal. The remaining tissue was minced with scissors in cold RPMI medium. After centrifugation, the resultant cell pellet was resuspended in RPMI with 10% FCS plus 1 mg/ml collagenase D and incubated for 45 min at 37 °C. Samples were dispersed by trituration, washed, filtered, and suspended in RPMI with 10% FCS. Cells were then pulsed with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (GolgiPlug; BD Pharmingen, San Diego, CA) for 4 h, fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.1% BSA and 0.05% Triton X-100 for intracellular cytokine staining with anti-IFN-γ and IL-17A.

2.9. T cell differentiation

2.9.1. Mouse—CD4⁺CD62L⁺ T cells were purified from p28 transgenic mice and their wild type littermates by using CD4⁺CD62L⁺ T Cell Isolation Kit II (Miltenyi Biotech, Cambridge, MA). Cells were stimulated by plate-bound anti-CD3 (2 µg/ml) and soluble

anti-CD28 (1 µg/ml). For Th1 polarization, cultures were supplemented either with 10 ng/ml IL-12 or 50 ng/ml IL-27 plus 10 µg/ml of anti-IL-4. For Th17 polarization, cultures were supplemented with 10 ng/ml IL-6, 1 ng/ml TGF-β, 10 µg/ml anti-IFN-γ and 10 µg/ml anti-IL-4. For Th0, cultures were supplemented with 10 µg/ml anti-IFN-γ and 10 µg/ml anti-IL-4. Where specified, 100 ng/ml of IL-27p28 was added to the cultures. On day 3, cells were pulsed with PMA/Ionomycin and stained for intracellular cytokine analysis, as described above.

2.9.2. Human—PBMCs were isolated from peripheral blood using Histopaque-1077 (Sigma–Aldrich). Naïve CD45RA⁺CD4⁺ T cells were isolated from the PBMCs by using naïve CD4⁺ T cell isolation kit II (Miltenyi Biotec). Cells were stimulated with anti-CD3/CD28 coated beads (Invitrogen) in a bead-to-cell ratio of 1:10. For Th1 polarization, 10 ng/ml IL-12 or 50 ng/ml IL-27 plus 10 ng/ml of anti-IL-4. For Th17 polarization, cultures were supplemented with 10 ng/ml of IL-1β, IL-6, IL-23, 1 µg/ml TGF-β, 10 ng/ml anti-IFN-γ and 10 µg/ml anti-IL-4. For Th0, cultures were supplemented with 10 µg/ml anti-IFN-γ and 10 µg/ml anti-IL-4. Where specified, 500 ng/ml of IL-27p28 was added to the cultures. Half of the culture medium was replaced with fresh cytokine-containing medium every 4–5 days. On day 14, cells were pulsed with PMA/Ionomycin and stained for intracellular cytokine analysis as described above.

2.10. Real time PCR

Total RNA extraction was carried out with RNeasy mini Kit (Qiagen, Valencia, CA) and cDNA was synthesized with Superscript III First Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed with a TaqMan 7500 sequence detection system (Applied Biosystems, Foster City, CA) using gene-specific primer/probe sets from Applied Biosystems. Data were normalized to GAPDH expression, and results are expressed relative to Th0.

2.11. Intracellular staining of phosphorylated STAT1, STAT3 and STAT4

To study IL-12 signaling, CD4⁺CD62L⁺ T cells were purified from C57BL/6 mice and were differentiated under Th1 conditions to induce IL12rβ2 expression as described above. After 2 days, cells were placed on ice for 1 h before pre-incubation with IL-27p28 (100 µg/ml) for 2 h. Cells were restimulated with IL-12 (10 ng/ml) and harvested after 30 min. Alternatively, resting CD4⁺ T cells that expressed IL27ra and IL6ra were isolated by magnetic beads (Miltenyi Biotec), pre-incubated with IL-27p28 (100 ng/ml) for 2 h and then stimulated with IL-27 (50 ng/ml) or IL-6 (10 ng/ml) for 30 min. Cells were fixed with Cytofix, permeabilized with Perm III buffer (BD Pharmingen) according to manufacturer's instructions, and stained with anti-CD4, anti-pSTAT1, anti-pSTAT3 or anti-pSTAT4.

2.12. Knockdown of IL27Ra and IL6Ra by siRNA

Cells obtained from R161H mice [60] were polarized under Th1 or Th17 conditions with the specific antigen, IRBP161-180, in the presence of Accell siRNA oligos that target IL27ra or IL6ra (Dharmacon, Lafayette, CO) and with 3% FCS as described by others [23]. Cells were harvested for adoptive transfer or cDNA synthesis.

2.13. Three-dimensional modeling

The model of the 3-dimensional structure of the mouse IL-27A/human gp130 complex was constructed by homology modeling based on crystal coordinates for the IL-6–IL-6R–gp130 protein complex (Protein Data Bank file: 1p9m) as a structural template. The amino acid sequences of the mouse p28 and B subunit of the hexameric human il-6/il-6 alpha receptor/gp130 complex) were aligned by the method of Needleman & Wunch [24], incorporated in the program Look version 3.5.2 [25]. The 3-dimensional structure of the mouse IL-27A was built by using the automatic segment matching algorithm incorporated in the same program [26] followed by 500 cycles of energy minimization. Finally, the structure of mouse IL-27p28 was docked to the human gp130 using the 1p9m biological assembly structure as a template to find a mouse IL-27A/human gp130 complex. The UCSF Chimera software package (<http://www.cgl.ucsf.edu/chimera/>) was used for the structure visualization.

2.14. Data reproducibility and statistical analysis

Mann–Whitney *U* test, unpaired *t*-test or paired *t*-test was used for two-group comparison. Two-way ANOVA was used for multi-group analysis. A *p* value 0.05 was considered statistically significant. Data are displayed as mean ± SEM. Experiments were repeated at least twice with essentially the same result. Depending on the experiment, Figures depict combined or representative data, as specified.

3. Results

3.1. IL-27p28 inhibits generation of Th1 and Th17 effector responses in vivo, and ameliorates EAU and EAE

To examine whether IL27-p28 affects susceptibility to EAU, we immunized IL-27p28 transgenic mice on the C57BL/6 background (p28-TG) [19] and their wild type (WT) littermates with the uveitogenic retinal antigen, IRBP. p28-TG mice developed significantly lower disease scores than their WT littermates and had fewer IL-17A and IFN- γ producing CD4⁺ T cells in their eyes (Fig. 1A and B). GM-CSF has recently been identified as the shared effector molecule of both Th1 and Th17 effector CD4⁺ T cells that are responsible for the pathology of EAE [27,28]. Notably, p28-TG mice had markedly reduced GM-CSF-producing CD4⁺ T cells in their ocular cell infiltrate (Fig. 1B). This finding demonstrates that the protective role of IL-27p28 in tissue specific autoimmunity is not restricted to uveitis.

Because the EAU and EAE resistance of p28-TG mice was compelling, we wished to examine the therapeutic potential of IL-27p28 in a more clinically relevant situation, by therapeutic overexpression of IL-27p28 in the adult mouse and by treatment with the recombinant protein itself. *In vivo* expression of IL-27p28 was induced by hydrodynamic injection of an IL-27p28 expression plasmid (pmIL27p28) in B10.RIII mice, a strain highly susceptible to EAU. After a single hydrodynamic injection, IL-27p28 was detectable by ELISA in the serum of mice for about one week (Fig. S1A). For induction of EAU, B10.RIII mice were immunized on the same day with a known pathogenic epitope of IRBP for this genotype, residues 161–180 (IRBP161-180) and received pmIL27p28 or vector control hydrodynamically on day 0 and 7. These 2 hydrodynamic injections induce serum IL-27p28

level that is comparable to EAU-immunized p28-TG during the priming phase but a lower level was observed during the effector phase (Fig. S1B and C). Mice that had been injected with pmIL27p28 developed significantly milder EAU when compared to the vector-injected control ($p < 0.05$, Fig. 1C). As with p28-TG mice (Fig. 1B), the ocular infiltrate of pmIL27p28 treated mice contained fewer IL-17A, IFN- γ and GM-CSF-producing CD4⁺ T cells (Fig. 1D). Furthermore, administration of a low dose of recombinant IL-27p28 protein to mice that had been immunized with IRBP also significantly ameliorated development of disease (Fig. 1E).

A number of studies have shown that gp130 signaling can also regulate B cell responses, including inhibition of germinal center formation and antibody production [19,29–32]. Although EAU is mediated by T cells and pathology does not require presence of antibodies [33], nevertheless, antibodies can modify disease severity [34]. We, therefore, examined anti-IRBP antibody titers in p28 Tg mice immunized for uveitis and found that they are considerably reduced (Fig. 1F). Thus, inhibition of antibody production may constitute a part of the protective mechanism in this model.

Decreased susceptibility was also noted to EAE induced by immunization with MOG_{35–55} peptide in p28 Tg mice as well as in mice in which p28 overexpression was induced by a hydrodynamic injection of pmIL27p28 (Fig. 1G and H). This is in agreement to observations previously reported by others [19].

It has been reported that IL-27 limits the number of regulatory T (Treg) cells leading to spontaneous inflammation in mice [35] and inhibits Foxp3 expression [36]. We therefore examined the possibility that, as an IL-27 antagonist, IL-27p28 could confer protection from autoimmunity by promoting Treg induction. However, in our experiments, the EAU-challenged p28-TG mice and mice hydrodynamically injected with pmIL27p28 both had significantly decreased Foxp3⁺ Treg cells compared to their respective controls, despite their lower EAU scores (Fig. S2). We therefore conclude that the most plausible mechanism for the protective effect of IL-27p28 is a direct suppressive effect on generation of IFN- γ and IL-17A effector responses.

3.2. IL-27p28 inhibits IL27-driven Th1 and IL6-driven Th17 polarization

To examine the effects of excess IL-27p28 at the molecular level on T cells undergoing polarization, we stimulated naïve CD4⁺CD62L⁺ T cells from WT donors or from p28-TG donors with anti-CD3/CD28 under Th17 polarizing conditions, or under Th1 conditions driven by either IL-12 or IL-27. IL-27-driven IFN- γ production was significantly reduced in p28-TG cells and in WT cells cultured with IL-27p28, but IL-27p28 had no effect on IL-12-driven IFN- γ production (Fig. 2A). Similarly, expression of *Tbx21* and *Il12rb2* transcripts was reduced in p28-TG cells and in cells treated with exogenous IL-27p28 under IL-27-driven, but not under IL-12-driven Th1 conditions (Fig. 2B). These results are compatible with the interpretation that IL-27p28 specifically blocks IL-27-driven Th1 polarization, but not IL-12-driven Th1 polarization, because IL-12 signaling does not require gp130.

To further confirm that IL-27p28 was suppressing IL-27 but not IL-12 signaling, we examined their respective downstream 2nd messengers in T cells stimulated in the presence

of IL-12 or IL-27 with or without IL-27p28. IL-12 signals through STAT4 to drive Th1 polarization, whereas IL-27 induces Th1 polarization through STAT1 [12,37]. Addition of IL-27p28 did not affect IL-12 signaling, as assessed by STAT4 phosphorylation (Fig. 2C). In contrast, IL-27-dependent STAT1 as well as STAT3 phosphorylation were both markedly suppressed in the presence of IL-27p28 (Fig. 2D). Under Th17 polarizing conditions, IL-27p28 inhibited production of IL-17A from TCR-stimulated CD4⁺CD62L⁺ T cells (Fig. 3A), correlating with the reduced expression levels of *Il23r* and *Rorc* (Fig. 3B). IL-27p28 also markedly inhibited IL-6-induced STAT3 phosphorylation, which drives ROR γ t expression [11] (Fig. 3C), as has been reported by others [19]. IL-27p28 did not affect IL-4 induced Th2 polarization (data not shown).

These data demonstrate that IL-27p28 is able to inhibit IL-27 signaling and IL-27-mediated Th1 polarization as well as IL-6 signaling and IL-6 mediated Th17 polarization, and provide a mechanistic basis for the protective role of IL-27p28 in EAU through concurrent inhibition of adaptive IFN- γ and IL-17A responses (Fig. 1).

3.3. IL-27p28 inhibits effector function of already primed Th1 and Th17 cells in vivo and confers protection from EAU

EAU induced by active immunization is a complicated model that involves both Th1 and Th17 immune responses and comprises both the induction and the effector phases of the disease [38]. To dissect the effect of IL-27p28 on individual effector lineages, Th1 or Th17, EAU was induced by adoptive transfer of *in vitro* polarized retina-specific Th1 or Th17 effector cells. Retina-specific T cells obtained from mice which express a transgenic T cell receptor specific for IRBP161-180, the major pathogenic epitope of IRBP recognized by B10.RIII mice (R161H mice) [60] were polarized with their specific peptide Ag under Th1 or Th17 conditions for 3 days (Fig. S3) and were then transferred to naïve B10.RIII recipients congenic for CD90.1 (so that CD90.2 donor cells could be retrieved for analysis). As treatment, recipients simultaneously received a hydrodynamic injection of pmIL27p28, or were treated with twice-daily injections of recombinant IL27p28. Disease development in recipient mice was followed by fundus examination and eyes were harvested at the end of the experiment for histology analysis.

Although disease incidence and time of onset were not obviously affected (data not shown), both regimens of IL-27p28 delivery significantly reduced retinal inflammation in Th1-driven as well as in Th17-driven EAU (Fig. 4A, B for Th1 and 5A, B for Th17), demonstrating that IL-27p28 could affect the effector function of already polarized uveitogenic Th1 and Th17 cells *in vivo*. To examine the effector status of these cells at the molecular level, donor cells from the inflamed eyes and the draining lymph nodes of hydrodynamically treated mice were analyzed on the day of disease onset (day 4–5) to determine the expression of signature cytokines and transcription factors for each lineage, i.e. IFN- γ and Tbet for Th1; and IL-17A and ROR γ t for Th17. The data showed that IL-27p28 treatment significantly suppressed expression of IFN- γ and Tbet in Th1 donor cells and of IL-17A and ROR γ t in Th17 donor cells (Fig. 4C, D and Fig. 5C, D, respectively). To further investigate the effects of IL-27p28 *in vivo*, adoptively transferred Th1 or Th17 donor cells were retrieved from the recipients by FACS and their gene

expression profile was studied by Th1/Th17 Taqman custom gene expression array. Th1 or Th17 signature genes were downregulated by pmIL27p28 in the respective Th1 or Th17 donor cells that had been retrieved from the recipients (Figs. 4 and 5E and Fig. S4). Interestingly, there was no significant difference in the gene expression level of IL-10, a suppressive cytokine that can be regulated by IL-27 and IL-6, in Th1 or Th17 cells after IL-27p28 treatment. Activation of IL-4, the signature Th2 cytokine gene, was not detected.

3.4. Th1 or Th17 polarized effector cells induce less severe EAU in the absence of IL-27 or IL-6 signaling

As described above, IL-27p28 suppressed the ability of both Th1 and Th17 uveitogenic effector cells to induce EAU (Figs. 4 and 5). It is known that IL-27 and IL-6 are important in initiating Th1 and Th17 immune response by promoting naïve T cells to express IL12R β 2 and IL23R respectively [12,37,39]. As a result, these cells become more responsive to IL-12 or IL-23 during Th1 or Th17 polarization and acquire their effector functions [40–42]. Based on our observations, IL-27 and IL-6 may also be required for the polarized Th1 and Th17 cells to maintain their effector functions for disease induction. To address this, we used siRNA to knock down IL27R α and IL6R α expression in Th1 and Th17 polarized R161H cells, respectively, and assessed their ability to induce EAU in naïve B10.RIII recipients.

IL27R α in Th1 and IL6R α in Th17 polarized R161H cells were knocked down by corresponding siRNAs (Fig. 6A and B), as described previously [23]. To eliminate the possibility that Th1 cells with IL27R α knockdown (Th1^{IL27ra-}) and Th17 cells with IL6R α knockdown (Th17^{IL6ra-}) had failed to polarize, we compared their expression levels of IFN- γ /Tbet or of IL-17A/ROR γ t, with those of Th1 or Th17 polarized cells that were transfected with control siRNA and confirmed that no significant difference was detectable (Fig. 6A and B). Polarized cells, sufficient or deficient in expression of IL27ra or IL6ra, were then transferred to naïve B10.RIII recipients and disease severity was monitored by histology. As shown in Fig. 6C and D, Th1^{IL27ra-} cells and Th17^{IL6ra-} cells induced significantly less disease compared to cells transfected with non-targeting siRNA control. These data suggests that IL-27 and IL-6 signaling is important for the Th1 and Th17 T cells, respectively, to maintain their pathogenicity *in vivo*, and hence their ability to induce EAU.

3.5. IL-27p28 inhibits IL27-Th1 and IL6-Th17 polarization by suppressing IL-27 and IL-6 signaling in human T cells

To extend the suppressive effects of IL-27p28 observed in mouse to human T cells, we isolated naïve CD4RA⁺CD4⁺ T cells from blood of healthy donors and polarized them to Th1 with IL-12 or IL-27, or to Th17 with IL-6, with or without IL-27p28 *in vitro*. Consistent with our findings in mice, IL-27p28 suppressed IL27-Th1, but not IL12-Th1, and IL6-Th17 polarization, with reduced production of IFN- γ and IL-17A, respectively (Fig. 7A and B). Similarly to the effect on mouse T cells, IL-27p28 inhibited expression of both Th1 and Th17 related genes during polarization to the respective lineages (Fig. 7C, Fig. S5) and inhibited the phosphorylation of STAT1 and STAT3 after IL-27 and IL-6 stimulation, respectively (Fig. 7D). These data suggest that, similarly to the mouse system, IL-27p28 also suppresses IL-27 and IL-6 signaling in human T cells by antagonizing gp130.

It should be noted that in these studies we used mouse IL-27p28, as the human cytokine is not available to us. Stumhofer et al. constructed a three-dimensional structure to study the possible interaction between human IL-27p28 with human gp130 and suggested that Leu81 and Glu85 from human IL-27p28 interact with Leu3 of gp130 (16). By constructing a similar three-dimensional structure with mouse IL-27p28 and human gp130, it appears that, instead of Leu81 and Glu85, mouse IL-27p28 uses amino Val189, Val 193 and Phe90 to form hydrophobic interaction with amino acid Leu3 of gp130 (Fig. S6). Nevertheless, the biological consequences of receptor engagement for the functions examined appear to be similar.

4. Discussion

Data from our laboratory as well as from others support the notion that autoimmunity in the CNS and possibly also other tissues involves both Th1 and Th17 effector responses, with each lineage separately able to drive specific pathology. The inherent plasticity of effector responses therefore raises a concern that targeting either Th1 or Th17 cells may shift the balance of effector response towards the alternative pathway without reducing pathology, and could possibly explain the disappointing results of anti-IL-17 therapy in some clinical immunotherapy trials [17,18].

This idea has been well supported in experimental models, such as EAU and EAE. Although stably polarized IFN- γ -producing Th1 lines are highly pathogenic [43], and IFN- γ producing pathogenic Th17 cells at the site of tissue damage are involved in the pathogenic process in mice and in humans [44,45], blocking of IFN- γ is known to exacerbates EAE [46,47] and EAU [6,48]. This may be due at least in part to an elevated pathogenic Th17 response [6]. Conversely, some studies report that blocking of IL-17A and/or IL-17F has little impact on EAE development [49,50] and neutralization of IL-17A only partially suppresses EAU development [6]. Blocking of upstream cytokines involved in the Th17 response, such as IL-6 and IL-23, may be a more promising approach than blocking IL-17 itself [51,52] but it still does not suppress Th1 response.

In contrast to therapeutic approaches that suppress a single lineage, IL-27p28 suppressed both, by concurrently affecting the expression of their master switches, Tbet and ROR γ t, through inhibition of IL-27 as well as IL-6 signaling. The inhibitory effects of IL-27p28 on the Th17 response may be attributed in part to suppression of phosphorylation of STAT3, which is used by a number of Th17-promoting cytokines. IL-6, IL-1 β and IL-23, which all are important for Th17 differentiation and function signal through STAT3 to enhance the expression of Th17 related genes, including *Il17*, *Il23r* and *Rorc* [42,53]. By inhibiting IL-6 mediated STAT3 phosphorylation, IL-27p28 suppressed Th17 differentiation *in vitro*, in line with data previously reported by others [19]. In parallel, inhibition of IL-27 mediated STAT1 phosphorylation contributed to suppression of Tbet, and thereby the Th1 effector response. IL-27p28 also suppressed GM-CSF, a pathogenic cytokine that is expressed by both effector lineages, which was recently shown to be involved in pathogenesis of EAE [27,28]. Importantly, similar effects were observed on human Th1 and Th17 cells, suggesting that such a double-targeting approach might be applicable to human diseases where these cytokines may play redundant roles in pathogenesis.

It has been suggested that heterodimeric IL-27 can be used clinically in Th17 driven diseases, because it inhibits Th17 responses by suppressing expression of ROR γ t, IL-17A and GM-CSF [27,54] and by promoting IL-10 production [55,56]. However, IL-27 can be a double-edged sword, because on the other hand it promotes Th1 responses by inducing expression of IFN- γ , Tbet and IL12R β 2 from naïve T cells [12,13,37]. IL-27R α -deficient mice showed a reduced susceptibility to EAU, with less pathogenic IFN- γ and Th1-related chemokines production [16]. In the present study we observed that knockdown of IL-27R α in primed retinal-specific Th1 cells reduced their ability to induce EAU (Fig. 6), indicating that IL-27 signaling promotes CNS autoimmunity. Additionally, IL-27 may promote inflammation by suppressing Treg cells [35,36]. Thus, Loss of IL-27 signaling ameliorated the T cell transfer model of colitis by promoting Foxp3 expression and Treg cells induction [36] whereas overexpression of IL-27 led to spontaneous inflammation due to limiting the Treg population [35]. Therefore, although IL-27 may inhibit Th17 response, its ability to promote the Th1 response and to suppress FoxP3⁺ Treg cells should be considered before using IL-27 to treat autoimmune disease. We suggest that IL-27p28 may constitute a safer treatment option than IL-27, as in addition to inhibiting Th17, it also suppresses Th1-mediated autoimmunity by antagonizing IL-27 signaling. All forms of p28 delivery (transgenic, hydrodynamic or as recombinant protein) were protective, speaking for the robustness of the effect and its clinical relevance. Importantly, IL-27p28 compromised not only induction but also the maintenance of autopathogenic effector T cells *in vivo*, so that not only the initiation phase, but also the effector phase of disease was ameliorated. This, together with the *in vitro* inhibitory effects on human T cell polarization, could bode well for a potential clinical use of this paradigm.

In line with our current report, Wang et al. [57] recently reported a suppressive role of IL-27p28 in the “classical” model of EAU, induced by active immunization with IRBP in CFA. However, notable differences emerge between the two studies. First, Wang et al. [57] did not address the concept of concurrent inhibition, because classical EAU is driven by a dominant Th17 response, and deficiency or inhibition of IFN- γ only exacerbates disease pathology [6]. This confirmed what was already known, that IL-27p28 inhibits the Th17 response [27,54]. By contrast, in the adoptive transfer model, we demonstrate direct suppression of the pathogenic Th1 response, inclusive of IFN- γ , Tbet, and other Th1 related genes *in vivo* (Fig. 4). Furthermore, IL-27p28 specifically inhibited only the IL-27 induced-, but not the IL-12-induced, Th1 response (Fig. 2), demonstrating that its regulatory role is gp130-dependent. Second, regulatory mechanisms in our studies appear to differ. In our hands IL-27p28 did not promote Foxp3⁺ Treg cells in p28-TG mice immunized for EAU, nor did it induce IL-10 expression in Th1 and Th17-polarized IRBP-specific T cells. In contrast, Wang et al. [57] reported that recombinant IL-27p28 treatment increased Foxp3⁺ Treg cells and induced production of IL-10 in a small proportion of CD4 T cells, however, functional significance of the changes they observed was not examined. Because they generated the recombinant IL-27p28 protein in the insect cell system and used a crude supernatant for treatment, there may have been different post-translational modifications which can alter protein function, as well as insect cell products that affect the immune response. This possibility is underscored by a recent study reporting that different expression systems impact IL-27p28 functions [58].

Although we specifically concentrated on changes in Th1/Th17 polarization and lineage stability, there could be additional mechanisms by which IL-27p28 affects pathogenesis. One is inhibition of serum antibody production by gp130 signaling [19, 29–32 and Fig. 1F]. An effect that could also play a role, but whose evaluation is beyond the scope of the present study, is direct effects on cells composing the CNS tissue. Gp130 is part of the IL-6 receptor, which is expressed by neurons and microglia [59]. It is therefore conceivable IL-27p28 could have neuroprotective effects on CNS resident cells through inhibition of IL-6 signaling.

5. Conclusion

In conclusion, we demonstrated that IL-27p28, a natural antagonist of gp130, modulated the autoimmunity in CNS by concurrently suppressing the commitment and the subsequent lineage stability of autoaggressive Th1 and Th17 cells. This effect is achieved at least in part by blocking the IL27-mediated STAT1 and IL6-mediated STAT3 phosphorylation, which leads to the inhibition of Tbet and ROR γ t expression in Th1 and Th17 cells. Finally, IL-27p28 suppressed both IL-27 mediated Th1 and IL-6 mediated Th17 polarization in human T cells. Overall, these results point to a therapeutic potential of IL-27p28 augmentation to control immunologically complex autoimmune diseases by targeting the IL-6 family cytokines that signal through gp130.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Overexpression of IL-27p28 suppresses EAU and EAE and reduces Th1 and Th17 immune responses. (A–B) p28-TG mice and WT littermates were immunized for EAU. (A) Average disease score and representative histopathology (day 21, hematoxylin-eosin, original magnification $\times 200$). (B) Expression of IFN- γ , IL-17A and GM-CSF in CD4⁺ T cells from the eyes by intracellular staining on day 21. (C–D) B10.RIII mice were immunized for EAU and treated hydrodynamically with 20 μ g of pmIL27p28 (d 0 and 7). (C) Average disease scores and representative histopathology (day 21, h&e, $\times 200$). (D) Expression of IFN- γ , IL-17A and GM-CSF by CD4⁺ cells in uveitic eyes by intracellular staining (d 21). (E) C57BL/6 mice were immunized for EAU and treated with IL-27p28 (5 μ g every other day) or PBS, starting from day 0 (9–10 mice per group), data shown is the average disease scores on day 21. (F) Anti-IRBP antibody titers in p28-TG mice and their WT littermates immunized for EAU. (G) EAE scores in IL-27p28 Tg mice and WT littermates immunized with MOG_{35–55}. (H) EAE in WT C57BL/6 mice immunized with MOG_{35–55} and hydrodynamically injected with pmIL27p28 (d 0 and 7). (A, C, G, H) Data shown as mean $\pm$ SEM from 2 independent experiments with total of at least 10 mice per group. *: $p < 0.05$.

Fig. 2.

IL-27p28 suppresses IL-27, but not IL-12, driven Th1 responses. (A) CD4⁺CD62L⁺ cells from p28-TG mice or from WT littermates (with or without added IL-27p28) were stimulated with anti-CD3/CD28 in the presence of IL-12 or IL-27 for 3 days. (B) The same cells were analyzed by real-time PCR for expression of *Il12rb2* and *Tbx21* (T-bet); (C) CD4⁺CD62L⁺ cells were polarized into Th1 cells by IL-12 and anti-CD3/CD28 for 2 days, rested and reincubated with IL-12 with or without IL-27p28 for 2 h. STAT4 phosphorylation was determined after 30 min and compared to unstimulated Th1 cells (Unstim, filled gray histogram). (D) CD4⁺CD62L⁺ cells were incubated in presence or absence of IL-27p28 for 2 h, then IL-27 was added for 30 min. STAT1 and STAT3 phosphorylation was determined and compared to unstimulated CD4⁺CD62L⁺ cells (Unstim, filled gray histogram). Data are representative of at least 2 independent experiments.

Fig. 3.

IL-27-p28 suppresses Th17 polarization by antagonizing IL-6 signaling. (A) CD4⁺CD62L⁺ cells from p28-TG mice and their WT littermates were stimulated with anti-CD3/CD28 under Th17 polarizing conditions for 3 days. IL-27p28 suppressed the ability of Th17 cells to produce IL-17A, as determined by intracellular staining. (B) IL-27p28 also suppressed *Il23r* and *Rorc* expression in Th17 cells, as determined by real time PCR. (C) Freshly isolated CD4⁺CD62L⁺ cells were preincubated with or without IL-27p28 for 2 h, followed by stimulation with IL-6 for 30 min. STAT3 phosphorylation was determined by flow cytometry and was compared to unstimulated CD4⁺CD62L⁺ cells (Unstim, filled gray histogram). Data are representative of at least 2 independent experiments.

Fig. 4.

IL27p28 suppresses Th1-mediated EAU and inhibits IFN- γ and Tbet expression in Th1 polarized R161H CD4⁺ T cells. Lymph node cells were obtained from CD90.1 R161H mice and were polarized into Th1 cells for 3 days. 4×10^6 polarized cells were transferred into WT B10.RIII mice. Disease severity was significantly reduced by the injection of (A) pmIL27p28 (at least 10 mice per group) and (B) recombinant IL-27p28 (6 mice per group), when compared to mice treated with vector control and PBS, respectively, as determined by histology. (C–D) Expression of IFN- γ and Tbet in CD90.1⁺CD4⁺ cells from eyes and draining lymph nodes was determined by flow cytometry. The expression of both IFN- γ and Tbet was significantly suppressed by IL-27p28 ($n = 6$). (E) Gene expression analysis of sorted Th1 polarized CD90.1⁺CD4⁺ cells from recipient. *: $p < 0.05$.

Fig. 5.

IL27p28 suppresses Th17-mediated EAU as well as IL-17 and ROR γ t expression in Th17-polarized R161H cells. Th17-polarized lymph node cells from CD90.1-congenic R161H mice were infused (4×10^6 cells) into WT B10.RIII recipients. Disease severity was significantly reduced by the injection of (A) pmIL27p28 (at least 10 mice per group) and (B) recombinant IL-27p28 (6 mice per group), when compared to the mice treated with vector control and PBS, respectively, as determined by histology. (C–D) Expression of IL-17A and ROR γ t in CD90.1⁺CD4⁺ cells from eyes and draining lymph nodes as determined by flow cytometry. The expression of both IL-17A and ROR γ t was significantly suppressed by IL27p28 ($n = 6$). (E) Gene expression analysis of sorted Th17 polarized CD90.1⁺CD4⁺ cells from recipient. *: $p < 0.05$.

Fig. 6.

IL-27 and IL-6 signaling are important for Th1 and Th17 polarized cells, respectively, to mediate EAU. Lymph node cells from R161H mice were polarized into Th1 or Th17 phenotype. siRNAs targeting *IL27Rα* and *IL6Rα* were used to block the corresponding genes in Th1 and Th17 cells, respectively. (A) Expression of *Il27ra*, *Ifng* and *Tbx21*; (B) Expression of *Il6ra*, *Il17* and *Rorc* by real time PCR in Th1 and Th17 polarized cells, respectively (representative of 3 independent experiments). (C,D) siRNA treated, Th1 (Th1^{*Il27ra*-/-}) or Th17 cells (Th17^{*Il6ra*-/-}), or cells treated with siRNA control, were transferred to WT B10.RIII recipients and disease severity was determined by histology on day 15. Data are combined from two independent experiments with total of at least 10 mice per group. *: $p < 0.05$.

Fig. 7.

IL-27p28 suppresses IL-27 and IL-6 induced Th1 and Th17 differentiation in human CD4 $^{+}$ T cells. (AeB) CD45RA $^{+}$ CD4 $^{+}$ naïve human T cells were isolated and polarized into Th1, with IL-12 or IL-27, and Th17, with IL-6, conditions with or without IL-27p28. Cells were harvested at day 14 for analysis. IL-27p28 significantly inhibited IL-27 induced Th1 and IL-6 induced Th17 polarization with lower production of IFN- γ and IL-17A, respectively. Data obtained from 4 independent individuals. *: p < 0.05. (C) Gene expression analysis of human IL27-induced Th1 and IL6-induced Th17 CD4 $^{+}$ T cells with or without IL-27p28 during polarization. (D) CD45RA $^{+}$ CD4 $^{+}$ naïve human T cells were preincubated with IL-27p28 for 2 h. Then, they were stimulated with IL-27 or IL-6 for 30 min. STAT1 and STAT3 phosphorylation was studied by intracellular staining. Representative of 3 independent individuals.