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Suppression of Ongoing Experimental Autoimmune Encephalomyelitis by Neutralizing the Function of the p28 Subunit of IL-27

Ruth Goldberg,*‡ Yaniv Zohar,* Gizi Wildbaum,* Yifat Geron,* Gila Maor,‡ and Nathan Karin2*†

IL-27 is a recently defined family member of the long-chain, four-helix bundle cytokines, which consist of EBI3, an IL-12p40-related protein, and p28, an IL-12p35-related polypeptide. The role of IL-27 in the regulation of experimental autoimmune encephalomyelitis has never been studied. We show in this study that neutralizing the in vivo function of IL-27 by Abs against IL-27 p28 rapidly suppressed an ongoing long-lasting disease in C57BL/6 mice. These Abs were then used to determine the mechanistic basis of disease suppression. We show in this study that IL-27 is involved not only in the polarization of naïve T cells undergoing Ag-specific T cell activation, but also in promoting the proliferation and IFN-γ production by polarized T cells, including the long term Th1 line that has been previously selected against the target encephalitogenic determinant. This may explain in part why neutralizing IL-27 suppresses an already established disease in a very rapid and significant manner. The Journal of Immunology, 2004, 173: 6465–6471.

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xperimental autoimmune encephalomyelitis (EAE)3 is an autoimmune disease of the CNS that serves as a model for the human disease, multiple sclerosis (MS). In both diseases circulating leukocytes penetrate the blood-brain barrier and damage myelin, resulting in impaired nerve conduction and paralysis (1, 2). Ag-specific T cells direct the initiation and progression of disease. Depending on their cytokine profile, CD4+ T cells fall into different subsets, including Th1 cells that produce large amounts of IFN-γ and TNF-α and low levels of IL-4; Th2 cells that mostly produce IL-4, IL-5, and IL-13 and, to a much lesser extent, IFN-γ and TNF-α (3, 4); Th3 cells that produce high levels of TGF-β and, to a much lesser extent, other cytokines (5, 6); Tr1 cells that produce high levels of IL-10 (7); and CD4+CD25+ suppressor T cells (8). The pivotal role of Th1 cells in the initiation and progression of the inflammatory process in several autoimmune diseases, including EAE, has been well documented. Thus, neutralization of IL-12, IL-18, or IFN-γ-inducible protein-10 (CXCL10) suppresses experimentally induced EAE (9–11) and other T cell-mediated autoimmune diseases (12–15), while shifting Ag-specific T cell polarization from Th1 to Th2.

IL-27 is a newly defined family member of the long-chain, four-helix bundle cytokine (16). It is a heterodimeric cytokine that consists of EBI3, an IL-12p40-related protein, and p28, an IL-12p35-related polypeptide (16). This cytokine is an early product of activated APCs and drives rapid clonal expansion of naïve CD4+ T cells. IL-27 synergizes with IL-12 to trigger IFN-γ production of naïve CD4+ T cells and mediates its biologic effects through the orphan cytokine receptor WSX-1/TCCR (16–20). WSX-1 signaling induces the induction of T-bet through activation of STAT1 during initial Th1 commitment (21–23). It is therefore possible, although has yet to be proven, that neutralization of IL-27 may affect the regulation of Th1-mediated inflammatory diseases. Very recently, its expression was identified in tissue samples from patients suffering from granulomatous diseases (24). In a very recent study we have demonstrated the ability of neutralizing Abs to IL-27 to suppress adjuvant-induced arthritis (25). However, the role of IL-27 in the regulation of other inflammatory autoimmune diseases, particularly EAE has never been elucidated.

Our laboratory was the first to use the DNA vaccination technology and neutralizing Abs generated by this technology for exploring the roles of different cytokines, chemokines, and Fas ligand in the regulation of EAE and other T cell-mediated autoimmune diseases (10, 14, 26–33). In this study we use, once again, this powerful technology to obtain highly specific neutralizing Abs to the p28 subunit of IL-27 and to explore the role of this newly defined cytokine in the regulation of EAE.

Materials and Methods

Animals

Lewis rats and C57BL/6 mice, −6 wk old, were purchased from Harlan (Jerusalem, Israel) and maintained under specific pathogen-free conditions in our animal facility.

Peptide Ags

Myelin oligodendrocyte glycoprotein (MOG) p33–55 was ordered from the PAN facility of the Beckman Center of Stanford University. After purification by HPLC, sequence was confirmed by amino acid analysis, and the correct mass was checked by mass spectroscopy. Purification of the peptide that was used in the current study was >95%.

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Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis.
Immunizations and active disease induction

EAE was induced by immunizing mice with MOGp33–55/CFA as described by Mendel et al. (34). Animals were then monitored for clinical signs daily by an observer blind to the treatment protocol. EAE was scored as follows: 0, clinically normal; 1, flaccid tail; 2, hind limb paralysis; 3, total hind limb paralysis, accompanied by an apparent front limb paralysis; and 4, total hind limb and front limb paralysis.

Induction of transferred EAE

Transferred EAE was induced by transferring 5–10 × 10^6 spleen T cells after in vitro priming with MOGp35–55, according to the protocol previously described (35).

Cloning of mouse IL-27 p28, IL-1β, and IL-18

Specific oligonucleotide primers were designed based on its published sequence of each of the above cytokines: mouse IL-27 p28: sense, 5′-ATG G0CAGGTAAGGAGACCTTGGC-3′; antisense, 5′-TTAGGAATTCCACAGCTGAGCTGCGGC-3′; mouse IL-18: sense, 5′-ATG GCTGCACTTGAAGAAGCTTGGCT-3′; antisense, 5′-CTAAGTTGAGTTAGTAAATG-3′; and IL-1β: sense, 5′-ATGGCAATCTGGTAATGACAGGTCAGAGG-3′; antisense, 5′-TTAGGAAGACACTCCTTCTCTGTTGAA-3′.

PCR products were cloned into a pUC57/T vector (T-cloning kit K1212; MBF Fermentas, Vilnius, Lithuania) and transformed into Escherichia coli according to the manufacturer’s protocol. Each clone was then sequenced (Sequenase version 2; Upstate Biotechnology, Cleveland, OH) according to the manufacturer’s protocol. Each clone was then sequenced by our sequencing services unit. The sequenced PCR product of mouse IL-27 p28, IL-18, and IL-1β was verified by gel electrophoresis, followed by sequencing (N terminus) by our sequencing services unit.

Western blot analysis

Our recombinant mouse IL-27 p28, produced as described above, and commercially available recombinant mouse IL-18, IL-12, and TNF-α (R&D Systems) were each subjected to Western blot analysis according to the protocol described in detail previously (29, 32), with the minor modification of using a 1% (rather than 8%) running gel. IgG from IL-27 p28 DNA-vaccinated rats or IgG from normal rat serum (final dilution of 1/500 each) were used as primary Abs. Goat anti-rat biotin-conjugated Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary Ab, followed by streptavidin-HRP (Jackson ImmunoResearch Laboratories). The Western blotting Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA) was then used as a substrate.

Selection of MOG-specific T cell lines

The MOG-specific T cell line was selected according to the protocol described previously (37) with the minor modification of using Con A supernatant as a source of IL-2.

Spot ELISA

Spot ELISAs were conducted according to the detailed protocol described by Min et al. (38). In brief, hemagglutinin multiscreen plates (Millipore, Bedford, MA) were coated with anti-mouse IFN-γ capture Ab (catalogue no. MAB785; R&D Systems) at a concentration of 5 μg/ml in PBS overnight at 4°C. Free sites were saturated with 5% BSA in PBS. Subsequently, 10^5 spleen cells or line T cells were added. After 24 h of incubation, plates were supplemented with a biotinylated anti-IFN-γ mAb (catalogue no. BAF485; R&D Systems), followed by streptavidin conjugated to avidin-peroxidase (Jackson ImmunoResearch Laboratories). Spots were then counted and measured under a dissecting microscope.

Histopathology

Histological examination of H&E-stained sections of formalin-fixed, paraffin-embedded sections of the lower thoracic and lumbar regions of the spinal cord was performed. Each section was examined without knowledge of the treatment status of the animal. The following scale was used: 0, no
mononuclear cell infiltration; 1, one to five perivascular lesions per section with minimal parenchymal infiltration; 2, five to 10 perivascular lesions per section with parenchymal infiltration; and 3, >10 perivascular lesions per section with extensive parenchymal infiltration. The mean histological score ± SE was calculated for each treatment group.

Statistical analysis
Significance of differences was examined using Student’s t test. A value of \( p < 0.05 \) was considered significant. The Mann-Whitney sum of ranks test was used to evaluate significance of differences in mean maximal clinical scores. A value of \( p < 0.05 \) was considered significant.

Results
Anti-IL-27 p28 Abs suppress ongoing EAE
A DNA vaccination-based anti-IL-27 p28 polyclonal Ab was constructed as described in Materials and Methods. First, we verified that this Ab is specific to its target. Fig. 1 shows that our Ab binds recombinant mouse IL-27 p28 (lane 1; 27 kDa) and not recombinant mouse IL-18, IL-12, or TNF-\( \alpha \) (lanes 2, 3, and 4, respectively). We also verified (by ELISA) that this Ab does not bind different type I cytokines, including IL-2, IL-4, and IL-15 (data not shown), nor did it bind recombinant soluble \( \beta \)-actin that we constructed under conditions identical with those used to generate rIL-27 (10), but it did bind a 27-kDa band in supernatant of activated primary cultured lymph node cells from EAE rats that were cultured in the presence of MOGp33–55 (not shown).

We then determined the competence of this Ab to affect the dynamics of an ongoing disease. Starting on day 17, at the onset of disease, mice were subjected to subsequent administration of anti-IL-27 Abs, IgG from preimmunized rats, or PBS. Although both control groups (IgG and PBS) continued to develop severe EAE (Fig. 2A; mean maximal score of six mice ± SE, 3.3 ± 0.6 and 3.1 ± 0.3, respectively), in those treated with purified anti-p28 Ab, the development and progression of disease were markedly attenuated (mean maximal score, 0.7 ± 0.23; \( p < 0.001 \) compared with each control group). Histological analysis (Fig. 3 and Table I) of the lumbar spinal cord of these mice (day 30) clearly showed that disease suppression was accompanied by a significant reduction in parenchymal mononuclear cell infiltration (Table I; mean histological score, 0.8 ± 0.3 compared with 2.8 ± 0.3 and 2.8 ± 0.3 in control groups). In a subsequent experiment (Fig. 2B) we compared the abilities of anti-IL-27 Abs to suppress ongoing EAE to
DNA vaccination-based Abs against IL-1β and IL-18 produced by the same protocol. Each of these Abs could significantly suppress adjuvant-induced arthritis (not shown). Our results (Fig. 2B) clearly show that neutralizing IL-18 or IL-27 rapidly suppresses EAE in a similar manner. In contrast, neutralizing IL-18 displays a much-moderated effect on the severity of disease that significantly differs (p < 0.001) from that obtained by neutralizing IL-18 or IL-27 (Fig. 2B). To determine whether the effects of these Abs are direct or indirect effect (i.e., suppression of the responses induced by the CFA with which active disease is induced), we determined the abilities of these Abs to suppress ongoing transferred EAE. Fig. 2C shows that similar to active disease, these Abs rapidly and most effectively suppress transferred EAE. Another permanent question referred to the abilities of these Abs to induce long term resistance against EAE. We therefore conducted an experiment in which C57BL/6 mice suffering from active EAE were treated with either a single dose or repeated administrations of anti-IL-27 neutralizing Abs. Fig. 4 shows that the beneficial effect of anti-IL-27 therapy in mice subjected to a single Ab administration was temporary and lasted for ~7–10 days.

**Therapy with anti-IL-27 Abs decreases in vivo polarization of CD4+ T cells into Th1 and suppresses IFN-γ production by Ag-specific T cells**

To determine the possibility that the adoptive transfer of anti-IL-27 p28 Abs suppresses EAE by altering the in vivo polarization of CD4+ T cells. C57BL/6 mice were subjected to active induction of EAE and then to repeated administration (days 12, 14, and 16) of anti-IL-27 p28 Abs, PBS, or normal rat IgG. On day 17, cervical lymph node cells (that drain the autoimmune site) were obtained from anti-IL-27 p28 Abs, PBS, or normal rat IgG. On day 17, cervical lymph node cells were subjected to FACS analysis (Fig. 5A). After 72 h of stimulation, supernatants were assayed for the protein levels of IFN-γ (Fig. 5B) and IL-4 (not shown). Our results clearly show that repeated administration of anti-IL-27 Abs led to a significant reduction in Th1 polarization (Fig. 5A; ~80% of CD4+ Th1 cells in both control groups compared with 47.5% in anti-IL-27 p28-treated mice) and to a significant increase in IL-4lowIFN-γlow-producing nonpolarized CD4+ T cells (~16% in control groups and 41.6% in treated mice). Analysis of cytokine production after stimulation in the presence of 100 μM MOGp35–55 showed a marked reduction in IFN-γ production (Fig. 5B; 710 ± 50 and 740 ± 60 pg/ml in control groups compared with 240 ± 30 pg/ml in anti-IL-27-treated mice). IL-4 levels were <10 pg/ml in all groups, and no significant change in levels of TNF-α was found (not shown). The reduction in IFN-γ production was accompanied by a marked reduction in Ag-specific proliferative response (Fig. 5C; 5200 ± 320 and 6200 ± 580 cpm with backgrounds of 1700 and 1760 cpm in control groups compared with 2600 ± 370 cpm, with background of 1700 cpm in anti-IL-27-treated mice; p < 0.0001).

It should be emphasized that intracellular staining of IFN-γ was conducted on freshly isolated cervical lymph node T cells, whereas IFN-γ levels were determined by ELISA after 3 days of activation with the target MOG Ag. It is plausible that after 3 days of in vitro priming, the relative number of Ag-specific T cells proliferating in response to their target Ag will dramatically increase; if they do produce IFN-γ, its level would be extremely high, and if they do not, the level of this cytokine would be relatively low. Finally, the dramatic effect of anti-IL-27 Abs on in vitro production of IFN-γ suggests that this cytokine promotes IFN-γ production not only during the initial polarization of naive T cells in response to the target Ag (16), but also of already primed T cells to increase their Ag-specific proliferative response and IFN-γ production. The paragraph below explores this possibility.

**IL-27 acts on activated/memory CD4+ T cells to increase their IFN-γ, but not TNF-α, production**

To determine whether IL-27 also acts on long term activated/memory T cells, we studied the effect of IL-27 on the proliferative response and cytokine production of our long term MOGp33–35-specific memory/effector (Th1) CD4+ T cell line. This line was cultured in the presence of rIL-27 p28 or recombinant β-actin, which were constructed under the same conditions. The addition of rIL-27 p28 could very effectively increase IFN-γ production by these cells (1580 ± 120 compared with 610 ± 70 pg/ml; p < 0.001). Our anti-IL-27 neutralizing Abs could successfully reverse
this effect (Table I). Control IgG had no effect on this response (not shown). Thus, IL-27 can act as a potent proinflammatory mediator not only on naive cells undergoing pro-Th1 polarization (16), but also on T cells that have previously been polarized into Th1. The addition of IL-27 also significantly increased their proliferative response (Table II; ~70% increase; \( p < 0.001 \)), but had no effect on TNF-\( \alpha \) production by these cells (Table II). As expected, IL-4 was produced at very low levels by these Th1 cells. The addition of IL-27 did not increase the production of this cytokine by these cells. Taken together, these results show for the first time that the function of IL-27 is not limited to naive T cells.

To further determine whether IL-27 potentiates the production of IFN-\( \gamma \) in T cells, including long-term memory T cells we have conducted 2 sets of complementary experiments in which the function of IL-27 was neutralized in vivo (Fig. 6A) or in long term cultured T cells (Fig. 6B), and the relative number of IFN-\( \gamma \)-producing T cells was determined by spot ELISA. In these experiments the size of each spot, representing the amount of cytokine produced by these cells, was also determined. We show in this study that blocking the in vivo function of IL-27 not only reduces the relative number of IFN-\( \gamma \)-producing cells (Fig. 6A; 25 ± 3 compared with 152 ± 18 and 144 ± 15 spots/10⁷ cells; \( p < 0.001 \)), but also the amount (i.e., size of the spot) of cytokine produced by each cell (see Fig. 6A). Likewise, the addition of neutralizing Abs to IL-27 to long term cultured CD4⁺ T cells responding to their target Ag (MOG) significantly decreased the spot size of IFN-\( \gamma \)-producing T cells (Fig. 6B; ~10-fold lower). This further substantiates the effect of IL-27 on the function of T cells, including those that have already been primed, and can explain in part the why neutralizing IL-27 rapidly and effectively suppresses an ongoing inflammatory autoimmune disease (Figs. 2 and 4).

### Discussion

The current study shows that anti-IL-27-based Ab therapy can be used effectively to suppress an ongoing autoimmune disease (Figs.

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**Table II. IL-27 elicits proliferation and IFN-\( \gamma \) production by MOGp33–55-specific CD4⁺ T cell line**

<table>
<thead>
<tr>
<th></th>
<th>No Ag</th>
<th>+Ag</th>
<th>+β-actin (50 ng/ml)</th>
<th>+IL-27 (50 ng/ml)</th>
<th>+IL-27 + anti-IL-27 Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4 (pg/ml)</td>
<td>15 ± 4</td>
<td>30 ± 6</td>
<td>25 ± 6</td>
<td>33 ± 5</td>
<td>30 ± 6</td>
</tr>
<tr>
<td>IFN-( \gamma ) (pg/ml)</td>
<td>530 ± 45</td>
<td>610 ± 70</td>
<td>650 ± 80</td>
<td>1,580 ± 120</td>
<td>720 ± 55</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>15 ± 3</td>
<td>20 ± 4</td>
<td>18 ± 3</td>
<td>20 ± 5</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>TNF-( \alpha )</td>
<td>350 ± 40</td>
<td>510 ± 70</td>
<td>480 ± 60</td>
<td>520 ± 80</td>
<td>550 ± 30</td>
</tr>
<tr>
<td>Proliferation (cpm)</td>
<td>1,320 ± 180</td>
<td>23,200 ± 1,930</td>
<td>22,700 ± 2,210</td>
<td>39,100 ± 2,400</td>
<td>25,100 ± 1,700</td>
</tr>
</tbody>
</table>

*IL-27 p28 elicits the proliferative response and IFN-\( \gamma \) production by long-term Ag-specific CD4⁺ T cells. MOGp33–55-specific CD4⁺ T cell line was cultured with or without the addition of 100 µM of MOGp35–55. Cultured cells were supplemented with 50 ng/ml of our IL-27 or with β-actin, which has been generated and purified under the same conditions. Some of the wells were also supplemented with 10 ng/ml of our purified IL-27 p28-specific Abs or control IgG from naive rats (not shown). After 72 h of stimulation levels of IFN-\( \gamma \), IL-4, and IL-10 were recorded. Results are shown as mean triplicates ± SE. At the same time proliferative response to MOGp33–55 was also recorded. Results are shown as mean cpm (triplicates) ± SE.
neutral CD4/H9262 Abs at a final concentration of 10
inflammatory effect that leads to a rapid suppression of an inflam-

show that neutralizing the p28 subunit of IL-27 exerts an anti-

subjected to active induction of EAE and then to repeated administration
of Ag-specific inflammatory T cells. The current study showing
that anti-IL-27 therapy does not significantly select Th2 cells, but,
reduces the relative number of Ag-specific inflammatory T cells further suggests that suppression of Th1 function would be
sufficient for suppressing EAE. We clearly show (Fig. 5) that neu-
tralizing IL-27 leads to a significant reduction not only in the num-
ber of IFN-γ-producing cells, but also in the amount of the cyto-
kine produced by these cells. It has yet to be explored, however,
whether the proinflammatory function of IL-27 is due to the direct
amplification of IFN-γ production by autoimmune T cells or is
associated with other effects, as yet to be determined, of this cy-
tokine on the function of these cells. Hence, from a clinically ori-
ented perspective, such a therapy could be favorable in shifting the
balance toward Th2, which might, over the long term, lead to the
development of allergic responses (45).

Neutralizing the function of IL-27 reduces IFN-γ produc-
tion by Ag-γ-producing T cells. A. C57BL/6 mice (three per group) were
subjected to active induction of EAE and then to repeated administration
(days 3 and 6) of 100 μg of anti-IL-27 p28 Abs (group 3), PBS (group 2),
or normal rat IgG (group 1). On day 9, spleen cells were subjected to spot
ELISA as previously described (38). A. Relative number of positive spots
per 10⁷ cultured cells. The average size of positive spots was analyzed.
B. The MOGp33–55-specific CD4⁺ T cell line was cultured with or without
100 μM MOGp33–55. Cultured cells were supplemented with anti-IL-27
Abs at a final concentration of 10 μg/ml (C), normal rat IgG (B), or PBS
(C). After 60 h of incubation, cells were plates in spot ELISA plates for an
additional 24 h for the detection of IFN-γ-positive spots (38). Number of
positive spots (y-axis) and spot sizes (x-axis; logarithmic scale) determined
as previously described (46).

IL-27 is a newly defined family member of the long chain, four-
helix bundle cytokines (16). This cytokine is an early product of
activated APCs and drives rapid clonal expansion of naive CD4⁺
T cells. IL-27 synergizes with IL-12 to trigger IFN-γ production of
naive CD4⁺ T cells and mediates its biologic effects through the
orphan cytokine receptor WSX-1/TCR (16–20). This may sug-
gest IL-27 as a potential relevant target for therapy of inflamma-
atory autoimmune disease, particularly those in which Th1 cells are
the key mediators of the inflammatory autoimmune process. In con-
trast, the finding that IL-27 acts primarily on naive, and not activated, T cells (16, 21, 22) may question the efficiency of anti-
IL-27 therapy after the onset of disease. Additionally, a very recent
study conducted in mice lacking the IL-27 p28 receptor (WSX-
1−/− mice) shows that the absence of this particular receptor does
not reduce the competence of these mice to provoke an inflamma-
tory response against Toxoplasma gondii (39). Our results clearly
show that neutralizing the p28 subunit of IL-27 exerts an anti-

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