Two-Sided Roles of IL-27: Induction of Th1 Differentiation on Naive CD4+ T Cells versus Suppression of Proinflammatory Cytokine Production Including IL-23-Induced IL-17 on Activated CD4+ T Cells Partially Through STAT3-Dependent Mechanism

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*J Immunol* 2006; 177:5377-5385; doi: 10.4049/jimmunol.177.8.5377

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Two-Sided Roles of IL-27: Induction of Th1 Differentiation on Naive CD4\(^+\) T Cells versus Suppression of Proinflammatory Cytokine Production Including IL-23-Induced IL-17 on Activated CD4\(^+\) T Cells Partially Through STAT3-Dependent Mechanism\(^1\)

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Recent lines of evidence have demonstrated that IL-27, a newly identified IL-12-related cytokine, has two apparently conflicting roles in immune responses: one as an initiator of Th1 responses and the other as an attenuator of inflammatory cytokine production. Although the IL-27-mediated Th1 initiation mechanism has been elucidated, little is known about the molecular basis for the suppression of cytokine production. In the present study, we demonstrated that IL-27 suppressed the production of various proinflammatory cytokines by fully activated CD4\(^+\) T cells while it had no effect on the cytokine production by CD4\(^+\) T cells at early phases of activation. IL-27 also suppressed IL-17 production by activated CD4\(^+\) T cells, thereby counteracting IL-23, another IL-12-related cytokine with proinflammatory effects. In fully activated CD4\(^+\) T cells, STAT3 was preferentially activated by IL-27 stimulation, whereas both STAT1 and 3 were activated by IL-27 in early activated CD4\(^+\) T cells. Lack of STAT3 in fully activated cells impaired the suppressive effects of IL-27. These data indicated that the preferential activation of STAT3 in fully activated CD4\(^+\) T cells plays an important role in the cytokine suppression by IL-27/WSX-1. The Journal of Immunology, 2006, 177: 5377–5385.

Interleukin 12, a composite cytokine made with p35 and p40 subunits, is a potent inducer of IFN-\(\gamma\) production by T cells and NK cells and is pivotal for the differentiation and maintenance of Th1 responses. Recently, two composite cytokines, IL-23 and IL-27, have been discovered, both of which are structurally and functionally similar to IL-12 (1). IL-23 is composed of p19 subunit plus p40 of IL-12 and has been reported to induce proliferation of and IFN-\(\gamma\) production by memory-type Th1 cells (2). IL-27 is made with the p28 subunit and EBI-3, a polypeptide structurally related to p40 of IL-12 (3, 4). IL-27 has originally been shown to initiate the differentiation of Th1-type CD4\(^+\) T cells (4). It has thus been suggested that IL-12, 23, and 27 now are members of the family of heterodimeric cytokines along with two other composite cytokines whose functions are not clear yet (5).

WSX-1 is a novel class I cytokine receptor with homology to the IL-12 receptors and is highly expressed in CD4\(^+\) T cells as well as NK/NKT cells and macrophages (6–8). Recent reports identified WSX-1 as a subunit of IL-27 receptor complex (4, 9). In an analysis of mice deficient for WSX-1, we demonstrated that normal production of IFN-\(\gamma\) by naive CD4\(^+\) T cells was impaired in the knockout mice infected with Leishmania major (8). We and others reported that STAT1 is activated downstream of WSX-1, leading to T-bet induction followed by IL-12R\(\beta2\) expression in naive CD4\(^+\) T cells (9–11). These results revealed that IL-27/WSX-1 is critical for initial Th1 commitment. In addition, WSX-1-deficient mice infected with a nematode, Trichuris muris, showed impaired Th1 responses with augmented Th2 responses resulting in more efficient expulsion of the worms in the knockout mice than in wild-type mice (12, 13).

Although the role of IL-27/WSX-1 signaling in the differentiation of naive CD4\(^+\) T cells into Th1 population has become clearer, a distinct role of IL-27/WSX-1 has recently been revealed. We have reported that, in the absence of WSX-1, CD4\(^+\) T cells as well as NK1.1\(^+\) CD3\(^+\) NKT cells and macrophages overproduced several inflammatory cytokines during Toxoplasma gondii or Trypanosoma cruzi infection, demonstrating a new role of WSX-1 as an inhibitory regulator of cell activation and cytokine production (14, 15). The suppressive role of IL-27/WSX-1 was also observed in experimental hepatitis model where WSX-1-deficient NKT cells and CD4\(^+\) T cells responded to Con A by overproduction of various proinflammatory cytokines (16). Thus, it has been demonstrated that IL-27/WSX-1 has a double-sided role during immune responses; one as an inducer of Th1 responses and the other as an attenuator of inflammatory responses (for review, see Ref. 17). The molecular basis of the IL-27/WSX-1-mediated suppression, however, has not been clarified and, more importantly, in
what milieu IL-27/WSX-1 exerts its suppressive function is not fully understood.

In this report, we examined the suppressive role of IL-27/WSX-1 in vitro by examining the signal pathways downstream of WSX-1. IL-27 suppressed the production by fully activated CD4+ T cells of various cytokines including IL-2, IL-4, and IFN-γ in vitro. In addition, IL-27 also suppressed the production by T cells of IL-17, a proinflammatory cytokine induced by IL-23. IL-27 showed its suppressive effects when added at earlier phases of cell activation but failed to suppress cytokine production when added at later phases. Downstream of the fully functional IL-27R, composed of WSX-1 plus gp130, STAT3 as well as STAT1 was activated in naive CD4+ T cells. Interestingly, the STAT1 activation was almost lost in fully activated CD4+ T cells and STAT3 activation was not affected. In STAT3-deficient T cells, the suppressive effect of IL-27 was significantly diminished over that of control T cells. These data suggested that IL-27/WSX-1 plays its suppressive role on fully activated CD4+ T cells through the preferential activation of STAT3.

Materials and Methods

Animals

Generation of WSX-1 knockout mice was reported previously (8). All of the mice used (8–10 wk old) were backcrossed into the C57BL/6 background (>10 generations). STAT3−/− mice were reported elsewhere (18) and crossed with Tie2-Cre-transgenic mice (19) to delete the STAT3 gene in lymphocytes and hematopoietic stem cells. Similarly, suppressor of cytokine signaling (SOCS3)3fl/+ mice (20) were crossed with Lck-Cre-transgenic mice (49). Mice were maintained in a specific pathogen-free condition at Kyushu University and experiments were performed under the control of the Guidelines for Animal Experiment at Kyushu University.

Cells

HEK293 and HeLa cells were cultured in DMEM containing 10% calf serum. Ba/F3 cells were maintained in RPMI 1640 medium containing 10% FCS and 1 ng/ml murine IL-3. Establishment of the stable WSX-1-expressing Ba/F3 cell lines was described elsewhere (9). Stable Ba/F3 transformants expressing both WSX-1 and gp130 were obtained similarly. CD4+ T cells were purified by magnetic beads sorting (MACS; Miltenyi Biotec) and were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics. For preparation of naive CD4+ T cells, CD4+ T cells were sorted by negative selection using magnetic beads plus anti-CD8a, anti-B220, anti-Mac-1, and anti-NK1.1 Abs (eBioscience). Purified CD4+ T cells were then positively selected using anti-CD62l ligand (CD62L) Ab (CD4+ CD62L+ cells >>99%). The murine macrophage cell line RAW264.7 cells were obtained from the RIKEN Cell Bank (Japan). The RAW264.7 cells were cultured in DMEM supplemented with 10% FBS and 1% nonessential amino acids solution (Invitrogen Life Technologies).

Reagents

Culture supernatants containing recombinant murine IL-27 was prepared by transfection of murine EB1-3 cDNA in pCMV-3xFLAG (Sigma-Aldrich) plus murine p28 cDNA in pcDNA4.0-myc (Invitrogen LifeTechnologies) into HEK293 cells by a conventional calcium phosphate method. Activity of IL-27 in the supernatants was confirmed by induction of STAT1 and STAT3 phosphorylation in CD4+ T cells (data not shown). IL-23 was purchased from eBioscience. Anti-T cell cytokine receptor (WSX-1) Ab was purchased from Abcam. Anti-STAT1, anti-phosphoysotyrosine (pY)-STAT1, and anti-pY-STAT3 Abs were purchased from New England Biolabs; anti-gp130, anti-STAT3, and anti-STAT5b Abs were obtained from Santa Cruz Biotechnology. Anti-rabbit IgG-HRP Abs were purchased from Jackson ImmunoResearch Laboratory. Purified murine rIL-27 was purchased from R&D Systems.

Proliferation assay

Ba/F3 cells (2.5 × 104/200 μl/well) were stimulated for 72 h in the presence or absence of IL-27. For Ba/F3 cell proliferation, [3H]thymidine was added to the cells (1 μCi/well) for the last 6 h of culture. [3H]Thymidine incorporation was determined by scintillation counting.

RT-PCR

Total RNA extracted from cells using TRIzol reagent (Invitrogen LifeTechnologies) was used as a template for cDNA synthesis. cDNA was obtained using murine leukemia virus reverse transcriptase (Applied Biosystems), and then PCR was performed. The following primer sets were used: SOCS1, sense, 5′-CAC TCA TTG CCT CAC CCT CC-3′ and antisense, 5′-GGC AGG GGC GTA TCT GAG AA-3′; SOCS3, sense, 5′-GTT GAG GCT CAA CAC CCA GT-3′ and antisense, 5′-GGG TGG CAA AGA AAA GGA GGA G-3′; β-actin, sense, 5′-TGG AAT CCA GTG GCA TCC ATG AAA C-3′ and antisense, 5′-TTA AAC GAC GCT CAG TAA CAC GCT GCA GCT G-3′.

Real-time quantitative PCR

Reverse transcription reactions and TaqMan PCR were performed according to the manufacturer’s instructions (Applied Biosystems Japan). Sequences of specific primers and an internal fluorescent reporter dye (FAM) signal for each primer were designed using the Primer Express program (Applied Biosystems Japan). Primers used were as follows: GAPDH, sense, 5′-TGG CCT CCA AGG AGT AAA CAC C-3′, antisense, 5′-GGG ATG AAG GCT CTC TCT GTG CT-3′; IL-17F, sense 5′-GAT AAC ACT GTG AGA GTT GAC-3′, antisense, 5′-AAT GCA GGC GTC ATG CCA-3′; IL-17F, sense 5′-GAT AAC ACT GAC GCC ATT CTA-3′, antisense, 5′-AAT GCA GGC GTC ATG CCA-3′; and TaqMan probe, 5′-AAC CAA AAC CAG GGC ATT TCT GTC CC-3′.

Activation of CD4+ T cells

Purified CD4+ T cells were activated with plate-bound anti-CD3 Ab (145-2C11, 1 μg/ml; BD Pharmingen) plus soluble anti-CD28 Ab (1 μg/ml; BD Pharmingen) for 2 days, transferred to a new plate without anti-CD3 Ab, and additionally cultured for 5 days as a total of 7 days either in the presence or absence of IL-27 and/or IL-23. Cells were then washed and restimulated with either anti-CD3 Ab plus anti-CD28 Ab for cytokine production or IL-27 for STAT activation.

For determination of time points when IL-27 suppresses cytokine production, purified CD4+ T cells were activated for a total of 7 days as above except in the absence of IL-23. IL-27 was added for 7 days, the initial 2 or 3 days followed by wash and addition of IL-2, or the last 6.5 or 3 days (see Fig. 3A for schematic demonstration). The cells were then washed and restimulated as above for cytokine production.

Measurement of cytokines

Cytokines in collected sera and culture supernatants of liver mononuclear cells (LMNC) or CD4+ T cells were analyzed using a micro bead-based ELISA system for detection of mouse cytokine (Multiplex Ab Bead kits; BioSource International) according to the manufacturer’s directions with Luminex 100 (Luminex). In some cases, ELISA was also used for detection of IL-2, IL-4, IL-17A, TNF-α, and IFN-γ using ELISA development kits (Genzyme, R&D Systems, and eBioscience) according to the manufacturer’s directions. IL-27-mediated suppression of cytokine production (percent suppression) was calculated as follows: percent suppression = 100 × (1 − cytokine production in the presence of IL-27)/(cytokine production in the absence of IL-27)).

Reporter assay

STAT3 activity was assayed in a reporter assay as described previously (21). Briefly, HeLa cells were plated at 2 × 106/well on 6-well dishes. Cells were transfected with various combinations of receptor-expressing plasmids plus an acute phase response element-containing, STAT3-responsive luciferase plasmid using FuGENE 6 (Roche). After stimulation with IL-27, luciferase activity was measured using the Promega luciferase assay system (Promega) according to the manufacturer’s directions. Luciferase activity was normalized to β-galactosidase activity.

Western blot

CD4+ T cells and Ba/F3 cells were lysed in a lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM vanadate, 50 mM NaF, 1 mM DTT, and 0.1% p-aminophenylmethanesulfonyl fluoride hydrochloride) and centrifuged at 12,000 × g for 10 min. The lysates were resolved on SDS-PAGE, immobiloblotted, and visualized with the indicated Abs. For tyrosine phosphorylation of STAT1 and 3, exposed films were scanned and band intensities were analyzed with the NIH Image (Scion Image) software (Scion).
IL-27 suppression of IL-17 production by activated CD4+ T cells. A. Sera and LMNC were prepared from wild-type (■) or WSX-1−/− (□) mice 11 days after T. cruzi infection. LMNC were cultured for 3 days without further stimulation. IL-17A production in sera and the culture supernatants was measured. B. CD4+ T cells from C57BL/6 mice were stimulated with plate-bound anti-CD3 Ab (1 μg/ml) plus soluble anti-CD28 Ab (1 μg/ml) in the absence or presence of titrated doses of IL-23 (0, 0.1, or 1.0 ng/ml)- and IL-27-containing media (0, 1, or 10%) for 7 days, washed twice, and restimulated with plate-bound anti-CD3 Ab (1 μg/ml) and soluble anti-CD28 Ab (1 μg/ml) for 24 h. Culture supernatants were analyzed by ELISA for the production of IL-17A. Shown are means ± SD. *p < 0.05. Experiments were repeated twice with similar results. C. Total RNA was prepared from cells stimulated as in B in the absence or presence of titrated doses of IL-23 (0 or 1.0 ng/ml) and IL-27. Expression of IL-17F and GAPDH was examined by real-time PCR. Shown are means ± SD of the IL-17F/GAPDH ratio. Experiments were repeated twice with similar results.

Flow cytometry

Purified splenic CD4+ T cells were stimulated with anti-CD3 Ab and anti-CD28 Abs. On days 0, 3, and 7, cells were stained with FITC-conjugated anti-CD4 Ab (eBioscience) and biotin-conjugated anti-gp130 Ab (R&D Systems). Then cells were stained with PE-conjugated streptavidin (eBioscience). The cells were analyzed for surface expression of gp130 using a FACS Calibur (BD Biosciences) and FlowJo software (Tree Star).

Results

IL-27 mediated suppression of IL-17 production by activated T cells

In our previous reports, we demonstrated that WSX-1 suppressed cytokine production by CD4+ T cells in addition to macrophages and NK/NKT cells (14–16). Recently, IL-23, another member of the IL-12 cytokine family, has been reported to induce the production of IL-17 (IL-17A and F) (22, 23), inflammatory cytokines that are produced by activated T cells, and play crucial roles in inflammation by cytokine production, granulopoiesis, chemotaxis, and angiogenesis (24–27). To examine the effect of IL-27 on IL-17 production, we first infected WSX-1-deficient mice with T. cruzi and examined IL-17A production. As shown in Fig. 1A, IL-17A was robustly overproduced in the sera of the infected mice and in the culture supernatants of liver-infiltrating mononuclear cells from infected WSX-1-deficient mice as compared with those of wild-type mice. To directly examine the effect of IL-27 on IL-17A production, CD4+ T cells were stimulated with anti-CD3 plus anti-CD28 Abs in the presence of IL-23 and/or IL-27 for 7 days and then restimulated. As reported previously (23), exogenous IL-23 augmented the production of IL-17 by activated T cells (Fig. 1B). The production of IL-17, however, was significantly suppressed by IL-27 in a dose-dependent manner (Fig. 1B). In addition, addition of IL-27 also suppressed the expression of IL-17F (Fig. 1C) in the cells cultured without IL-23. Thus, in addition to suppressing proinflammatory cytokine production by CD4+ T cells, NK/NKT cells, and macrophages, IL-27 suppressed IL-17 production by activated T cells, which presumably adds to IL-27-mediated anti-inflammatory effects. More importantly, it was revealed that IL-27 counterregulates the activity of IL-23 in terms of IL-17A production.

IL-27 mediated suppression of cytokine production by fully activated CD4+ T cells

We previously reported IL-27 augmented IFN-γ production by naive CD4+ T cells in synergy with IL-12 (9) while IL-27 also has an inhibitory function on cytokine production including IFN-γ by CD4+ T cells (14, 15). Of great interest therefore is how and in what situations IL-27/WSX-1 discriminate their anti- and proinflammatory (Th1-promoting) functions. To address this issue, we examined the effect of IL-27 on IFN-γ production by 7-day activated (fully activated) CD4+ T cells. Just contrary to the effect of IL-27 on naive CD4+ T cells (9), the addition of IL-27 significantly suppressed IFN-γ production by fully activated T cells (Fig. 2). IL-27 also suppressed IL-2 production by fully activated T cells, indicating its antiproliferative/antiactivation effect on T cells (12). Various other cytokines produced by activated T cells, such as IL-4 and TNF-α, were also suppressed by IL-27. IL-23 appeared to augment the production of IFN-γ by activated CD4+ T cells at a low dose. However, it had little, if any, effect on the production of other cytokines (excluding IL-17; Fig. 1, B and C).
Differential roles of IL-27 on naive and activated CD4+ T cells

In our previous studies, we reported that WSX-1 and IL-27 were required for initial commitment of Th1 differentiation by T-bet induction (9). Thus, the suppression of cytokines including IFN-γ by IL-27 is seemingly contradictory. To address this issue, we purified CD4+CD62L+ naive T cells and stimulated them with anti-CD3 Ab for cytokine production. As has been reported recently (28, 29), IL-27 suppressed IL-2 production by stimulated naive CD4+ T cells (Fig. 3A). IL-27 also slightly suppressed IL-17 production. In contrast, IL-27 only slightly increased IFN-γ and TNF-α production. However, IL-27 had almost no effect on the production of IL-10 by naive T cells. The IFN-γ-inducing effect of IL-27 was far less than that of IL-12 plus IL-27 (Fig. 3B), as has been reported (9). Note that in these experiments, we used purified

FIGURE 3. Differential effects of IL-27 on naive T cell and activated T cells. A, Purified CD4+CD62L+ T cells were stimulated with plate-bound anti-CD3 Ab plus soluble anti-CD28 Ab in the presence of titrated doses of IL-27 (0, 1.0, or 10 ng/ml) for 3 days. Culture supernatants were analyzed for indicated cytokine production. B, Purified CD4+CD62L+ T cells were stimulated as in A in the absence or presence of titrated doses of IL-12 (0 or 1.0 ng/ml) and IL-27 (0, 1.0, or 10 ng/ml) for 3 days. IFN-γ production in culture supernatants was analyzed by ELISA. C, CD4+ T cells were stimulated as in Fig. 1B in the presence of titrated doses of IL-27 (0, 1.0, or 10 ng/ml) for 7 days, washed, and restimulated with anti-CD3 and anti-CD28 Abs for 24 h. Culture supernatants were analyzed for indicated cytokine production.

FIGURE 4. Initial requirement of IL-27 for suppression of cytokine production. A, Schematic presentation of CD4+ T cell activation protocols. Purified CD4+ T cells from C57BL/6 mice were stimulated with plate-bound anti-CD3 Ab plus anti-CD28 Ab as in Fig. 1B in the presence of 10% IL-27-containing medium and IL-2 (20 IU/ml) for a total of 7 days (see Materials and Methods for details). Cells were then recovered and restimulated for 24 h as in Fig. 1B, and culture supernatants were examined for cytokine production. B, Cytokine production was measured and percent suppression was calculated as described in Materials and Methods. Experiments were repeated three times with similar results. C, Purified CD4+ T cells were stimulated according to protocol b in the additional presence of IL-23. Culture supernatants were analyzed for indicated cytokine production. Experiments were repeated twice with similar results.
IL-27 (0–10 ng/ml) instead of culture supernatants containing IL-27. The purified IL-27 showed just the same cytokine suppressive effect on activated T cells cultured as in Fig. 2 (Fig. 3C).

**IL-27 is required at the early phases of cell activation for suppression of cytokine production**

To further examine the suppressive effect of IL-27, we cultured CD4+ T cells with IL-27 in various protocols as depicted in Fig. 4A, and cytokine production was measured. Since the amounts of cytokines produced in each culture protocol were considerably different, percent suppression was calculated as in Fig. 1B and Fig. 2 by comparing the amounts of cytokines produced in the presence or absence of IL-27. The presence of IL-27 at the initial phases of cell activation (protocols a, b, and c) showed evident suppressive effects on cytokine production, including IL-2, -4, -17 (Fig. 4B) and IL-5, -6, -10, TNF-α and GM-CSF (data not shown). However, addition of IL-27 on day 2 or later (protocols d, e, and f) showed little, if any, suppressive effects. The suppressive effects of IL-27 on IFN-γ production appeared different from ones on other cytokines, since only protocol a (7 days with IL-27) suppressed IFN-γ production. In other protocols, such as b and c, the suppressive effect of IL-27 may be counterbalanced by its Th1 differentiation activity. Interestingly, when purified CD4+ T cells were stimulated with anti-CD3 plus anti-CD28 Abs for 1, 2, or 3 days, addition of IL-27 to the culture showed no suppressive effect on cytokine production by these cells, either before or after restimulation (data not shown), indicating that cells may need the expansion and/or differentiation phase in the presence of IL-2 (protocols b and c) or IL-27 (protocol a) to be subject to IL-27-mediated cytokine suppression. Addition of IL-23 in protocols b and c (Fig. 4C and data not shown) showed virtually no effect on cytokine production except for IL-17, just as demonstrated in Fig. 2. Taken together, IL-27 stimulation at the initial phases of cell activation followed by an incubation period is required for the suppression of various cytokine production except for IFN-γ.

**Signal transduction pathways downstream of IL-27R**

To clarify the suppressive mechanisms by IL-27/WSX-1, we next tried to elucidate the signal transduction pathways downstream of WSX-1. First, the counterreceptor for WSX-1, with which WSX-1 conforms to a fully functional IL-27R, was searched in STAT1 and STAT3 reporter assays. Among various type I cytokine receptors examined, including leukemia inhibitory factor receptor and gp130 of the IL-6R, only gp130 when coexpressed with WSX-1 in HEK293 cells induced STAT1 activation upon IL-27 stimulation (data not shown). Accordingly, WSX-1 plus gp130 induced transcriptional activation of STAT3 upon IL-27 stimulation in a STAT3-responsive reporter assay (Fig. 5A), as expected from previous findings that STAT3 is activated downstream of gp130 (30).

In Ba/F3 cells constitutively expressing both WSX-1 and gp130, IL-27 also induced transient STAT1 and STAT3 phosphorylation (Fig. 5B). IL-27-induced STAT1 and STAT3 phosphorylation was also confirmed in RAW264.7 cells (Fig. 5C) and in purified CD4+ T cells (see below in Fig. 7A). WSX-1 plus gp130, but not WSX-1 only, expressed in Ba/F3 cells was also functional in supporting IL-27-mediated proliferation of cells (Fig. 5D). In addition, among various genes known to be induced by STAT1 or STAT3, SOCS1 and 3, both attenuators of cytokine signaling, were expressed in Ba/F3 cells expressing both WSX-1 and gp130 and in CD4+ T cells as well as in macrophage cell lines, RAW264.7, upon IL-27 stimulation (Fig. 5E).

**STAT3 is required for IL-27-mediated cytokine suppression downstream of WSX-1**

STAT3 is known to deliver anti-inflammatory signals downstream of IL-10R. Given the STAT3 activation in addition to STAT1 activation downstream of IL-27R, we then addressed the question whether STAT3 also delivers anti-inflammatory signals downstream of IL-27R by using mice deficient for STAT3 specifically in cells of hemopoietic lineage. Just as shown in Figs. 1B and 2,
FIGURE 6. IL-27-mediated cytokine suppression in STAT3-deficient CD4\(^+\) T cells. A, CD4\(^+\) T cells from T cell-specific STAT3-deficient mice (Tie2-Cre \(\times\) STAT3\(^{flox/flox}\), Stat3KO) or control mice (STAT3\(^{flox/flox}\), Ctrl) were stimulated with plate-bound anti-CD3 Ab plus soluble anti-CD28 Ab in the presence or absence of IL-27 as in Fig. 2. Cells were likewise washed and restimulated. Cytokine production was measured and percent suppression was calculated as described in Materials and Methods. Inset, Western blot analysis of the STAT3 expression in CD4\(^+\) T cells used. B, CD4\(^+\) T cells from T cell-specific SOCS3-deficient mice (Lck-Cre \(\times\) SOCS3\(^{flox/flox}\), SOCS3KO) or control mice (SOCS3\(^{flox/flox}\), Ctrl) were stimulated likewise and cytokine production measured. Shown are means \(\pm\) SD. Experiments were repeated twice with similar results.

IL-27 suppressed cytokine production, such as IL-2, IL-4, and IL-17A, by fully activated control CD4\(^+\) T cells. However, the suppressive effect of IL-27 on STAT3-deficient CD4\(^+\) T cells was significantly lower than in control cells (Fig. 6A). Note that the suppression in STAT3-deficient CD4\(^+\) T cells was not complete and also that IFN-\(\gamma\) production was less affected in the absence of STAT3. Reciprocally, in CD4\(^+\) T cells deficient for SOCS3, which preferentially attenuates STAT3 activation (31), IL-27 suppressed cytokine production more effectively than in control CD4\(^+\) T cells (Fig. 6B). Taken together, these data demonstrated that IL-27 exerts its suppressive effect on fully activated CD4\(^+\) T cells through, albeit partially, STAT3 activation.

**Discussion**

In the current study, we demonstrated that the IL-27 via IL-27R complex (WSX-1 plus gp130) has a suppressive function on cytokine production by fully activated CD4\(^+\) T cells. IL-27 suppressed production of IL-17 by activated CD4\(^+\) T cells, thereby counteracting IL-23. In fully activated CD4\(^+\) T cells, STAT3 activation was induced upon IL-27 stimulation while IL-27 induced cytokine suppression was significantly impaired. These data demonstrated the significant contribution of STAT3 to IL-27-mediated cytokine suppression in fully activated CD4\(^+\) T cells.

We demonstrated that both STAT1 and 3 were activated in WSX-1/gp130-expressing Ba/F3 cells and RAW264.7 macrophage cell lines as well as in CD4\(^+\) T cells at their early phases of activation (Figs. 5B, 5C, and 7A). In CD4\(^+\) T cells at an early phase of activation, STAT1 is important for the induction of T-bet (9). Kamiya et al. (10) also reported that in STAT1-deficient T cells neither T-bet induction nor IFN-\(\gamma\) production was properly induced. Although STAT1 is important in Th1 differentiation downstream of WSX-1, other STATs, especially STAT3, are activated downstream of WSX-1. Among these STATs, although STAT2 and 5 activation appeared secondary to STAT1 activation (10), STAT3 activation is directly induced by IL-27-mediated WSX-1 ligation. Since we confirmed the observation by others that gp130 of the IL-6R is a counterpartner of WSX-1 to conform to a STAT1 phosphorylation, IL-27 stimulation in STAT3-deficient T cells is not clear, STAT3 is probably important for the IL-27-mediated proliferation of CD4\(^+\) T cells. Proproliferative roles of STAT3 are also revealed in mice with STAT3-deficient T cells (34).

IL-27 and its receptor WSX-1 have originally been reported as critical factors for initial commitment of Th1 differentiation (4, 7, 8). The Th1-promoting effect of IL-27 has been reported both in vivo and in vitro experiments (9, 10, 35–37). However, recent lines of evidence have also demonstrated that IL-27 has a suppressive effect on proinflammatory cytokine production and/or cell activation (14–16, 38). IL-2 suppression by IL-27 (Refs. 28 and 29 and Fig. 3A) may be at least partially responsible for this effect. Thus, IL-27/WSX-1 have two-sided roles; one is for Th1 differentiation and the other for cytokine suppression (reviewed in Ref. 17).
our current study, we demonstrated that IL-27 has an IFN-γ-inducing effect on CD62L− naive T cells (Fig. 3, A and B). The same cytokine suppresses various cytokine production by 7-day cultured cells upon restimulation. IL-27 therefore gave distinct results on early activated and fully activated CD4+ T cells. On molecular basis, in fully activated CD4+ T cells, preferential STAT3 activation was induced by IL-27, which seems responsible for the suppressive effects of IL-27. Although STAT3 is activated downstream of IL-6R/gp130 to deliver proinflammatory signals, it is also activated downstream of IL-10R to deliver anti-inflammatory signals. Anti-inflammatory effect of STAT3 is most evident in mice with macrophage-selective STAT3 deficiency resulted in robust inflammation and cytokine overproduction relative to wild-type mice (39, 40), a phenotype similar to those observed in IL-10-deficient mice (41). Thus, overproduction of multiple cytokines and robust inflammation observed in WSX-1-deficient mice was quite similar to those observed in IL-10-deficient mice or mice with STAT3-deficient macrophages. Interestingly, STAT3 delivers anti-inflammatory signals even downstream of IL-6R in SOCS3-deficient macrophages (20). STAT3 activation in a given molecular milieu thus delivers anti-inflammatory signals. In the current study, we demonstrated CD4+ T cells deficient for STAT3 were less sensitive to IL-27-mediated cytokine suppression (Fig. 6A). Additionally, in CD4+ T cells deficient for SOCS3, a preferential negative regulator of STAT3 activation, IL-27-mediated suppressive effect was augmented over control cells (Fig. 6B). It is thus strongly indicated that the STAT3 activation induced by IL-27 is critical for IL-27/WSX-1-mediated suppression of proinflammatory cytokines. Presumably, appropriate equilibrium between STAT1 and STAT3 activation is required for physiological functions of IL-27/IL-27R, either Th1 promotion or cytokine suppression. The augmented expression of WSX-1 and reduced expression of gp130 in fully activated CD4+ T cells as compared with early activated cells (Fig. 7, B and C, and a report by Villarino et al. (32)) should also affect the equilibrium between STAT1 and STAT3 activation. Recently, STAT3 target genes responsible for IL-10-mediated immunosuppression were identified (42). The search for genes responsible for IL-27-mediated immunosuppression, including ones described in the report above, is in progress now. Importantly, even in STAT3-deficient CD4+ T cells, there remained detectable IL-27-mediated suppression. Additionally, some cytokines were less affected than others by the absence of either STAT3 or SOCS3 for IL-27-mediated suppression (Fig. 6). It is thus suggested that other signal pathways exist that mediate suppressive signals downstream of IL-27R.

Another important issue to be addressed is how STAT1 activation was selectively reduced in fully activated CD4+ T cells. For some cytokine receptors, SOCS molecules are known to be induced to inhibit the cytokine signaling in a feedback way (43). For STAT1, SOCS1 (also termed as JAB) is induced for feedback inhibition (44). SOCS1-deficient dendritic cells are more activated and potent as APCs by higher STAT1 activation than wild-type dendritic cells (45, 46). However, a critical contribution of SOCS1 in STAT1 activation is unlikely, because, in CD4+ T cells that had been fully activated, SOCS1 mRNA was barely induced by IL-27 stimulation (our unpublished observation). Although precise mechanisms are unknown, SOCS-independent mechanisms specific for persistent inhibition of IFN-γ-mediated STAT1 activation in activated T cells were reported previously (47). It is possible that some phosphatases, such as SHP-1 or -2 may be responsible for the STAT1 attenuation.

IL-23, another IL-12 cytokine family member, has recently been described to induce the production of IL-17 by activated T cells (22, 23, 48). Reportedly, exposure of activated T cells to exogenous IL-23 rendered the cells to preferentially produce IL-17 but not IFN-γ (referred to as Th IL-17 or Th17). Thus, IL-23 has now been a potent inducer of inflammation in, for instance, rheumatoid arthritis and experimental autoimmune encephalomyelitis by IL-17 induction. IL-27 has the ability to counteract IL-23 and suppresses the production of IL-17 production by activated CD4+ T cells. It would be fascinating to assume that IL-23 vs IL-27 is for Th1/Th2 differentiation just as IL-12 vs IL-4 is for Th1/Th2 differentiation. The suppressive effect of IL-27, however, is not selective for IL-17 production but appears to cover a broad group of proinflammatory cytokines produced by activated T cells. Of importance is that,
besides inflammatory cytokines. IL-27 also suppresses IL-2 production by activated CD4⁺ T cells. This may partly explain the higher proliferative responses of WSX-1-deficient T cells over wild-type cells (8, 12). Thus IL-27 in toto attenuates the magnitude of immune responses/inflammation by down-regulating cytokine production and cell proliferation/activation.

In summary, we demonstrated that STAT3 activation is at least partially involved in IL-27-mediated cytokine suppression in CD4⁺ T cells. By preferentially activating STAT3, IL-27 plays suppressive roles in fully activated CD4⁺ T cells, but not in early activated cells. Further elucidation of molecules involved in the selective inhibition of STAT1 activation as well as identification of genes induced by STAT3 for cytokine suppression will help us in understanding the precise mechanisms by which IL-12-related cytokines regulate immune reaction.

Acknowledgments

We thank Drs. Christopher Hunter, Christaana Saris, and Daniel Cua for technical comments, Dr. Hiroimitsu Nakachi for reagents, Sawako Muroi for animal husbandry, and members of Project W for helpful discussion.

Disclosures

The authors have no financial conflict of interest.

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