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STAT3 Is Indispensable to IL-27-Mediated Cell Proliferation but Not to IL-27-Induced Th1 Differentiation and Suppression of Proinflammatory Cytokine Production

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IL-27, a member of the IL-6/IL-12 family, activates both STAT1 and STAT3 through its receptor, which consists of WSX-1 and gp130 subunits, resulting in augmentation of Th1 differentiation and suppression of proinflammatory cytokine production. In the present study, we investigated the role of STAT3 in the IL-27-mediated immune functions. IL-27 induced phosphorylation of STAT1, -2, -3, and -5 in wild-type naive CD4+ T cells, but failed to induce that of STAT3 and STAT5 in STAT3-deficient cohorts. IL-27 induced not only proinflammatory responses including up-regulation of ICAM-1, T-box expressed in T cells, and IL-12Rβ2 and Th1 differentiation, but also anti-inflammatory responses including suppression of proinflammatory cytokine production such as IL-2, IL-4, and IL-13 even in STAT3-deficient naive CD4+ T cells. In contrast, IL-27 augmented c-Myc and Pim-1 expression and induced cell proliferation in wild-type naive CD4+ T cells but not in STAT3-deficient cohorts. Moreover, IL-27 failed to activate STAT3, augment c-Myc and Pim-1 expression, and induce cell proliferation in pro-B BaF/3 transfectants expressing mutant gp130, in which the putative STAT3-binding four Tyr residues in the YXXQ motif of the cytoplasmic region was replaced by Phe. These results suggest that STAT3 is activated through gp130 by IL-27 and is indispensable to IL-27-mediated cell proliferation but not to IL-27-induced Th1 differentiation and suppression of proinflammatory cytokine production. Thus, IL-27 may be a cytokine, which activates both STAT1 and STAT3 through distinct receptor subunits, WSX-1 and gp130, respectively, to mediate its individual immune functions. The Journal of Immunology, 2008, 180: 2903–2911.

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5 Abbreviations used in this paper: T-bet, T-box expressed in T cells; SOCS, suppressor of cytokine signaling; pY, phosphotyrosine; sIL-6R, soluble IL-6R.

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signaling (SOCS)-3 expression (19, 20), the possibility that IL-27 induces anti-inflammatory response in a mechanism similar to the IL-10-induced STAT3-mediated one was discussed previously (21, 22). However, the precise role of STAT3 and its molecular mechanism in not only IL-27-induced cell proliferation but also IL-27-induced anti-inflammatory response remain to be elucidated.

To explore the role of STAT3 in exertion of immune functions by IL-27, we used STAT3-deficient naive CD4+ T cells isolated from mice in which the STAT3 gene is specifically disrupted in T cells by condition gene targeting (23), and transfectants expressing wild-type gp130 and its mutant, in which Tyr residues in the putative STAT-3 binding phosphotyrosine sites of its cytoplasmic region were replaced by Phe.

Materials and Methods

Cell culture and mice

Naive CD4+ T cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 50 µM 2-ME, and 10% conditioned medium from WEHI3B cells as a source of IL-3. Mice lacking STAT3 specifically in T cells (Lck-Cre/STAT3lox/lox) in a mixed background of 129/Sv and C57BL/6 were generated by mating STAT3flox/flox mice (23), in which the Cre recombinase transgene is regulated by the T cell-specific Lck promoter. STAT3flox/flox mice were used as controls. All animal experiments were performed in accordance with our institutional guidelines.

Reagents

Anti-STAT1, anti-STAT3, anti-STAT5, anti-T-bet (4B10), anti-gp130, anti-c-Myc, and anti-Pim-1 (19F7) were purchased from Santa Cruz Biotechnology. Anti-STAT2 and anti-phosphotyrosine (pY)-STAT2 were from Upstate Biotechnology. Anti-pY-STAT1, anti-pY-STAT3 and anti-pY-STAT5 were from Cell Signaling Technology. Anti-CD3 (145–2C11), anti-IL-2 (S4B6), anti-IL-4 (11B11), anti-IFN-γ (XMG1.2), and anti-IL-12 (C17.6) were from American Type Culture Collection (Manassas, VA). Anti-c-CD28 (37.51), anti-IL-12Rβ2 (HAMI10B9), PE-anti-hamster IgG, and mouse rIL-2 were from BD Biosciences. Anti-FLAG (M2), control rat IgG, and anti-actin were from Sigma-Aldrich. Anti-iCAM-1 (Y1N/1.74), anti-hamster IgG, and FITC-anti-rat IgG were from eBioscience. Mouse IL-12 and human TGF-β were from R&D Systems and PeproTech, respectively. Human IL-2 and mouse IFN-γ were provided by Shionogi & Company. Curcumin (25) was purchased from Calbiochem.

Preparation of purified IL-27 and soluble IL-6R/IL-6 proteins

Purified mouse IL-27 was prepared as a FLAG-tagged single chain protein by flexibly linking EBV-induced gene 3 p28 with HEK293-F cells (Invitrogen Life Technologies) as described before (26). Mouse IL-6R and IL-6 cDNAs were isolated by RT-PCR using total RNA prepared from liver and Con A-activated spleen cells, respectively. A single-chain soluble (s)IL-6R/IL-6 expression vector, in which the extracellular domain of IL-6R is followed by the (Gly4Ser)3 linker and by IL-6, was then generated by standard PCR methods and cloned into a p3xFLAG-CMV-9 vector (Sigma-Aldrich), which has preprotryptsin signal peptide and 3xFLAG-epitope-tag sequences at the N-terminal. Purified mouse soluble IL-6R (sIL-6R/IL-6) was prepared using HEK293-F cells similarly as described before (26).

RT-PCR

Total RNA was extracted using a guanidine thiocyanate procedure, cDNA was prepared using oligo(dT) primer and Superscript RT (Invitrogen Life Technologies), and RT-PCR was performed using TaqDNA polymerase (28). Cycle conditions were 94°C for 40 s, 60°C for 20 s, and 72°C for 40 s. Primers used for gp130, WSX-1 and hypoxanthine phosphoribosyl transferase were previously described (20, 29). The following primers were also used: c-Myc antisense primer, 5′-GGGTCCGTGCTGCTCGACCGGATGAAGG-3′; c-Myc sense primer, 5′-GGTCCGTGCTGCTCGACCGGATGAAGG-3′; Pim-1 sense primer, 5′-GATCATCAAGGGCCAGAATTCTC-3′; Pim-1 antisense primer, 5′-CCCTCTGTGACCGAAGTTCT-3′.

Preparation of transfectants

Mouse gp130 cDNA was isolated by RT-PCR using total RNA prepared from Con A-activated spleen cells, and cloned into p3xFLAG-CMV-14 vector (Sigma-Aldrich) but without expression of a 3xFLAG-tag by insertion of a stop codon just after the gp130 sequence. Mouse/human chimeric gp130 wild-type construct and its mutants, in which Tyr-767, −814, −905, and −915 were replaced by Phe, were generated by standard PCR methods using the targeting vectors for gp130(WT) and gp130(WT)3xFLAG(26, 30). BalB/c3 mice were transfected with these wild-type and mutant expression vectors together with respective empty vectors as control by electroporation, and selected with Geneticin (G418).

Western blotting

Cells were lysed in a lysis buffer containing protease inhibitors, and resultant cell lysates were separated on an SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membrane (Millipore) as described previously (31). The membrane was then blocked, probed with primary Ab and then with appropriate secondary Ab conjugated to HRP, and visualized with the ECL detection system (Amersham Biosciences) according to the manufacturer’s instructions.

Preparation of naive CD4+ T cells

Primary T cells were purified by passing spleen cells depleted of erythrocytes through nylon wool. The flow-through fraction was incubated with anti-CD8α conjugated anti-CD8α, anti-B220, anti-I-A, anti-Ter-119, and anti-DX5, followed by incubation with anti-biotin magnetic beads (Miltenyi Biotec) and passed through a magnetic cell sorting column (Miltenyi Biotec). The negative fraction was collected (CD4+ T cells >95%). These purified T cells were then incubated with anti-CD28 conjugated magnetic beads (Miltenyi Biotec), and the positive fraction was collected as purified naive CD4+ T cells (CD26L- cells >99%).

Th differentiation assay

Naive CD4+ T cells (5 × 10^5 cells/ml) were primed with plate-coated anti-CD3 (2 µg/ml) and anti-CD28 (0.5 µg/ml) or soluble anti-CD3 (1 µg/ml) and irradiated splenic DC (4 × 10^6 cells/ml) as APC in the presence of IL-27 under Th1 polarizing conditions with or without IL-12 (10 ng/ml) including anti-IL-4, neutral conditions including anti-IL-4, anti-IFN-γ, and anti-IL-12. Th2 polarizing conditions with IL-4 (10 ng/ml) including anti-IFN-γ and anti-IL-12, or Th17 polarizing conditions with sIL-6R/IL-6 (20 ng/ml) including anti-IL-4, neutral conditions including anti-IL-4, anti-IFN-γ, and anti-IL-17 were tested.
ng/ml) and TGF-β1 (5 ng/ml) including anti-IL-4 and anti-IFN-γ. All these anti-cytokine neutralizing mAbs were used at 10 μg/ml. Splenic DCs were positively purified with anti-CD11c (N418) magnetic beads and AutoMACS from adherent cells of collagenase-digested spleen (CD11c+/T-A+ DCs >90%). On day 3, cells were split 1/4 and expanded in human IL-2 (50 U/ml)-containing complete medium. On day 5, cells were collected, washed, and restimulated at 5 × 10⁶ cells/ml with plate-coated anti-CD3 (2 μg/ml). After 24 h, culture supernatants were harvested and assayed for IL-2, IL-4, IL-13, and IL-17 production by ELISA according to the manufacturer’s instructions (BD Biosciences or R&D Systems).

**Proliferation assay**

For measurement of proliferation, naive CD4⁺ T cells (5 × 10⁵ cells/ml) were stimulated with plate-coated anti-CD3 (2 μg/ml) and IL-27 (0.1, 1 and 10 ng/ml) in the presence of anti-IL-2 (100 μg/ml) for 72 h and pulsed with [³H]-thymidine for last 24 h. For studying cell division, naive CD4⁺ T cells were labeled with 10 μM CFSE (Molecular Probes, Invitrogen Life Technologies) and stimulated with plate-coated anti-CD3 (0, 0.2, 2, and 20 μg/ml) and IL-27 (10 ng/ml) in the presence of anti-IL-2 (100 μg/ml) for 72 h. Cells were then analyzed on a flow cytometer.

**Results**

IL-27 induces activation of STAT1 and STAT2, but not STAT5, in addition to STAT3 in STAT3-deficient CD4⁺ T cells

To investigate the role of STAT3 in IL-27-mediated immune responses, we first used naive CD4⁺ T cells from mice, in which the STAT3 gene is specifically disrupted in T cells by conditional gene targeting (23). Wild-type and STAT3-deficient naive CD4⁺ T cells were activated with plate-coated anti-CD3 for 16 h, stimulated with IL-27 for 20 min, and analyzed for the phosphorylation of STAT3 by Western blotting (Fig. 1A). As expected, STAT3 phosphorylation was not detected in STAT3-deficient naive CD4⁺ T cells in response to IL-27 or sIL-6R/IL-6, which can activate gp130/STAT3 signaling (32). Phosphorylation of STAT1 and STAT2 was similarly observed between wild-type and STAT3-deficient naive CD4⁺ T cells in response to IL-27 or sIL-6R/IL-6, which can activate gp130/STAT3 signaling (32). Phosphorylation of STAT1 and STAT2 was similarly observed between wild-type and STAT3-deficient naive CD4⁺ T cells in response to IL-27, whereas unexpectedly STAT5 phosphorylation was largely reduced in STAT3-deficient naive CD4⁺ T cells. The expression levels of IL-27R subunits, WSX-1 and gp130, were comparable between wild-type and STAT3-deficient naive CD4⁺ T cells, which was determined by RT-PCR (Fig. 1, B and C). These results suggest that IL-27 induces activation of STAT1 and STAT2, but not STAT5 in addition to STAT3, in STAT3-deficient CD4⁺ T cells.

STAT3 is not required for IL-27-induced Th1 differentiation

We then examined the role of STAT3 in IL-27-induced proinflammatory responses such as up-regulation of ICAM-1, T-bet, and IL-12Rβ2 expression, synergistic primary IFN-γ production with

**FIGURE 2.** STAT3 is not required for IL-27-induced Th1 differentiation. Wild-type and STAT3-deficient naive CD4⁺ T cells were stimulated with plate-coated anti-CD3 and anti-CD28 in the presence or absence of IL-27 (10 ng/ml) under the Th1 polarizing conditions without IL-12 for 16 and 48 h and analyzed for cell surface expression of ICAM-1 by FACS using anti-ICAM-1 (solid line) and control rat IgG (plain line with shading) (A). After 48 h, these stimulated cells were also analyzed for expression of T-bet by Western blotting using anti-T-bet and anti-actin (B), and for IL-12Rβ2 expression by FACS using anti-IL-12Rβ2 (solid line) and control hamster IgG (plain line with shading) (C). Similar results were obtained in three independent experiments. Wild-type and STAT3-deficient naive CD4⁺ T cells were also stimulated as described above in the presence or absence of IL-27 and/or IL-12 for 48 h. Culture supernatants were analyzed for IFN-γ production in triplicate by ELISA (D). Data are shown as means ± SD of three independent experiments. No significant difference was observed between STAT3⁺/⁺ and STAT3⁺/⁻. Primed cells as described in (A) were further expanded in IL-2-containing medium on day 3, restimulated with plate-coated-anti-CD3 for 24 h on day 5, and analyzed for IFN-γ production in triplicate by ELISA (E). Data are shown as means ± SD of five independent experiments. No significant difference was observed between STAT3⁺/⁺ and STAT3⁺/⁻.

No significant difference was observed between STAT3⁺/⁺ and STAT3⁺/⁻.
IL-12, and Th1 differentiation, which were previously demonstrated to be mediated by STAT1 using STAT1-deficient mice (6, 8). Wild-type and STAT3-deficient naive CD4\(^+\) T cells were stimulated with plate-coated anti-CD3 and anti-CD28 in the presence or absence of IL-27 and/or IL-12 for 16 or 48 h and analyzed for expression of ICAM-1 (Fig. 2A) and IL-12R\(\beta_2\) (Fig. 2C) by FACS and T-bet by Western blotting (Fig. 2B), and IFN-\(\gamma\) production by ELISA (Fig. 2D). These primed cells were further expanded in IL-2-containing medium on day 3, restimulated with plate-coated anti-CD3 for 24 h on day 5, and analyzed for IFN-\(\gamma\) production by ELISA (Fig. 2E). IL-27-induced rapid up-regulation of ICAM-1 expression and augmentation of T-bet and IL-12R\(\beta_2\) expression was similarly observed between wild-type and STAT3-deficient naive CD4\(^+\) T cells. In addition, comparable primary IFN-\(\gamma\) production by IL-27 in synergy with IL-12 was detected. Similar Th1 differentiation induced by IL-27 was also observed between wild-type and STAT3-deficient naive CD4\(^+\) T cells. These results suggest that STAT3 is not necessary for IL-27-induced proinflammatory responses including up-regulation of ICAM-1, T-bet, and IL-12R\(\beta_2\) expression, synergistic primary IFN-\(\gamma\) production with IL-12, and Th1 differentiation.

**STAT3 is not necessary for IL-27-induced suppression of proinflammatory cytokines**

It was previously demonstrated using WSX-1-deficient mice that IL-27/WSX-1 is important for suppression of proinflammatory cytokine production in certain infections with *Toxoplasma gondii* (5, 14), *Trypanosoma cruzi* (15), and *Trichuris muris* (16), Con A-induced hepatitis (17), and experimental autoimmune encephalomyelitis (18). We and other groups recently demonstrated that IL-27 inhibits primary IL-2 production mediated through CD28 signaling from naive CD4\(^+\) T cells (19, 20) and that SOCS3, whose expression is induced by IL-27, plays a critical role in the inhibition in a negative feedback mechanism (20). Therefore, we next examined the role of STAT3 in IL-27-mediated suppression of production of various cytokines from Th cells. Wild-type and STAT3-deficient naive CD4\(^+\) T cells were stimulated with plate-coated anti-CD3 and anti-CD28 or soluble anti-CD3 and irradiated splenic DC as APC in the presence of IL-27 under neutral conditions, and Th2 and Th17 polarizing conditions. These cells were expanded with IL-2 on day 3, and restimulated with plate-coated anti-CD3 for 24 h on day 5. Culture supernatant was collected and assayed for IL-2 (Fig. 3A), IL-4 (B), IL-13 (C), and IL-17 (D) production in triplicate by ELISA. IL-2 and IL-4 production were shown as relative values to that in the absence of IL-27. The amounts of IL-2 produced from restimulated wild-type and STAT3-deficient CD4\(^+\) T cells in the absence of IL-27, which were calculated as 100%, were 92.3 \(\pm\) 39.0 and 31.6 \(\pm\) 32.2 ng/ml, respectively, under neutral conditions, and 75.0 \(\pm\) 27.1 and 63.5 \(\pm\) 62.8 ng/ml, respectively, under Th2 polarizing conditions. The amounts of IL-4 produced from restimulated wild-type and STAT3-deficient CD4\(^+\) T cells in the absence of IL-27, which were calculated as 100%, were 221.9 \(\pm\) 131.8 and 40.6 \(\pm\) 30.3 ng/ml, respectively. Data are shown as means \(\pm\) SD of three to six (IL-2), three (IL-4), three (IL-13) and four (IL-17) independent experiments. *p < 0.05, compared with STAT3\(^{+/+}\).

**IL-27 augments the expression of c-Myc and Pim-1 and induces cell proliferation in a STAT3-dependent mechanism**

Because gp130/STAT3 signaling was previously demonstrated to be critically important for IL-6-induced cell proliferation (23), we...
next explored the role of STAT3 in IL-27-induced proliferation. The original cloning paper of IL-27 showed that IL-27 induces IL-2-independent proliferation (1), which was further shown in a recent paper demonstrating that IL-2 suppresses the expression of WSX-1 in activated CD4\(^+\) T cells and that IL-27 responses are enhanced in the absence of IL-2 (19). Wild-type and STAT3-deficient naive CD4\(^+\) T cells were stimulated with plate-coated anti-CD3 and various concentrations of IL-27 or sIL-6R/IL-6 in the presence of anti-IL-2 for 72 h and \[^{[3]}\text{H}\]-thymidine incorporation was determined for the last 24 h (Fig. 4A). In wild-type naive CD4\(^+\) T cells, IL-27 enhanced proliferation in a dose-dependent manner, whereas IL-27 failed to increase proliferation in STAT3-deficient naive CD4\(^+\) T cells as did sIL-6R/IL-6. We also analyzed the cell division using CFSE (Fig. 4B). The lower concentration of anti-CD3 appeared to increase the more difference of cell division between the presence and absence of IL-27 in wild-type CD4\(^+\) T cells. A significant increase of the cell division in the presence of IL-27 compared with that in the absence of IL-27 (83.3 \(\pm\) 6.1\% vs 69.1 \(\pm\) 7.6\%, \(p < 0.05\)) was observed in wild-type CD4\(^+\) T cells, but not (65.0 \(\pm\) 13.4\% vs 60.3 \(\pm\) 13.1\%, \(p > 0.05\)) in STAT3-deficient cells, at 0.2 \(\mu\)g/ml anti-CD3 among four independent experiments. These results suggest that STAT3 is important for IL-27-induced cell proliferation.

To further examine the role of STAT3 in cell proliferation, we next used a potent inhibitor of STAT3 phosphorylation, curcumin, which was reported to inhibit IL-6-induced phosphorylation of STAT3, and also STAT1 but not STAT5, and suppress cell proliferation in human multiple myeloma cells (25). Naive CD4\(^+\) T cells were activated with plate-coated anti-CD3 for 16 h, then treated with curcumin (10 \(\mu\)M) for 1 h, stimulated with IL-27 or sIL-6R/IL-6 for 20 min and subjected to Western blotting using anti-pY-STATs and anti-total STATs (Fig. 4C). Naive CD4\(^+\) T cells were also treated with curcumin and then stimulated with plate-coated anti-CD3 and IL-27 or sIL-6R/IL-6 for 72 h, and \[^{[3]}\text{H}\]-thymidine incorporation was determined (Fig. 4D). As reported for IL-6 (25), curcumin inhibited phosphorylation of STAT3 induced by IL-27, and slightly suppressed that of STAT1 but not STAT5. Moreover, curcumin inhibited cell proliferation induced by IL-27 as well as sIL-6R/IL-6 in a dose-dependent manner. These results further support the concept that STAT3 is important for IL-27-induced cell proliferation.

It was previously demonstrated that Pim-1, a protooncogenic serine/threonine protein kinase, and c-Myc, a critical regulator of cell growth for cell cycle progression from the G1 to S phase, synergistically regulate gp130/STAT3-mediated cell proliferation and antiapoptosis in response to IL-6 (36). Therefore, we next examined whether IL-27 can induce the expression of c-Myc and Pim-1. Wild-type and STAT3-deficient naive CD4\(^+\) T cells were stimulated with plate-coated anti-CD3 in the presence or absence of anti-total STATs. D. Naive CD4\(^+\) T cells were also treated with various amounts of curcumin for 1 h, then stimulated with plate-coated anti-CD3 and IL-27 (10 ng/ml) or sIL-6R/IL-6 (1 ng/ml) in the presence of anti-IL-2 for 72 h and were pulsed with \[^{[3]}\text{H}\]-thymidine for the last 24 h. \[^{[3]}\text{H}\]-thymidine incorporation was measured in triplicate. Data are shown as means \(\pm\) SD. E and F. Wild-type and STAT3-deficient naive CD4\(^+\) T cells were stimulated with plate-coated anti-CD3 in the presence of anti-IL-2 for 72 h (10 ng/ml) or sIL-6R/IL-6 (1 ng/ml) for 24 h. Total RNA and cell lysates were prepared and analyzed for mRNA expression of c-Myc, Pim-1, and hypoxanthine phosphoribosyl transferase as control by RT-PCR (E) and for Pim-1 expression at protein level by Western blotting using anti-Pim-1 and anti-actin (F). Pim-1 is expressed as two isoforms as reported previously (55). Similar results were obtained in three independent experiments.
of IL-27 for 24 h, and analyzed for expression of c-Myc and Pim-1 by RT-PCR (Fig. 4E) and Western blotting (Fig. 4F). Augmentation of c-Myc and Pim-1 expression was clearly detected in wild-type naive CD4⁺ T cells but not in STAT3-deficient naive CD4⁺ T cells. We could not constantly detect c-Myc expression in stimulated naive CD4⁺ T cells by Western blotting (data not shown), probably due to the lower level of expression or the lower sensitivity of the Ab we used. These results suggest that IL-27 augments the expression of c-Myc and Pim-1 in STAT3-dependent mechanism, presumably resulting in enhanced cell proliferation.

**Tyr residues in the YXXQ motif of the cytoplasmic region of gp130 are required for IL-27-induced STAT3 activation and cell proliferation with enhanced expression of c-Myc and Pim-1**

To finally investigate the molecular mechanism underlying the STAT3 activation-mediated proliferation by IL-27, we used BaF/3 cells, which were determined to express WSX-1 but lack gp130 expression by RT-PCR analyses (data not shown) as reported before (2). We prepared BaF/3 transfectants expressing vector alone as control, wild-type gp130, and mutant gp130, in which all four Tyr residues in the YXXQ motif were previously demonstrated to be critically important for the cytoplasmic region was replaced by Phe. These Tyr residues in the YXXQ motif were previously demonstrated to be critically important for IL-6-induced cell proliferation and antiapoptosis in response to IL-6 (37). As expected, IL-27 induced phosphorylation of both STAT1 and STAT3 in BaF/3 transfectants expressing wild-type gp130 (Fig. 5A). In contrast, IL-27 induced phosphorylation of only STAT1 but not STAT3 in BaF/3 transfectants expressing mutant gp130. We then examined the effect of mutation in these four Tyr residues on IL-27-induced proliferation and expression of c-Myc and Pim-1 (Fig. 5B and C). IL-27 induced cell proliferation in BaF/3 transfectants expressing wild-type gp130, while IL-27 failed to induce it in BaF/3 transfectants expressing mutant gp130 as in those expressing control vector. Moreover, expression of c-Myc and Pim-1 was rapidly enhanced by IL-27 in BaF/3 transfectants expressing wild-type gp130, but not in those expressing mutant gp130. In response to IL-3, all these transfectants showed proliferative responses (data not shown) and augmented expression of c-Myc and Pim-1 (Fig. 5C). Similar results were obtained using a different transfectant clone (data not shown). These results suggest that Tyr residues in the YXXQ motif of the cytoplasmic region of gp130 are required for IL-27-induced STAT3 activation and proliferation with enhanced expression of c-Myc and Pim-1 independently of STAT1 activation.

**Discussion**

In the present study, we have demonstrated that STAT3 is dispensable to not only IL-27-induced augmentation of Th1 differentiation, but also IL-27-induced suppression of proinflammatory cytokine production. In contrast, STAT3 was required for IL-27-induced up-regulation of c-Myc and Pim-1 and consequent cell proliferation. In addition, IL-27-induced STAT3 activation was mediated through four Tyr residues in the YXXQ motif of the cytoplasmic region of gp130 as in the case of IL-6 (38). These results are consistent with our previous results using STAT1-deficient mice, showing that STAT1 activation is important for IL-27-induced augmentation of Th1 differentiation but not for IL-27-induced cell proliferation (6, 8).

In STAT3-deficient naive CD4⁺ T cells, IL-27-induced phosphorylation of STAT3 in addition to STAT3 was reduced (Fig. 1). Therefore, we also used a potent inhibitor of STAT3 phosphorylation, curcumin, which was reported to inhibit IL-6-induced phosphorylation of STAT3 and also STAT1 but not STAT5 and suppress cell proliferation in human multiple myeloma cells (25). Curcumin inhibited IL-27-induced phosphorylation of STAT3 and slightly STAT1 but almost not STAT5, and suppressed IL-27-induced cell proliferation (Fig. 4, C and D). Moreover, although IL-27-induced augmentation of STAT5 phosphorylation was hardly observed in BaF/3 transfectants expressing wild-type gp130 (data not shown) as reported before (39), these cells proliferated...
well in response to IL-27 (Fig. 5C). Reduced phosphorylation of STAT5 in response to IL-27 was also observed in STAT1-deficient naive CD4+/T cells, whereas these cells proliferate as well as wild-type naive CD4+/T cells do in response to IL-27 (6). Taken together, these facts further support our conclusion that STAT3 plays a critical role in IL-27-induced proliferation, although we cannot formally rule out the possibility of involvement of STAT5 in IL-27-induced proliferation. Because STAT3 is also known to be involved in cell proliferation in response to cytokines such as IL-2 (40), further studies using STAT5-deficient mice are necessary to elucidate the precise role of STAT5 in IL-27-mediated immune functions including cell proliferation.

IL-27 was first demonstrated to enhance proliferation of naive, but not memory, CD4+/T cells (1), whereas IL-27/IL-27R appears not to be required for T cell proliferation in vivo. Later studies rather revealed that WSX-1-deficient T cells more vigorously proliferate than wild-type counterparts in vivo and in vitro (5, 11). Therefore, the physiological relevance of the IL-27-mediated T cell proliferation remains unknown. To try to directly address the importance of IL-27-mediated proliferation during in vivo T cell responses, mice were given anti-CD3 mAb (2C11, 10 μg/mouse) (41) and IL-27 (1 μg/mouse, twice after 24 h) or PBS i.p. Thirty-six hours later and 12 h before sacrifice, mice were pulsed with 1 mg of BrdU, and spleen cells were stained for CD4 and intracellular BrdU. However, no significant increase was observed in the percentage of CD4+/BrdU+ cells between mice given IL-27 and PBS (data not shown). Recently, it was demonstrated that SOCS3-deficient CD8+/T cells show enhanced responses to TCR ligation due to increased IL-27 signaling, which drives unregulated proliferation in the absence of SOCS3, but not aberrant TCR signaling (42). Therefore, it might be possible that, under stressed or pathological circumstances, IL-27 may play a role in the induction of T cell proliferation in vivo. Further studies are necessary to clarify the physiological significance of IL-27 in T cell proliferation.

Although STAT1 and STAT3 are similar proteins often activated by the same stimuli, they have very different effects on cell growth and survival. It is considered that STAT1 is a tumor suppressor (43, 44), while STAT3 is an oncogene (45). One of the most typical cytokines, which activate STAT1 as a critical signaling molecule to mediate their immune functions, is IFN-γ. IFN-γ is a pluripotent cytokine that has a crucial role in several processes, including host defense against viruses and microorganisms, anti-proliferative effect, phagocyte activation, control of apoptosis, promotion of Ag processing and presentation, and Th1 differentiation (46). Although STAT3 and STAT5 are also activated by IFN-γ in certain cell types, STAT1 plays a major role in mediating these actions by IFN-γ. In contrast, the most prominent cytokine, which activates STAT3 as a crucial signaling molecule, is IL-6. IL-6 is also a multifunctional cytokine that regulates inflammatory responses, hematopoiesis, and the acute phase response (38).

Greater production of IL-6 is associated with immune-mediated diseases, such as rheumatoid arthritis. Gene deletion studies indicated that STAT3 has a primary role in determining the cellular responses to IL-6, although IL-6 activates STAT1 and STAT3. Because IL-27 activates both STAT1 and STAT3 through its distinct receptor subunits, WSX-1 and gp130, as shown in this study and previously (3), physiological consequences in response to IL-27 could be more complicated, and their relative abundance, which may vary substantially in different cell types, under different conditions appear to have a major impact on how cells behave in response to IL-27.

In addition, the reciprocal regulation of STAT1 and STAT3 activation was demonstrated previously. In mouse embryo fibroblasts lacking STAT3, IL-6 mediates an IFN-γ-like response including prolonged activation of STAT1, the induction of multiple IFN-γ-inducible genes, the expression of MHC class II Ags, and antiviral state (47). On the contrary, in STAT1-null mouse embryo fibroblasts, IFN-γ activates STAT3 strongly, and the activated STAT3 can drive the expression of some genes that normally respond to activated STAT1 in IFN-γ-treated wild-type cells (48). Therefore, it was suggested that much caution is necessary for the interpretation of the phenotypes of knockout mice due to the possible switching of one cytokine response to one closely mimicking another as a result of the loss of a single signaling component (47, 49). Because our present results as to the role of STAT3 obtained using STAT3-deficient mice are well consistent with the results obtained using STAT1-deficient mice (6, 8), our interpretation of these results seems reasonable.

However, there is a recent study demonstrating that the suppressive effect of IL-27 in STAT3-deficient CD4+/T cells is significantly lower than that in wild-type cohorts, and it concluded that the preferential activation of STAT3 in fully activated CD4+/T cells plays an important role in the cytokine suppression by IL-27 (22). The results of this article appear to be consistent with the mechanism by which IL-10 induces anti-inflammatory responses through STAT3 in macrophages (21, 49, 50), but not with the present results. This discrepancy might result from the differences in the Cre promoter used for generation of STAT3-deficient mice, Tie2 vs Lck promoters. Tie2 gene promoter drives Cre expression in bone marrow and endothelial cells (51), while Lck gene promoter drives it in immature T cells (24). Tissue-specific disruption of STAT3 in bone marrow cells of Tie2-Cre/STAT3floxt/lox mice during hematopoiesis was reported to cause death within 4–6 wk after birth with Crohn’s disease-like conditions including dramatic expansion of myeloid lineages, and massive infiltration of the intestine with neutrophils, macrophages, and eosinophils (52). This pathogenesis is considered to be caused by a pseudoactivated innate immune response to LPS as a result of the STAT3 deletion during hematopoiesis. In marked contrast, no such pathogenesis was observed in Lck-Cre/STAT3floxt/lox mice (23) (data not shown). In addition, it was demonstrated that Th17 differentiation is impaired in naive CD4+/T cells obtained from Lck-Cre/ STAT3floxt/lox mice, indicating that STAT3 is essential for Th17 differentiation (35, 53). Moreover, it was recently shown that IL-27 inhibits Th17 differentiation and this inhibition is impaired in STAT1-deficient naive CD4+/T cells (14, 18). We also reported that IL-27 inhibits primary IL-2 production in STAT1-dependent mechanism (20). These results suggest a critical role for STAT1 in IL-27-mediated suppression of cytokine production such as IL-17 and IL-2. However, another group reported that IL-27 inhibits secondary IL-2 production in a STAT1-independent mechanism, which was detected by intracellular staining of cells stimulated with anti-CD3/anti-CD28 for 48 h and then restimulated with PMA/ionomycin (19). Because it might be possible that IL-27 regulates the production of each cytokine via different mechanisms under different conditions, and the possibility of a shift in STAT signaling in the absence of another STAT as discussed above, cannot be formally ruled out, further studies on the roles of STAT1 and STAT3 in IL-27-mediated regulation of production of each cytokine are currently under investigation.

In macrophages stimulated with either IL-10 or IL-6, a seemingly identical process is activated, whereas the downstream readouts are distinct (49). The major function of IL-10 is to negatively regulate inflammatory responses by blocking the stimulatory effects of TLR agonists such as LPS. In contrast, IL-6 is a proinflammatory cytokine. Both cytokines can activate STAT3 and some STAT3-dependent genes in common, such as SOCS3. However, only IL-10 can induce the anti-inflammatory response.
Therefore, the STAT3 activated by IL-10 is considered to differ from STAT3 activated by IL-6 (21, 49, 50). The only obvious distinction between these receptor types is that the IL-6R is regulated by SOCS3, whereas the other anti-inflammatory STAT3-activating receptors, IL-10R, is not (49). Indeed, in the absence of SOCS3, IL-6 becomes capable of inducing anti-inflammatory response, but not inflammatory response, in the same way as IL-10 (54). However, the molecular mechanism of this effect remains to be established. Because one of the IL-27R subunit is the common gp130 and IL-27 can activate STAT3 and induce SOCS3 expression (19, 20), it might be highly expected that IL-27 induces anti-inflammatory response in a mechanism similar to the IL-10-induced STAT3-mediated one (21). However, the present results may exclude this possibility and rather support the concept that IL-27 induces STAT1-mediated anti-inflammatory response, the molecular mechanism of which is currently under investigation.

In conclusion, IL-27 is considered to possess immune functions similar to both IFN-γ and IL-6 by selectively using its receptor subunits/STATs. That is, IFN-γ-like functions including up-regulation of ICAM-1, T-bet, IL-12Rβ2, and MHC class I leading to Th1 differentiation and presumably also suppression of proinflammatory cytokine production are mediated by WSX-1/STAT1 signaling. In contrast, IL-6-like functions including augmentation of cell proliferation and c-Myc and Pin-1 expression are mediated by gp130/STAT3 signaling. Therefore, IL-27 may be a cytokine that activates both STAT1 and STAT3 and selectively uses its receptor subunits/STATs to exert its distinct immune functions: WSX-1/STAT1 for induction of proinflammatory responses and gp130/STAT3 for cell proliferation.

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Disclosures

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References


