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IL-27, a novel 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 positive and negative regulations of immune responses. We recently demonstrated that IL-27 induces Th1 differentiation through ICAM-1/LFA-1 interaction in a STAT1-dependent, but T-bet-independent mechanism. In this study, we further investigated the molecular mechanisms by focusing on p38 MAPK and ERK1/2. IL-27-induced Th1 differentiation was partially inhibited by lack of T-bet expression or by blocking ICAM-1/LFA-1 interaction with anti-ICAM-1 and/or anti-LFA-1, and further inhibited by both. Similarly, the p38 MAPK inhibitor, SB203580, or the inhibitor of ERK1/2 phosphorylation, PD98059, partially suppressed IL-27-induced Th1 differentiation and the combined treatment completely suppressed it. p38 MAPK was then revealed to be located upstream of T-bet, and SB203580, but not PD98059, inhibited T-bet-dependent Th1 differentiation. In contrast, ERK1/2 was shown to be located downstream of ICAM-1/LFA-1, and PD98059, but not SB203580, inhibited ICAM-1/LFA-1-dependent Th1 differentiation. Furthermore, it was demonstrated that STAT1 is important for IL-27-induced activation of ERK1/2, but not p38 MAPK, and that IL-27 directly induces mRNA expression of growth arrest and DNA damage-inducible 45g, which is known to mediate activation of p38 MAPK. Finally, IL-12Rβ2 expression was shown to be up-regulated by IL-27 in both T-bet- and ICAM-1/LFA-1-dependent mechanisms. Taken together, these results suggest that IL-27 induces Th1 differentiation via two distinct pathways, p38 MAPK/T-bet- and ICAM-1/LFA-1/ERK1/2-dependent pathways. This is in contrast to IL-12, which induces it via only p38 MAPK/T-bet-dependent pathway.

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Materials and Methods
Cell culture and mice
Naive CD4+ T cells were cultured in RPMI 1640 supplemented with 10% FBS and 50 μM 2-ME. Wild-type BALB/c mice were purchased from Japan SLC. STAT4-deficient mice (16) and T-bet-deficient mice (17) of BALB/c background were purchased from The Jackson Laboratory. Wild-type 129Sv mice and STAT1-deficient mice (18) of 129Sv background were purchased from Taconic Farms. All animal experiments were performed in accordance with our institutional guidelines.

Reagents
SB203580 and PD98059 were purchased from Calbiochem. Anti-CD3 (145-2C11), anti-IL-4 (11B11), and anti-LFA-1 (FD441.8) were from American Type Culture Collection. Anti-T-bet (4B10), anti-STAT1, anti-STAT4, anti-ERK1, anti-ERK2, and anti-p38 MAPK were from Santa Cruz Biotechnology. Anti-actin was from Sigma-Aldrich. Anti-phospho-p38 MAPK (Thr180/Tyr182) (3D7) were from Cell Signaling Technologies, according to the manufacturer’s instructions. Anti-actin was from Sigma-Aldrich. Anti-phospho-STAT4, anti-ERK1, anti-ERK2, and anti-p38 MAPK were from Santa Cruz Biotechnology. Anti-actin was from Sigma-Aldrich. Anti-phospho-p38 MAPK (Thr180/Tyr182) (3D7) were from Cell Signaling Technology. Anti-CD28 (37.51) and anti-IL-12Rβ2 (HAMI089) were from BD Biotechnologies. Anti-ICAM-1 (YN1/1.7.4) was from eBioscience. Mouse rIL-12 (10 ng/ml), including anti-IL-4 (10 ng/ml) and mouse IL-12 (10 ng/ml) were provided by Shionogi.

Preparation of purified rIL-27 protein
rIL-27 was prepared as a FLAG-tagged single chain protein, by a method described here, using EBV-induced gene 3 to d28 using HEK293 F cells (Invitrogen Life Technologies), as described before (19).

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 biotin-conjugated anti-CD3ε, anti-CD25, anti-Mac-1, 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), and the negative fraction was collected (CD4+ T cells >95%). These purified T cells were then incubated with anti-CD62 ligand magnetic beads (Miltenyi Biotec), and the positive fraction was collected as purified naive CD4+ T cells (CD62 ligand+ cells >99%).

Th1 differentiation assay
Naive CD4+ T cells (5 x 10^5 cells/ml) were primed with plate-coated anti-CD3 (2 μg/ml) and anti-CD28 (0.5 μg/ml) in the presence or absence of IL-27 (10 ng/ml) under the Th1-polarizing conditions or those without IL-12 (10 ng/ml), including anti-IL-4 (10 μg/ml). On day 3, cells were split 1:4 and expanded in IL-2 (50 U/ml)-containing complete medium. On day 6, cells were collected, washed, and resuspended at 5 x 10^6 cells/ml with plate-coated anti-CD3 (2 μg/ml). After 24 h, culture supernatants were harvested and assayed for IFN-γ production by ELISA.

Western blotting
Cells were trypsinized and subjected to Western blotting using anti-T-bet and anti-actin. In our experiments, we observed differences in T-bet expression between control and experimental groups. As shown in the figure, T-bet expression is reduced in experimental groups compared to control groups.

Statistical analysis
Statistical analysis was performed by Student’s t test. A p value of <0.05 was considered to indicate statistical significance.

Results
IL-27 induces Th1 differentiation in T-bet- and ICAM-1/LFA-1-dependent mechanisms
We recently demonstrated that IL-27 differentiates naive CD4+ T cells into Th1 cells under Th1-polarizing conditions, but without IL-12 through ICAM-1/LFA-1 interaction in a STAT1-dependent, but T-bet-, IFN-γ-, and STAT4-independent mechanism (13). To further elucidate the molecular mechanisms underlying IL-27-induced Th1 differentiation, we first explored the role of T-bet and compared it with that of ICAM-1. Naive CD4+ T cells from wild-type BALB/c and T-bet-deficient mice were stimulated with plate-coated anti-CD3 and anti-CD28 in the presence or absence of IL-27 for 16 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). Mean fluorescence intensity (MFI) of each histogram is shown as the mean ± SD of three independent experiments (B), C, ICAM-1/LFA-1 is not required for augmentation of T-bet expression by IL-27. Wild-type naive CD4+ T cells were stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 in the presence or absence of blocking Abs against ICAM-1 and LFA-1 (10 μg/ml each) for 48 h. Total cell lysates were prepared and subjected to Western blotting using anti-T-bet and anti-actin. D, IL-27-induced Th1 differentiation is reduced in T-bet-deficient naive CD4+ T cells and further inhibited by blocking ICAM-1/LFA-1 interaction, Wild-type and T-bet-deficient naive CD4+ T cells were primed with plate-coated anti-CD3/anti-CD28 and IL-27 in Th1-polarizing conditions without IL-12 in the presence or absence of anti-ICAM-1/anti-LFA-1 (10 μg/ml each). Primed CD4+ T cells were expanded with IL-2 on day 3 and restimulated with plate-coated anti-CD3 for 24 h on day 6, and culture supernatants were analyzed for IFN-γ production in triplicate by ELISA. Data are shown as means ± SD. * Indicates p < 0.05. Similar results were obtained in three independent experiments.

FIGURE 1. IL-27 induces Th1 differentiation in T-bet- and ICAM-1/LFA-1-dependent mechanisms. A, T-bet is not required for rapid up-regulation of ICAM-1 expression by IL-27. Wild-type BALB/c and T-bet-deficient naive CD4+ T cells were stimulated with plate-coated anti-CD3 and anti-CD28 in the presence or absence of IL-27 under Th1-polarizing conditions without IL-12 for 16 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). Mean fluorescence intensity (MFI) of each histogram is shown as the mean ± SD of three independent experiments (B). C, ICAM-1/LFA-1 is not required for augmentation of T-bet expression by IL-27. Wild-type naive CD4+ T cells were stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 in the presence or absence of blocking Abs against ICAM-1 and LFA-1 (10 μg/ml each) for 48 h. Total cell lysates were prepared and subjected to Western blotting using anti-T-bet and anti-actin. D, IL-27-induced Th1 differentiation is reduced in T-bet-deficient naive CD4+ T cells and further inhibited by blocking ICAM-1/LFA-1 interaction, Wild-type and T-bet-deficient naive CD4+ T cells were primed with plate-coated anti-CD3/anti-CD28 and IL-27 under Th1-polarizing conditions without IL-12 in the presence or absence of anti-ICAM-1/anti-LFA-1 (10 μg/ml each). Primed CD4+ T cells were expanded with IL-2 on day 3 and restimulated with plate-coated anti-CD3 for 24 h on day 6, and culture supernatants were analyzed for IFN-γ production in triplicate by ELISA. Data are shown as means ± SD. * Indicates p < 0.05. Similar results were obtained in three independent experiments.
T cells, but also T-bet-deficient cohorts. Wild-type naive CD4⁺ T cells were also stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 in the presence or absence of blocking Abs against ICAM-1 and LFA-1 for 48 h, and analyzed for T-bet expression by Western blotting (Fig. 1C). Blocking ICAM-1/LFA-1 interaction by their Abs did not affect the augmentation of T-bet expression by IL-27. We then examined the effect of lack of T-bet expression and/or blocking ICAM-1/LFA-1 interaction on IL-27-induced Th1 differentiation. Wild-type and T-bet-deficient naive CD4⁺ T cells were primed with plate-coated anti-CD3/anti-CD28 and IL-27 under Th1-polarizing conditions without IL-12 in the presence or absence of anti-ICAM-1/anti-LFA-1, expanded with IL-2 on day 3, restimulated with plate-coated anti-CD3 for 24 h on day 6, and analyzed for IFN-γ production in culture supernatants by ELISA (Fig. 1D). Lack of T-bet expression partially (~50%) inhibited the ability of IL-27 to induce Th1 differentiation. Blocking ICAM-1/ LFA-1 interaction also partially (~50%) inhibited the ability, as reported previously (13). Both lack of T-bet expression and blocking ICAM-1/LFA-1 interaction further inhibited the ability of IL-27 to induce Th1 differentiation. These results suggest that IL-27 induces Th1 differentiation via T-bet- and ICAM-1/LFA-1-dependent pathways, which are presumably regulated by distinct mechanisms.

**IL-27-induced Th1 differentiation is mediated by p38 MAPK and ERK1/2 independently**

p38 MAPK and ERK1/2 are well known to be involved in the regulation of various cytokine signalings (14, 15). It was demonstrated previously that IL-12 activates p38 MAPK, but not ERK1/2, and that activation of p38 MAPK is required for Th1 differentiation in a STAT4-independent manner (23). Therefore, we next explored the role of these MAPKs in the signaling of IL-27. Naive CD4⁺ T cells were primed with plate-coated anti-CD3 and either IL-27 or IL-12 for 48 h, then restimulated with IL-27 or IL-12, and analyzed for phosphorylation of p38 MAPK and ERK1/2 by Western blotting (Fig. 2A). IL-27 induced phosphorylation of both p38 MAPK and ERK1/2, whereas IL-12 induced that of p38 MAPK, but not ERK1/2, as reported previously (23). Then the effect of their inhibitors on IL-27-induced Th1 differentiation was examined. Wild-type naive CD4⁺ T cells were pretreated with SB203580 or PD98059 for 1 h, then primed with plate-coated anti-CD3/anti-CD28 and IL-27, expanded with IL-2 on day 3, restimulated with plate-coated anti-CD3 for 24 h on day 6, and analyzed for IFN-γ production in culture supernatants by ELISA (Fig. 2B). Pretreatment with either SB203580 or PD98059 partially, but dose dependently inhibited IL-27-induced Th1 differentiation to the level that corresponds to ~50% of that without inhibitors. In contrast, pretreatment with SB203580, but not PD98059, inhibited IL-12-induced Th1 differentiation, as reported previously (23). Moreover, the combined pretreatment with SB203580 and PD98059 completely inhibited IL-27-induced Th1 differentiation (Fig. 2C). These results suggest that IL-27 activates p38 MAPK and ERK1/2, and that IL-27-induced Th1 differentiation is mediated by both independently.

**T-bet-dependent Th1 differentiation by IL-27 is mediated by p38 MAPK, but not ERK1/2**

Next, we investigated the role of these two MAPKs in T-bet-dependent Th1 differentiation induced by IL-27. We first explored the effect of MAPK inhibitors on augmentation of T-bet expression by IL-27. Wild-type naive CD4⁺ T cells were pretreated with SB203580 or PD98059 for 1 h, stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 for 48 h, and analyzed for T-bet expression by Western blotting (Fig. 3A). IL-27- or IL-12-induced augmentation of T-bet expression was greatly suppressed by SB203580, but not PD98059. We then examined the effect of lack of T-bet expression on activation of MAPKs by IL-27. Wild-type and T-bet-deficient naive CD4⁺ T cells were primed with plate-coated anti-CD3 and either IL-27 or IL-12 for 48 h, then restimulated with IL-27 or IL-12 for 20 min, and analyzed for phosphorylation of p38 MAPK and ERK1/2 by Western blotting (Fig. 3B). Lack of T-bet expression did not affect activation of p38 MAPK and ERK1/2 induced by IL-27 and also activation of p38 MAPK by IL-12. Furthermore, the effect of MAPK inhibitors on IL-27-induced Th1 differentiation from wild-type and T-bet-deficient naive CD4⁺ T cells was examined (Fig. 3C). IL-27-induced Th1...
explored the effect of MAPK inhibitors on ICAM-1 expression LFA-1-dependent Th1 differentiation induced by IL-27. We first investigated the role of these two MAPKs in ICAM-1/LFA-1-dependent Th1 differentiation by IL-27 is mediated by p38 MAPK, but not ERK1/2, as shown in Fig. 1A. Notably, PD98059, but not SB203580, further suppressed the Th1 differentiation. These results suggest that p38 MAPK is located upstream of T-bet, and that T-bet-dependent Th1 differentiation by IL-27 is mediated by p38 MAPK, but not ERK1/2.

ICAM-1/LFA-1-dependent Th1 differentiation by IL-27 is mediated by ERK1/2, but not p38 MAPK

Next, we investigated the role of these two MAPKs in ICAM-1/LFA-1-dependent Th1 differentiation induced by IL-27. We first explored the effect of MAPK inhibitors on ICAM-1 expression up-regulated by IL-27. Wild-type naive CD4+ T cells were pretreated with SB203580 or PD98059 (10 μM each) for 1 h, and stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 under Th1-polarizing conditions without IL-12 for 48 h. Total cell lysates were then prepared and subjected to Western blotting using anti-T-bet and anti-actin. B, T-bet is not required for IL-27-induced activation of p38 MAPK and ERK1/2 and for IL-12-induced activation of p38 MAPK. Wild-type and T-bet-deficient naive CD4+ T cells were primed, restimulated, and analyzed for phosphorylation of p38 MAPK and ERK1/2 by Western blotting, as described in Fig. 2A. C, IL-27-induced Th1 differentiation in T-bet-deficient naive CD4+ T cells is further inhibited by PD98059, but not SB203580. Wild-type and T-bet-deficient naive CD4+ T cells were pretreated with SB203580 or PD98059 (10 μM each) for 1 h, primed, expanded, restimulated, and analyzed for IFN-γ production by ELISA, as described in Fig. 2C. Data are shown as means ± SD. * Indicates p < 0.05. Similar results were obtained in three independent experiments.

ICAM-1/LFA-1-dependent Th1 differentiation by IL-27 is mediated by ERK1/2, but not p38 MAPK

FIGURE 3. T-bet-dependent Th1 differentiation by IL-27 is mediated by p38 MAPK, but not ERK1/2. A, IL-27- or IL-12-induced augmentation of T-bet expression is inhibited by SB203580, but not PD98059. Wild-type naive CD4+ T cells were pretreated with SB203580 or PD98059 (10 μM each) for 1 h, and stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 under Th1-polarizing conditions without IL-12 for 48 h. Total cell lysates were then prepared and subjected to Western blotting using anti-T-bet and anti-actin. B, T-bet is not required for IL-27-induced activation of p38 MAPK and ERK1/2 and for IL-12-induced activation of p38 MAPK. Wild-type and T-bet-deficient naive CD4+ T cells were primed, restimulated, and analyzed for phosphorylation of p38 MAPK and ERK1/2 by Western blotting, as described in Fig. 2A. C, IL-27-induced Th1 differentiation in T-bet-deficient naive CD4+ T cells is further inhibited by PD98059, but not SB203580. Wild-type and T-bet-deficient naive CD4+ T cells were pretreated with SB203580 or PD98059 (10 μM each) for 1 h, primed, expanded, restimulated, and analyzed for IFN-γ production by ELISA, as described in Fig. 2C. Data are shown as means ± SD. * Indicates p < 0.05. Similar results were obtained in three independent experiments.

FIGURE 4. ICAM-1/LFA-1-dependent Th1 differentiation by IL-27 is mediated by ERK1/2, but not p38 MAPK. A, Activation of p38 MAPK and ERK1/2 is not required for IL-27-induced up-regulation of ICAM-1 expression. Wild-type naive CD4+ T cells were pretreated with SB203580 or PD98059 (10 μM each) for 1 h, stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 under Th1-polarizing conditions without IL-12 for 16 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). MFI of each histogram is shown as the mean ± SD of three independent experiments (B). C, ICAM-1/LFA-1 is required for IL-27-induced activation of ERK1/2, but not p38 MAPK. Wild-type naive CD4+ T cells were primed with plate-coated anti-CD3 and either IL-27 or IL-12 for 48 h in the presence or absence of anti-ICAM-1/anti-LFA-1 (10 μg/ml each) or after pretreatment with SB203580 or PD98059 (10 μM each), restimulated, and analyzed for phosphorylation of p38 MAPK and ERK1/2 by Western blotting, as described in Fig. 2A. D, IL-27-induced Th1 differentiation in the presence of anti-ICAM-1/anti-LFA-1 is further inhibited by SB203580, but not PD98059. Wild-type naive CD4+ T cells were pretreated with SB203580 or PD98059 (10 μM each) for 1 h, primed in the presence or absence of anti-ICAM-1/anti-LFA-1 (10 μg/ml each), expanded, restimulated, and analyzed for IFN-γ production by ELISA, as described in Fig. 2C. Data are shown as means ± SD. ** Indicates p < 0.01. Similar results were obtained in three independent experiments.

ICAM-1/anti-LFA-1 greatly suppressed IL-27-induced activation of ERK1/2, but not p38 MAPK. Consistent with these results, we also confirmed that ICAM-1/Fc chimera induced phosphorylation of ERK1/2, but not p38 MAPK in naive CD4+ cells stimulated with plate-coated anti-CD3 (data not shown). Furthermore, the effect of
STAT1-deficient naive CD4 T cells were pretreated with SB203580 or PD98059 (10 μM each) for 1 h and primed with plate-coated anti-CD3/anti-CD28 for 16 h. After washing, these cells were stimulated with IL-27 for 20 min. Total cell lysates were then prepared and subjected to Western blotting using anti-phosphotyrosine STAT1. Each blot was stripped and reprobed with anti-total STAT1 to control for loading. B, IL-27-induced activation of ERK1/2, but not p38 MAPK is abolished in STAT1-deficient naive CD4 T cells. Wild-type and STAT1-deficient naive CD4 T cells were primed, restimulated, and analyzed for phosphorylation of p38 MAPK and ERK1/2 by Western blotting, as described in Fig. 2A. Similar results were obtained in three independent experiments.

SB203580 or PD98059 on IL-27-induced Th1 differentiation from naive wild-type CD4 T cells in the presence or absence of anti-ICAM-1/anti-LFA-1 was examined (Fig. 4D). Blocking by anti-ICAM-1/anti-LFA-1 partially (~50%) suppressed IL-27-induced Th1 differentiation, as shown in Fig. 1D. Notably, SB203580, but not PD98059, further inhibited the Th1 differentiation to a level similar to that without IL-27. These results suggest that ERK1/2 is located downstream of ICAM-1/LFA-1, and that ICAM-1/LFA-1-dependent Th1 differentiation by IL-27 is mediated by ERK1/2, but not p38 MAPK.

STAT1 is important for IL-27-induced activation of ERK1/2, but not p38 MAPK

We previously demonstrated that STAT1 is important for IL-27 signaling to induce T-bet and ICAM-1 expression and subsequent Th1 differentiation (6, 13). Therefore, we next investigated the role of STAT1 in the activation of p38 MAPK and ERK1/2 by IL-27. Wild-type naive CD4 T cells were pretreated with SB203580 or PD98059 for 1 h, stimulated with plate-coated anti-CD3 for 16 h, restimulated with IL-27 for 20 min, and analyzed for tyrosine phosphorylation of STAT1 (Fig. 5A). Pretreatment with SB203580 or PD98059 did not affect tyrosine phosphorylation of STAT1. Analysis for phosphorylation of STAT1 (Fig. 5A). Pretreatment with SB203580 or PD98059 did not affect tyrosine phosphorylation of STAT1. Then wild-type and STAT1-deficient naive CD4 T cells were stimulated with plate-coated anti-CD3 and either IL-27 or IL-12 for 48 h, and analyzed for tyrosine phosphorylation of STAT1. Then wild-type and STAT1-deficient naive CD4 T cells were stimulated with plate-coated anti-CD3 and either IL-27 or IL-12 for 48 h, and analyzed for tyrosine phosphorylation of STAT1.

PD98059, further inhibited the Th1 differentiation to a level similar to that without IL-27. These results suggest that ERK1/2 is located downstream of ICAM-1/LFA-1, and that ICAM-1/LFA-1-dependent Th1 differentiation by IL-27 is mediated by ERK1/2, but not p38 MAPK.

STAT1 is important for IL-27-induced activation of ERK1/2, but not p38 MAPK

FIGURE 5. STAT1 is important for IL-27-induced activation of ERK1/2, but not p38 MAPK. A, STAT1 is not located downstream of p38 MAPK and ERK1/2. Wild-type naive CD4+ T cells were pretreated with SB203580 or PD98059 (10 μM each) for 1 h and primed with plate-coated anti-CD3/anti-CD28 for 16 h. After washing, these cells were stimulated with IL-27 for 20 min. Total cell lysates were then prepared and subjected to Western blotting using anti-phosphotyrosine STAT1. Each blot was stripped and reprobed with anti-total STAT1 to control for loading. B, IL-27-induced activation of ERK1/2, but not p38 MAPK is abolished in STAT1-deficient naive CD4+ T cells. Wild-type and STAT1-deficient naive CD4+ T cells were primed, restimulated, and analyzed for phosphorylation of p38 MAPK and ERK1/2 by Western blotting, as described in Fig. 2A. Similar results were obtained in three independent experiments.

FIGURE 6. STAT4 is not required for IL-27-induced activation of p38 MAPK and ERK1/2 and up-regulation of ICAM-1 expression. A, STAT4 is not necessary for IL-27-induced activation of p38 MAPK and ERK1/2. Wild-type and STAT4-deficient naive CD4+ T cells were primed, restimulated, and analyzed for phosphorylation of p38 MAPK and ERK1/2 by Western blotting, as described in Fig. 2A. Similar results were obtained in three independent experiments. B, STAT4 is not necessary for IL-27-induced rapid up-regulation of ICAM-1 expression. Wild-type and STAT4-deficient naive CD4+ T cells were stimulated with plate-coated anti-CD3/anti-CD28 in the presence or absence of IL-27 under Th1-polarizing conditions without IL-12 for 16 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). MFI of each histogram is shown as the mean ± SD of three independent experiments (C).

FIGURE 7. IL-27 directly induces GADD45 expression, which presumably mediates activation of p38 MAPK. A, IL-27 induces GADD45 expression independently of p38 MAPK, ERK1/2, and ICAM-1/LFA-1. Naive CD4+ T cells were stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 under Th1-polarizing conditions without IL-12 in the presence or absence of anti-ICAM-1/anti-LFA-1 (10 μg/ml each) or after pretreatment with SB203580 or PD98059 (10 μM each) for 1 h. Forty-eight hours later, total RNA was prepared and subjected to RT-PCR analysis for GADD45, GADD45, and hypoxanthine phosphoribosyl transferase mRNA expression. B–D, STAT1, STAT4, and T-bet are not required for IL-27-induced enhancement of GADD45 expression. Wild-type and STAT1-, STAT4-, and T-bet-deficient naive CD4+ T cells were stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 under Th1-polarizing conditions without IL-12 for 48 h, and total RNA was prepared and subjected to RT-PCR analysis, as described above. Similar results were obtained in three independent experiments.

GADD45 expression, which presumably mediates activation of p38 MAPK. A, IL-27 induces GADD45 expression independently of p38 MAPK, ERK1/2, and ICAM-1/LFA-1. Naive CD4+ T cells were stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 under Th1-polarizing conditions without IL-12 in the presence or absence of anti-ICAM-1/anti-LFA-1 (10 μg/ml each) or after pretreatment with SB203580 or PD98059 (10 μM each) for 1 h. Forty-eight hours later, total RNA was prepared and subjected to RT-PCR analysis for GADD45, GADD45, and hypoxanthine phosphoribosyl transferase mRNA expression. B–D, STAT1, STAT4, and T-bet are not required for IL-27-induced enhancement of GADD45 expression. Wild-type and STAT1-, STAT4-, and T-bet-deficient naive CD4+ T cells were stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 under Th1-polarizing conditions without IL-12 for 48 h, and total RNA was prepared and subjected to RT-PCR analysis, as described above. Similar results were obtained in three independent experiments.

We previously demonstrated that STAT1 is important for IL-27 signaling to induce T-bet and ICAM-1 expression and subsequent Th1 differentiation (6, 13). Therefore, we next investigated the role of STAT1 in the activation of p38 MAPK and ERK1/2 by IL-27. Wild-type naive CD4+ T cells were pretreated with SB203580 or PD98059 for 1 h, stimulated with plate-coated anti-CD3 for 16 h, restimulated with IL-27 for 20 min, and analyzed for tyrosine phosphorylation of STAT1 (Fig. 5A). Pretreatment with SB203580 or PD98059 did not affect tyrosine phosphorylation of STAT1. Then wild-type and STAT1-deficient naive CD4+ T cells were stimulated with plate-coated anti-CD3 and either IL-27 or IL-12 for 48 h, and then restimulated with IL-27 or IL-12 for 20 min, and analyzed for phosphorylation of p38 MAPK and ERK1/2 by Western blotting (Fig. 5B). In STAT1-deficient naive CD4+ T cells, activation of ERK1/2 by IL-27 was abolished, whereas that of p38 MAPK was still observed. These results suggest that STAT1 is important for IL-27-induced activation of ERK1/2, but not p38 MAPK.
STAT4 is not required for IL-27-induced activation of p38 MAPK and ERK1/2 and up-regulation of ICAM-1 expression

STAT4 is required for IL-12-induced Th1 differentiation (16, 24, 25). It was reported previously that IL-27 also induces tyrosine phosphorylation of STAT4 (26). However, we have demonstrated recently that STAT4 is not necessary for IL-27-induced up-regulation of T-bet and also for IL-27-induced Th1 differentiation (13). Therefore, we further investigated the role of STAT4 in IL-27-induced activation of p38 MAPK and ERK1/2 and up-regulation of ICAM-1 expression. Wild-type and STAT4-deficient naive CD4+ T cells were primed with plate-coated anti-CD3/anti-CD28 and IL-27 under Th1-polarizing conditions without IL-12 in the presence or absence of anti-LFA-1 (10 μg/ml) for 16 and 48 h, and analyzed for expression of ICAM-1 and IL-12Rβ2 by FACS. Numbers in the panels indicate percentages of cells in each quadrant. Percentage of ICAM-1+IL-12Rβ2+ cells after the stimulation for 48 h of each dot plot is shown as the mean ± SD of three independent experiments (B). *, and **, indicate p < 0.05 and p < 0.01, respectively. C, p38 MAPK and ERK1/2 are not essential for IL-27-induced up-regulation of IL-12Rβ2 expression. Wild-type and T-bet-deficient naive CD4+ T cells were pretreated with SB203580 or PD98059 (10 μM each) for 1 h, stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 for 48 h, and analyzed for cell surface expression of IL-12Rβ2 in triplicate by FACS using anti-IL-12Rβ2 (solid line) and control hamster IgG (plain line with shading). Numbers represent the MFI of each histogram. MFI of each histogram is shown as the mean ± SD (D). Similar results were obtained in three independent experiments.

IL-27 directly induces GADD45γ expression, which presumably mediates activation of p38 MAPK

It was demonstrated previously that GADD45β expression in naive CD4+ T cells activates p38 MAPK and selectively increases cytokine-induced IFN-γ production (27). It was also reported that GADD45γ mediates activation of the p38 MAPK pathway to increase IFN-γ production in effector Th1 cells (28). Because IL-27 activated p38 MAPK even in STAT1-deficient naive CD4+ T cells (Fig. 5B), we next investigated whether IL-27 can induce expression of GADD45β and/or GADD45γ. Wild-type naive CD4+ T cells were stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 for 48 h, and analyzed for mRNA expression of GADD45β and GADD45γ by RT-PCR (Fig. 7A). IL-27 enhanced mRNA expression of GADD45γ, but not GADD45β, the expression of which appears to be regulated by signaling through TCR, as reported (28, 29). IL-27-induced enhancement of GADD45γ expression was not inhibited by SB203580, PD98059, or anti-ICAM-1/anti-LFA-1. Then the role of STAT1, STAT4, and T-bet in IL-27-induced enhancement of GADD45γ expression was examined using respective deficient mice. Enhancement of GADD45γ expression by IL-27 was still observed in naive CD4+ T cells from all these deficient mice (Fig. 7, B–D). These results suggest that IL-27 directly induces GADD45γ expression, independently of p38 MAPK, ERK1/2, ICAM-1/LFA-1, STAT1, STAT4, and T-bet.
IL-27-induced up-regulation of IL-12Rβ2 expression is mediated by both T-bet- and ICAM-1/LFA-1-dependent mechanisms

IL-27 induces expression of T-bet and subsequent IL-12Rβ2 on naive CD4+ T cells, resulting in synergistic IFN-γ production with IL-12 (3, 6). In addition, IL-27 rapidly up-regulates ICAM-1 expression on naive CD4+ T cells (13). Therefore, we finally investigated the interaction among ICAM-1, T-bet, and IL-12Rβ2. Wild-type and T-bet-deficient naive CD4+ T cells were stimulated with plate-coated anti-CD3/anti-CD28 and IL-27 in the presence or absence of anti-LFA-1 for 16 and 48 h, and analyzed for expression of ICAM-1 and IL-12Rβ2 by FACS (Fig. 8, A and B). Sixteen hours after the stimulation with IL-27, ICAM-1 expression was rapidly up-regulated on naive CD4+ T cells regardless of the presence or absence of T-bet expression, whereas IL-12Rβ2 expression was not increased. Forty-eight hours later, IL-12Rβ2 expression was also increased on wild-type naive CD4+ T cells. In contrast, lack of T-bet expression or blocking by anti-LFA-1 reduced the IL-12Rβ2 expression to a level corresponding to ~50% of that on wild-type naive CD4+ T cells without treatment by anti-LFA-1. Notably, both lack of T-bet expression and blocking by anti-LFA-1 almost completely inhibited the up-regulation of IL-12Rβ2 expression. Moreover, pretreatment with SB203580 or PD98059 did not affect IL-27-induced up-regulation of IL-12Rβ2 expression on wild-type and T-bet-deficient naive CD4+ T cells (Fig. 8, C and D). These results suggest that IL-27-induced up-regulation of IL-12Rβ2 expression is mediated by both T-bet- and ICAM-1/LFA-1-dependent mechanisms, and that p38 MAPK and ERK1/2 are not essential for it.

Discussion

In this study, we demonstrated that IL-27 induces Th1 differentiation via two distinct pathways, p38 MAPK/T-bet- and ICAM-1/LFA-1-dependent pathways, as schematically shown in Fig. 9. Ligation of IL-27R by IL-27 immediately activates STAT1, which mediates rapid up-regulation of ICAM-1 and thereafter augmentation of T-bet expression on naive CD4+ T cells (6, 13). Independently of STAT1, IL-27 also induces GADD45γ expression, which presumably mediates p38 MAPK activation (28), followed by augmentation of T-bet expression. ICAM-1 then ligates LFA-1 on CD4+ T cells, resulting in activation of ERK1/2 and consequent Th1 differentiation. Notably, ICAM-1/LFA-1 interaction, as well as T-bet, cooperatively enhances IL-12Rβ2 expression, leading to Th1 differentiation. Thus, IL-27 activates two MAPKs, p38 MAPK and ERK1/2, which play critical roles in the T-bet- and ICAM-1/LFA-1-dependent pathways, respectively. Although IL-27 activates STAT1-5 (3–6), STAT1 is critically important for IL-27 signaling, in particular, leading to up-regulation of ICAM-1 and T-bet expression on naive CD4+ T cells and consequent Th1 differentiation (6, 13). However, augmentation of T-bet expression by IL-27 appears to be also regulated by the GADD45γ/p38 MAPK pathway, independently of STAT1. IL-27 induced GADD45γ expression and activation of p38 MAPK even in STAT1-deficient naive CD4+ T cells. Moreover, p38 MAPK inhibitor, SB203580, greatly inhibited IL-27-induced augmentation of T-bet expression. These results imply that both signals mediated by STAT1 and p38 MAPK may be required for IL-27-induced augmentation of T-bet expression. In contrast, in T-bet-deficient naive CD4+ T cells or in the presence of blocking Ab against LFA-1, the up-regulation of IL-12Rβ2 expression by IL-27 was reduced, but was still significantly present. These results suggest that either T-bet or ICAM-1/LFA-1 may be necessary for up-regulation of IL-12Rβ2 expression by IL-27. In contrast, IL-12 induces Th1 differentiation via only the p38 MAPK/T-bet-dependent

FIGURE 9. IL-27 signaling pathways leading to Th1 differentiation. When naive CD4+ T cells are stimulated with IL-27, the following two molecular events are initiated: one is activation of STAT1, and the other is induction of GADD45γ expression, which is also induced by IL-12/STAT4 signaling. Activation of STAT1 leads to not only augmentation of T-bet expression, but also up-regulation of ICAM-1 expression in T-bet-independent manner. ICAM-1 interacts with LFA-1 on the adjoining CD4+ T cells, resulting in ERK1/2 activation and differentiation into effector Th1 cells. In contrast, GADD45γ activates p38 MAPK and subsequently T-bet, which directly enhances IFN-γ production. T-bet also up-regulates IL-12Rβ2 expression, which contributes to Th1 differentiation through IL-12/STAT4 signaling. Furthermore, we found that IL-27 also augments IL-12Rβ2 expression through ICAM-1/LFA-1 signaling in T-bet-independent manner as an alternative pathway. Thus, IL-27 activates two MAPKs, p38 MAPK and ERK1/2, for Th1 differentiation by multiple pathways as compared with IL-12, and the p38 MAPK/T-bet pathway appears to be shared by IL-27 and IL-12.
expression, as shown in this study, it could be conceivable that IL-27 may play a role in generating IFN-γ-producing Ag-specific CD4+ T cells for the resistance to L. monocytogenes and M. tuberculosis infection in T-bet-deficient mice.

In vitro study also showed that T-bet is not always necessary for IFN-γ production in CD4+ T cells differentiated into Th1 cells. Usui et al. (35) reported that CD4+ T cells from T-bet-deficient mice still produce IFN-γ if both IL-12Rβ2 and STAT4 are stably expressed in the CD4+ T cells. The present study showed that IL-27 up-regulates IL-12Rβ2 expression via ICAM-1/LFA-1 signaling even in the absence of T-bet (Fig. 8, A and B), suggesting that T-bet is not essential for IL-12-mediated Th1 differentiation in the presence of IL-27. Moreover, we demonstrated that IL-27 itself induces Th1 differentiation of CD4+ T cells from T-bet-deficient mice through STAT1/ICAM-1/LFA-1 signaling (Fig. 3C), followed by ERK1/2 activation (Figs. 4 and 5). These results suggest that IL-27/STAT1 is an alternative signaling pathway leading to Th1 differentiation in addition to IL-12/STAT4.

Previous studies using mice lacking one of the IL-27R subunits, TCCR (8)/WSX-1 (9), revealed that IL-27 is required for the early initiation of Th1 responses, and that WSX-1/TCCR-deficient mice have enhanced susceptibility to infection with intracellular pathogens such as L. major (9, 10) and L. monocytogenes (8). In contrast, WSX-1 is not essential to develop the protective Th1 responses against T. gondii parasites, and rather acts to attenuate the inflammatory responses induced by protozoan infection, including cellular hyperactivation and overproduction of proinflammatory cytokines (5). One of the key differences between infection with L. major or L. monocytogenes and that with T. gondii might be considered to be the induction level of IL-12, a principal mediator of strong type 1 immunity (36). T. gondii promotes strong innate immune responses that lead to high IL-12 levels early during infection, whereas acute L. major induces much less IL-12 production (37). Consistent with these observations, we previously demonstrated that the ability of IL-27 to induce Th1 differentiation is most pronounced under Th1-polarizing conditions, but without IL-12 and overruled by IL-12 dose dependently (13). The IL-27-induced Th1 differentiation is partially mediated by rapid and marked up-regulation of ICAM-1 expression on naive CD4+ T cells through ICAM-1/LFA-1 interaction in STAT1-dependent, but T-bet-, IFN-γ-, and STAT4-independent mechanism. In the present study, we further clarified the signaling molecules leading to Th1 differentiation induced by IL-27, and demonstrated that IL-27 uses more complicated mechanisms than IL-12. Namely, IL-27 activates both p38 MAPK/T-bet and ICAM-1/LFA-1/ERK1/2 pathways, whereas IL-12 activates only p38 MAPK/T-bet pathway. Although these results may imply that IL-27 has a greater effect on Th1 differentiation than IL-12, comparison of phenotypical analyses of IL-12-deficient mice and WSX-1/TCCR-deficient mice clearly reveals that IL-12 is the Th1-driving cytokine (9, 38). This difference could be explained by the growing evidences that IL-27 possesses not only proinflammatory property, but also anti-inflammatory property, although the molecular mechanisms remain unknown (12, 39). We recently demonstrated that IL-27 plays an important role to suppress excessive progression of CD28-mediated IL-2 production and IL-2 responses by inducing suppressor of cytokine signaling (SOCS3) expression in a negative feedback mechanism (40). Because SOCS3 was reported to inhibit IL-12-mediated Th1 differentiation through STAT4 activation as well (41), IL-27 may play a role in inhibition of Th1 differentiation induced by IL-12 through SOCS3, and therefore, synergistic induction of Th1 differentiation by IL-12 and IL-27 cannot be seen (13). These possibilities are currently under investigation.

Taken together, it is highly conceivable that IL-12 dominantly regulates Th1 differentiation through p38 MAPK/T-bet pathway under the circumstances that IL-12 is abundantly produced and the IL-12 signaling pathway is intact. However, when IL-12 production is limited or the IL-12 signaling pathway is impaired by infection with certain pathogens, the IL-27-mediated alternative signaling pathway leading to Th1 differentiation may emerge and compensate for it.

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Disclosures

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References


