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A Role for IL-27 in Early Regulation of Th1 Differentiation¹

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IL-27 is a novel IL-6/IL-12 family cytokine that is considered to play a role in Th1 differentiation, whereas the exact role of IL-27 in Th1 differentiation and its molecular mechanism remain unclear. In this study we demonstrate a role for IL-27 in the early regulation of Th1 differentiation and its possible molecular mechanism. The ability of IL-27 to induce Th1 differentiation was most prominent under Th1-polarizing conditions, but without IL-12 in a STAT4- and IFN- γ -independent manner, and was overruled by IL-12 dose dependently. IL-27 rapidly up-regulated the expression of ICAM-1 on naive CD4⁺ T cells, but not on APCs, and blocking Abs against ICAM-1 and LFA-1 inhibited the IL-27-induced Th1 differentiation. Although IL-27 augmented T-bet expression in naive CD4⁺ T cells as previously reported, T-bet was not necessary for the IL-27-induced rapid up-regulation of ICAM-1 expression and Th1 differentiation. In contrast, STAT1 was revealed to be required for the rapid up-regulation of ICAM-1 expression and Th1 differentiation by directly mediating the transcriptional enhancement of ICAM-1 gene expression. These results indicate that IL-27 efficiently induces Th1 differentiation under Th1-polarizing conditions, but without IL-12, and that the rapid up-regulation of ICAM-1 expression on naive CD4⁺ T cells is important for the IL-27-induced Th1 differentiation. Considering that IL-27 is produced from macrophages and DCs earlier than IL-12, the present results suggest that IL-27 may play a pivotal role in early efficient induction of Th1 differentiation until sufficient IL-12 is produced. *The Journal of Immunology*, 2005, 175: 2191–2200.

Interleukin-27, a novel member of the IL-6/IL-12 family, is a heterodimeric cytokine that consists of an IL-12 p40-related protein, EBV-induced gene 3, and a newly discovered IL-12 p35-related protein, p28 (1). The orphan cytokine receptor WSX-1/T cell cytokine receptor (TCCR),³ which is homologous to the IL-12R β 2 subunit, and gp130 constitute a functional signal-transducing receptor for IL-27 (1, 2). IL-27 activates JAK1, JAK2, TYK2, STAT1, STAT2, STAT3, STAT4, and STAT5 in naive CD4⁺ T cells (3–6) and induces proliferation in naive, but not memory, CD4⁺ T cells. IL-27 also induces the expression of T-bet, a master transcriptional regulator for Th1 differentiation (7), and subsequent IL-12R β 2 and synergizes with IL-12 in primary IFN- γ production (1, 3, 4, 6).

Previous studies of mice lacking one subunit of IL-27R, 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 *Leishmania major* (9, 10) and *Listeria monocytogenes* (8). However, WSX-1/TCCR is not essential to develop the protective Th1 responses against *Toxoplasma gondii* parasites (5). Moreover,

in vitro analyses of the effect of IL-27 on Th1/Th2 development revealed that IL-27 is not able to synergize with IL-12 to increase the production of IFN- γ by Th1 cells (11). Therefore, although IL-27 has been implicated in Th1 development, the exact role of IL-27 and its molecular mechanism remain unclear.

We have recently evaluated the adjuvant activity of single-chain IL-27 expression plasmid in the prime-boost immunization and elucidated that IL-27 has a potent adjuvant activity for the induction of hepatitis C virus-specific CTLs (12). We have also evaluated the antitumor activity of IL-27 against a murine tumor model of colon carcinoma, colon 26 (C26), by transducing with single-chain IL-27 cDNA and found that IL-27 has potent abilities to induce tumor-specific antitumor activity and protective immunity (13). These studies suggest that IL-27 can augment type 1 cell-mediated immunity in vivo. In the latter study we also noticed that the induction of antitumor activity by IL-27 is still observed in STAT4-deficient mice, suggesting that IL-12 may not be necessary for the induction of type 1 protective immunity by IL-27.

To better define the role of IL-27 in regulation of Th1/Th2 differentiation and its molecular mechanism, we examined the effect of IL-27 on in vitro Th1 differentiation in the presence and the absence of IL-12. We found that the ability of IL-27 to induce Th1 differentiation was most prominent under Th1-polarizing conditions, but without IL-12, and was overruled by IL-12 dose dependently. The IL-27-induced Th1 differentiation was mainly mediated by rapid and marked up-regulation of ICAM-1 expression on naive CD4⁺ T cells through ICAM-1/LFA-1 interaction in a STAT1-dependent, but a T-bet-, IFN- γ -, and STAT4-independent, mechanism.

Materials and Methods

Mice

BALB/c and C57BL/6 mice were purchased from Japan SLC. STAT4-deficient mice (14), IL-12 p40-deficient mice (15), and T-bet-deficient mice (16) of the BALB/c background were purchased from The Jackson Laboratory. IFN- γ -deficient mice were provided by Dr. Y. Iwakura (University of Tokyo, Tokyo, Japan). STAT1^{+/-} and STAT1^{-/-} mice (17) of

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³ Abbreviations used in this paper: TCCR, T cell cytokine receptor; C26, colon 26; GAS, IFN- γ -activating site.

a mixed background of 129/Sv and C57BL/6 were provided by Dr. R. D. Schreiber (Washington University, St. Louis, MO). All animal experiments were performed in accordance with our institutional guidelines.

Reagents

Anti-actin was purchased from Sigma-Aldrich. Anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-IL-4 (11B11), anti-IFN- γ (XMG1.2), anti-Thy1.2 (30-H12), and anti-LFA-1 (FD441.8) were obtained from American Type Culture Collection. Mouse rIL-4, anti-CD28 (37.51), FITC-anti-rat IgG, and PE-anti-CD4 (GK1.5) were obtained from BD Biosciences. Anti-ICAM-1 (YNI1/1.7.4) was purchased from eBioscience. Anti-STAT1, anti-STAT4, and anti-T-bet (4B10) were obtained from Santa Cruz Biotechnology. Mouse rIL-12 and mouse ICAM-1-Fc fusion protein were purchased from R&D Systems. Anti-IL-12 (C17.8) was provided by Dr. G. Trinchieri (Schering-Plough Research Institute, Dardilly, France). Human rIL-2 and mouse rIFN- γ were provided by Shionogi.

Preparation of C26 transfectants

C26 cells were transfected with single-chain IL-27 expression vector (C26-IL-27) or the empty vector (C26-Vector) using FuGene 6 (Roche) and were selected with geneticin (G418) as described previously (13). Mice were injected s.c. with C26-Vector or C26-IL-27 (2×10^5 cells), and tumor volume was monitored. Tumor volume was calculated using the following volume equation: $0.5(ab^2)$, where a is the long diameter, and b is the short diameter. After in vitro restimulation of spleen cells obtained from mice inoculated with C26 transfectants, by irradiated parental C26 cells, culture supernatants were analyzed for IFN- γ production by ELISA (13).

Preparation of purified rIL-27 protein

Recombinant IL-27 was prepared as a soluble tagged fusion protein by flexibly linking EBV-induced gene 3 to p28 as described previously (18).

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-CD8 α , anti-B220, anti-Mac-1, anti-Ter-119, and anti-DX5, followed by incubation with anti-biotin magnetic beads (Miltenyi Biotec) and was 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-CD62L magnetic beads (Miltenyi Biotec), and the positive fraction was collected as purified naive CD4⁺ T cells (CD62L⁺ cells > 99%).

Th1/Th2 differentiation assay

Naive CD4⁺ T cells (1×10^6 cells/ml) were primed with plate-coated anti-CD3 (2 μ g/ml) and anti-CD28 (0.5 μ g/ml) or soluble anti-CD3 (1 μ g/ml) and irradiated T/NK cell-depleted spleen cells (3×10^6 cells/ml). T/NK cell-depleted spleen cells were prepared as follows. Spleen cells depleted of erythrocytes were incubated with anti-Thy1.2, followed by incubation with anti-rat IgG magnetic beads (Miltenyi Biotec) together with anti-DX5 magnetic beads (Miltenyi Biotec) and passed through a magnetic cell sorting column. The negative fraction was used as the T/NK cell-depleted spleen cells. To induce Th1/Th2 differentiation, the above cultures were supplemented with 10 μ g/ml anti-IL-4 and 10 ng/ml IL-12 (Th1-polarizing conditions); 10 μ g/ml anti-IL-4, 10 μ g/ml anti-IL-12, and 10 μ g/ml anti-IFN- γ (neutral conditions); or 10 μ g/ml anti-IL-12, 10 μ g/ml anti-IFN- γ , and 10 ng/ml IL-4 (Th2-polarizing conditions). On day 3, cells were split 1:4 and expanded in human IL-2 (50 U/ml)-containing complete medium. On day 6, cells were collected, washed, and restimulated at 1×10^6 cell/ml with plate-coated anti-CD3 (2 μ g/ml). After 24 h, culture supernatants were harvested and assayed for production of IFN- γ and IL-4 by ELISA.

Western blotting

Cells were lysed in a lysis buffer containing protease inhibitors, and resultant cell lysates were separated by SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membrane (Millipore) as described previously (19). The membrane was 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.

Reporter gene assay

The 5'-flanking region of the mouse *ICAM-1* gene from nucleotide -1001 (numbered from the putative transcription initiation site) to +57 was ob-

tained from genomic DNA of C57BL/6 mouse liver by standard PCR techniques, confirmed by sequencing, and subcloned into a luciferase reporter plasmid pGL3 (Promega) (20–22). Mutated (GACCTCTTAGA at nucleotides -63 to -53) construct in the IFN- γ -activating site (GAS) (wild-type; TTTCCCGGAAA at nucleotide -63 to -53) of 5'-flanking region of *ICAM-1* gene was also prepared and subcloned into pGL3. T cell hybridoma 68-41 cells were transiently transfected by electroporation with these reporter plasmid constructs and pRL-TK (sea pansy (*Renilla reniformis*) luciferase expression plasmid under control of the thymidine kinase promoter) as an internal control. After ~40 h, these cells were stimulated with IL-27 for 6 h and harvested for measurement of activities of firefly and sea pansy luciferases using a Dual-Luciferase Reporter Assay System (Promega), following the manufacturer's instructions. Firefly luciferase activity in each sample was normalized by the corresponding sea pansy luciferase activity to correct transfection efficiency. Data are shown as normalized firefly luciferase activity.

EMSA

Oligonucleotide corresponding to the 5'-flanking sequence of mouse *ICAM-1* from nucleotide -72 (numbered from the putative transcription initiation site) to -43 (5'-GGCGCGAGGTTTCCCGGAAAAGTGGCCCGA-3'), which contains the GAS (-63 to -53; underlined) and its mutant (5'-GGCGCGAGGGACCTCTTAGAGTGGCCCCGA-3') (20–22), were labeled with ³²P. These radiolabeled probes were then incubated with 1 mg of the nuclear extract in 25 ml of binding buffer (10 mM HEPES (pH 8.0), 50 mM KCl, 1.25 mM MgCl₂, 0.1 mM EDTA, 0.5 mg of poly(deoxyinosinic-deoxycytidylic acid), 0.5 mM DTT, and 10% glycerol) at room temperature for 20 min. Sample were analyzed by 4.5% nondenaturing PAGE and were visualized with a BAS 2000 bioimaging analyzer (Fuji). For competition assays, 100-fold molar excess amounts of unlabeled wild-type oligonucleotide or its mutated oligonucleotide in the GAS were added to the mixture before incubation with the radiolabeled probe. For blocking experiments with Ab, nuclear extracts were preincubated with Abs (0.5 mg) against STAT1 or STAT4 as a control for 1 h on ice before addition of the radiolabeled probe.

Results

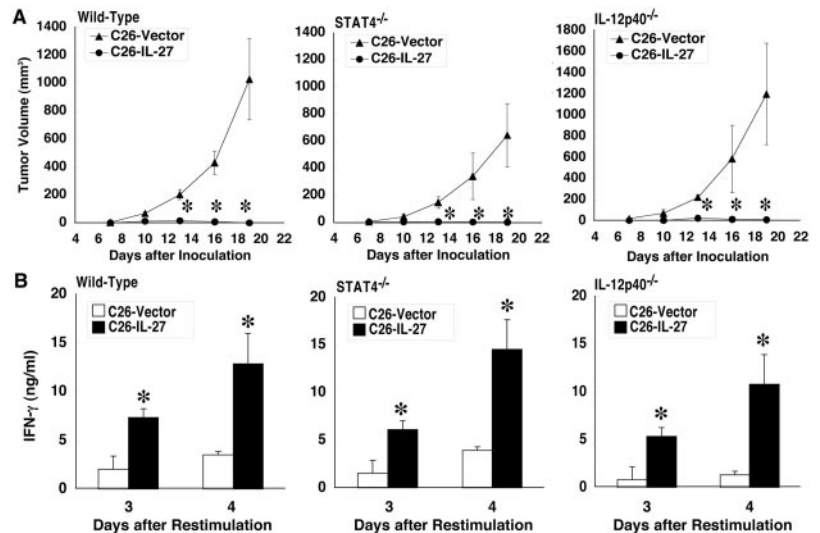
IL-12 is not essential to IL-27-induced type 1 cell-mediated immunity in vivo

To elucidate the role of IL-12 in IL-27-induced antitumor activity in vivo, we first of all examined whether IL-27 induces an antitumor activity even in IL-12 p40-deficient mice as in wild-type BALB/c and STAT4-deficient mice and enhances IFN- γ production as well. C26-Vector and C26-IL-27 were injected s.c. into these mice, and tumor volume was monitored. As in wild-type and STAT4-deficient mice, IL-27 still showed antitumor activity even in IL-12 p40-deficient mice (Fig. 1A). In addition, IL-27 significantly enhanced IFN- γ production from spleen cells restimulated in vitro with irradiated parental C26 in STAT4-deficient and IL-12 p40-deficient mice as in wild-type mice (Fig. 1B). These results suggest that IL-27 can induce an antitumor activity by augmenting type 1 cell-mediated immunity in vivo in an IL-12-independent manner.

IL-27 has an ability to induce in vitro Th1 differentiation in the absence of IL-12, but this Th1-inducing ability is overruled by IL-12

Therefore, we next examined the effect of IL-27 on in vitro Th1 differentiation with various concentrations of IL-12. Naive CD4⁺ T cells were primed with plate-coated anti-CD3 and anti-CD28 in the presence and the absence of IL-27 under Th1-polarizing conditions with various concentrations of IL-12 and also under neutral and Th2-polarizing conditions. Because IL-27 induces the expression of T-bet, a master transcriptional regulator for Th1 differentiation by directly activating Th1-associated genetic programs and repressing Th2 cytokine production (7, 16, 23), total cell lysates were prepared after culture for 48 h and subjected to Western blotting for detection of T-bet expression. IL-27 greatly enhanced T-bet expression under Th1-polarizing conditions regardless of the

FIGURE 1. IL-12 is not essential to IL-27-induced type 1 cell-mediated immunity in vivo. *A*, Inhibition of tumor growth by IL-27 even in STAT4- or IL-12 p40-deficient mice. Wild-type (BALB/c), STAT4-deficient, and IL-12 p40-deficient mice ($n = 3-5$) were injected s.c. with C26-Vector or C26-IL-27, and tumor volume was monitored. Data are shown as the mean \pm SD. *, $p < 0.05$ compared with C26-Vector. *B*, Enhanced IFN- γ production from spleen cells by IL-27 even in STAT4- or IL-12 p40-deficient mice. Three weeks after tumor inoculation, spleen cells from each mouse were restimulated with irradiated parental C26 cells for 3 and 4 days, and culture supernatants were analyzed for IFN- γ production by ELISA. Data are shown as the mean \pm SD. *, $p < 0.05$, compared with C26-Vector. Similar results were obtained in two independent experiments.



presence or the absence of IL-12 and also under neutral and Th2-polarizing conditions (Fig. 2A). Then, these primed CD4⁺ T cells were expanded and restimulated with plate-coated anti-CD3 on day 6 for 24 h, and culture supernatants were analyzed for IFN- γ and IL-4 production by ELISA. Consistent with recent results on the effect of IL-27 on in vitro Th1 differentiation (11), IL-27 failed to enhance Th1 differentiation under Th1-polarizing conditions with a high concentration of IL-12 (Fig. 2B). The lower concentration of IL-12 in the absence of IL-27 resulted in the lower production of IFN- γ from restimulated CD4⁺ T cells, that is, the lower efficiency to induce Th1 differentiation as expected. However, notably, the lower concentration of IL-12 led to the stronger Th1 polarization by IL-27. Even under neutral and Th2-polarizing conditions, IL-27 induced less efficient, but significant, Th1 differentiation. In contrast, IL-4 production from restimulated CD4⁺ T cells, that is, Th2 differentiation was not greatly affected by IL-27 regardless of the presence or the absence of IL-12. Under Th1-polarizing conditions without IL-12, IL-27 dose dependently induced T-bet expression (Fig. 2C) and Th1 differentiation (Fig. 2D). These results suggest that IL-27 induces in vitro Th1 differentiation most efficiently in the absence of IL-12 and that the presence of a higher concentration of IL-12 overrules the ability of IL-27 to induce Th1 differentiation.

STAT4 is not required for IL-27-induced Th1 differentiation

We then examined the requirement for STAT4, which is an essential signal-transducing molecule to IL-12/IL-12R signaling (14, 24), in IL-27-induced Th1 differentiation using STAT4-deficient naive CD4⁺ T cells. Regardless of the presence or the absence of STAT4, IL-27 greatly induced T-bet expression under Th1-polarizing, neutral, and Th2-polarizing conditions (data not shown). In contrast, IL-12 failed to induce T-bet expression in the absence of STAT4 as expected (data not shown). Correlating with T-bet expression, IL-27 was still able to induce Th1 differentiation even in the absence of STAT4, whereas IL-12 was not able to induce it (Fig. 2E). These results suggest that STAT4 is not required for IL-27-induced Th1 differentiation.

IFN- γ is not required for IL-27-induced Th1 differentiation

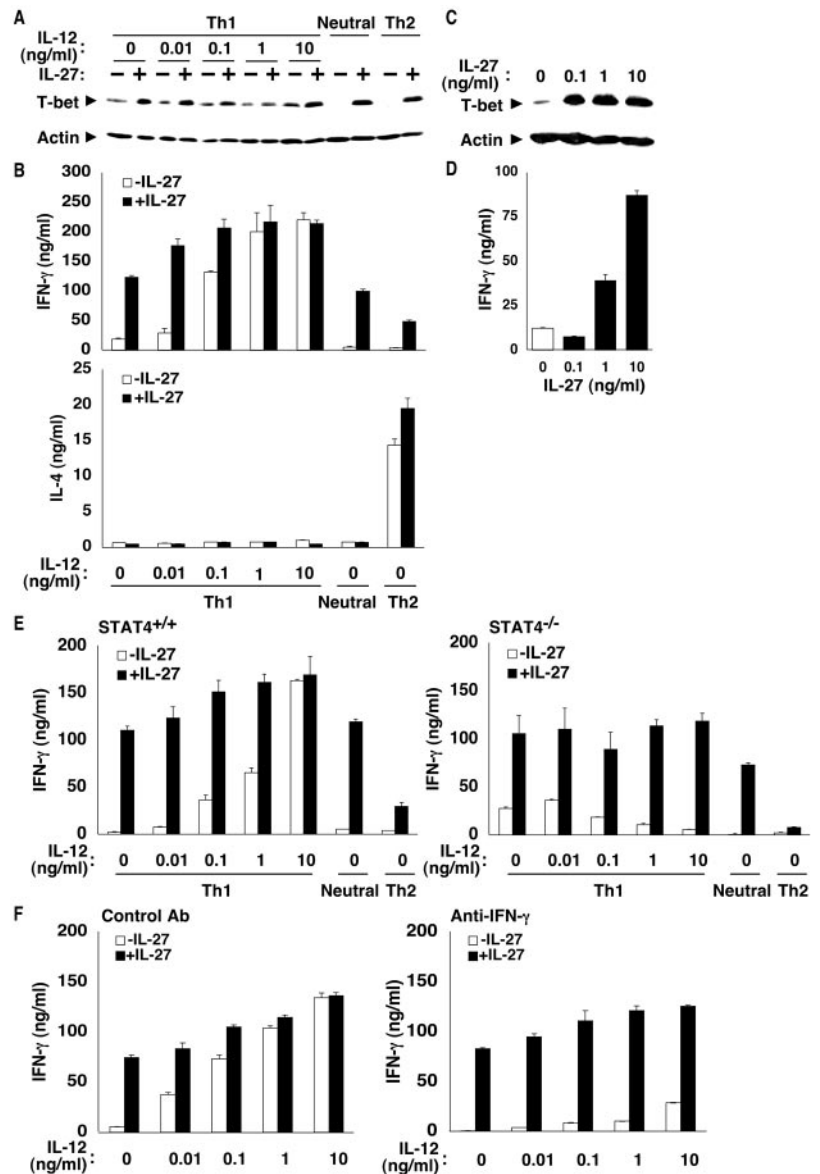
Because IL-27 synergizes with IL-12 in primary IFN- γ production (1, 3, 4, 6) and IFN- γ is well known to play a critical role in Th1 differentiation through induction of T-bet and subsequent IL-12R β 2 expression (25, 26), we next examined the requirement for

IFN- γ in IL-27-induced Th1 differentiation using IFN- γ -deficient naive CD4⁺ T cells and neutralizing mAb against IFN- γ . Regardless of the presence or the absence of IFN- γ , IL-27 greatly induced T-bet expression under Th1-polarizing, neutral, and Th2-polarizing conditions (data not shown). In contrast, IL-12 failed to induce T-bet expression in the absence of IFN- γ as expected (data not shown). Correlating with T-bet expression, IL-27 was able to induce Th1 differentiation even in the presence of neutralizing mAb against IFN- γ , whereas IL-12 was not able to induce it (Fig. 2F). These results suggest that IFN- γ is not required for IL-27-induced Th1 differentiation.

IL-27 induces rapid and marked up-regulation of ICAM-1 expression on naive CD4⁺ T cells stimulated with plate-coated anti-CD3 and anti-CD28, which is important for IL-27-induced Th1 differentiation

Several previous studies revealed that ICAM-1/LFA-1 interaction is important for Th1 polarization (27, 28) and that ICAM-1 expressed on T cells can provide a costimulatory signal for T cell activation and Th1 cytokine production (29). More recently, it has been demonstrated that ICAM-1/LFA-1 interaction favors human Th1 development, and this Th1 differentiation is overruled by IL-12 in a dose-dependent manner (30). Therefore, we next examined the cell surface expression of ICAM-1 and its counterpart, LFA-1, on naive CD4⁺ T cells. Naive CD4⁺ T cells were stimulated with plate-coated anti-CD3 and anti-CD28 in the presence or the absence of IL-27 under Th1-polarizing conditions without IL-12, and ICAM-1 and LFA-1 expressions were analyzed by FACS (Fig. 3A). ICAM-1 expression was rapidly and markedly induced in the presence of IL-27 at ~ 16 h after the stimulation, whereas IL-12 neither induced ICAM-1 expression nor affected the enhancement of ICAM-1 expression by IL-27. After 48 h, however, a comparable level of ICAM-1 expression was observed between the presence and the absence of IL-27. Thus, ICAM-1 expression appears to be more rapidly decreased in the presence of IL-27 than in its absence. In contrast, LFA-1 expression was up-regulated by the stimulation, but no marked difference in the LFA-1 expression level was observed between the presence and the absence of IL-27. To determine the role of ICAM-1 expression in IL-27-induced Th1 polarization, we next examined the effect of blocking mAbs against ICAM-1 and LFA-1 on IL-27-induced Th1 polarization (Fig. 3B). The ability of IL-27 to induce Th1 polarization was markedly inhibited by anti-ICAM-1 or anti-LFA-1 and was

FIGURE 2. IL-27 has the ability to induce in vitro Th1 differentiation in the absence of IL-12 with a STAT4- and IFN- γ -independent mechanism, but this Th1-inducing ability is overruled by IL-12. **A**, Augmentation of T-bet expression by IL-27. Naive CD4⁺ T cells from BALB/c mice were primed with plate-coated anti-CD3 and anti-CD28 in the presence and the absence of IL-27 under Th1-polarizing conditions with various concentrations of IL-12 and under neutral and Th2-polarizing conditions. After culture for 48 h, total cell lysates were prepared and subjected to Western blotting using anti-T-bet and anti-actin. **B**, Induction of Th1 differentiation by IL-27 in the absence of IL-12, which is overruled by IL-12. Primed CD4⁺ T cells, as described in **A**, were expanded and restimulated with plate-coated anti-CD3 on day 6 for 24 h, and culture supernatants were analyzed for IFN- γ and IL-4 production in triplicate by ELISA. Data are shown as the mean \pm SD. **C**, Dose-dependent augmentation of T-bet expression by IL-27. Naive CD4⁺ T cells were primed with plate-coated anti-CD3 and anti-CD28 in the presence of various concentrations of IL-27 under Th1-polarizing conditions without IL-12, and after 48 h, total cell lysates were prepared and subjected to Western blotting. **D**, Dose-dependent induction of Th1 differentiation by IL-27. Primed CD4⁺ T cells, prepared as described in **C**, were expanded, restimulated, and analyzed for IFN- γ production as described in **B**. **E**, STAT4 is not required for IL-27-induced Th1 differentiation. Wild-type (BALB/c) and STAT4-deficient naive CD4⁺ T cells were primed, expanded, restimulated, and analyzed for IFN- γ and IL-4 as described in **B**. **F**, IFN- γ is not required for IL-27-induced Th1 differentiation. Wild-type (C57BL/6) naive CD4⁺ T cells were primed with plate-coated anti-CD3 and anti-CD28 in the presence and the absence of IL-27 under Th1-polarizing conditions with various concentrations of IL-12 and anti-IFN- γ or control rat IgG (20 μ g/ml each). Primed CD4⁺ T cells were expanded, restimulated, and analyzed for IFN- γ and IL-4 production as described in **B**. Similar results were obtained in three independent experiments.



slightly but further inhibited by combined treatment with these mAbs, whereas these treatments did not induce Th2 polarization (data not shown). In contrast, these treatments did not affect IL-12-induced Th1 polarization regardless of the presence or the absence of IL-27. As reported previously (30), ligation of LFA-1 on naive CD4⁺ T cells by ICAM-1-Fc fusion protein induced Th1 polarization in a dose-dependent manner (Fig. 3C). These results suggest that IL-27 induces rapid and marked up-regulation of ICAM-1 expression on naive CD4⁺ T cells stimulated with plate-coated anti-CD3 and anti-CD28, which is important for IL-27-induced Th1 differentiation.

IL-27 induces Th1 differentiation from naive CD4⁺ T cells stimulated with soluble anti-CD3 and spleen APCs in the absence of IL-12, and rapid up-regulation of ICAM-1 expression by IL-27 plays an important role in IL-27-induced Th1 differentiation

We next examined the ability of IL-27 to induce Th1 polarization from naive CD4⁺ T cells stimulated with soluble anti-CD3 and irradiated T/NK cell-depleted spleen cells as APCs under Th1-polarizing conditions with and without IL-12 and under neutral

and Th2-polarizing conditions (Fig. 4A). As in naive CD4⁺ T cells stimulated with plate-coated anti-CD3 and anti-CD28, IL-27 efficiently induced Th1 polarization in the absence of IL-12, and the IL-27-induced Th1 differentiation was overruled by IL-12 (10 ng/ml). Similar, but less efficient, induction of Th1 differentiation was observed under neutral and Th2-polarizing conditions. Then naive CD4⁺ T cells were stimulated as described above but with non-irradiated T/NK cell-depleted spleen cells as APCs, and the effect of IL-27 on ICAM-1 expression on CD4⁺ T cells and T/NK cell-depleted spleen cells was analyzed by FACS (Fig. 4B). ICAM-1 expression on CD4⁺ T cells was up-regulated by stimulation with soluble anti-CD3 and spleen APCs, and IL-27 further greatly enhanced it. In contrast, on nonirradiated T/NK cell-depleted spleen cells, ICAM-1 expression was efficiently up-regulated by stimulation with soluble anti-CD3 and spleen APCs, and IL-27 minimally enhanced it. Similar results were obtained when irradiated T/NK cell-depleted spleen cells were used instead of nonirradiated cells, but the level of up-regulation of ICAM-1 expression on these irradiated cells was much less than that on nonirradiated cells (data not shown). Finally, the effect of blocking mAbs against ICAM-1 and LFA-1 on IL-27-induced Th1 differentiation was examined

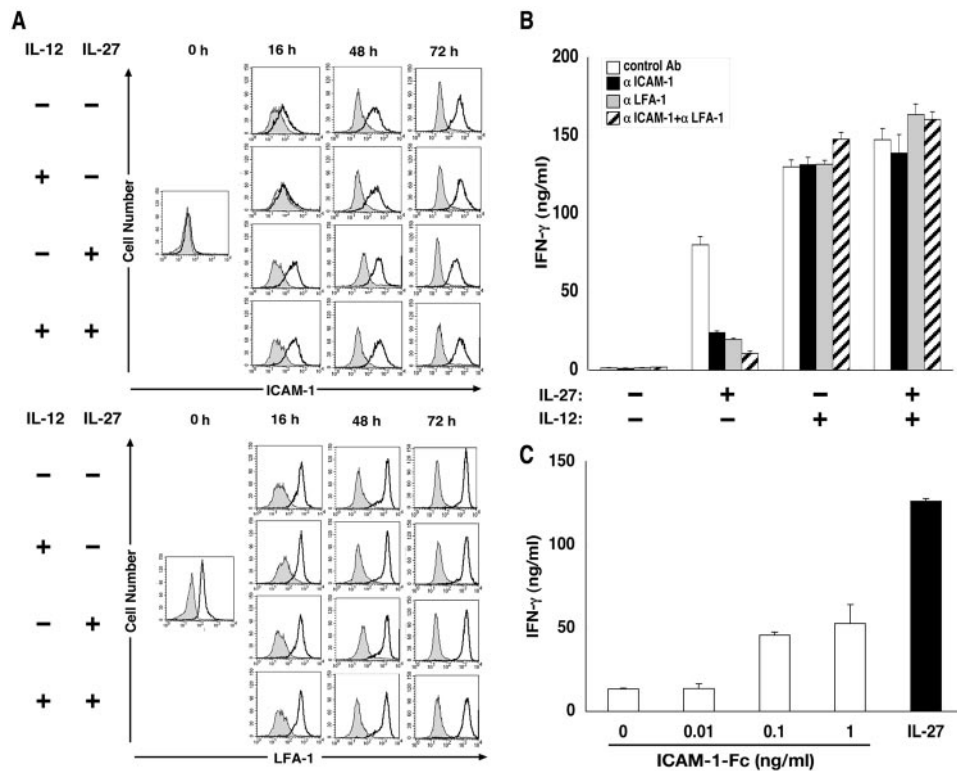


FIGURE 3. IL-27 induces rapid and marked up-regulation of ICAM-1 expression on naive CD4⁺ T cells stimulated with plate-coated anti-CD3 and anti-CD28, which is important for IL-27-induced Th1 differentiation. *A*, Rapid and marked up-regulation of ICAM-1 expression by IL-27. Wild-type (BALB/c) naive CD4⁺ T cells were primed with plate-coated anti-CD3 and anti-CD28 in the presence or the absence of IL-27 (10 ng/ml) under Th1-polarizing conditions without IL-12. After culture for various times, cells were analyzed for cell surface expression of ICAM-1 and LFA-1 by FACS using anti-ICAM-1 (solid line), anti-LFA-1 (solid line), and control rat IgG (plain line with shading). *B*, Inhibition of IL-27-induced Th1 differentiation by anti-ICAM-1 and anti-LFA-1. Naive CD4⁺ T cells were primed with plate-coated anti-CD3, anti-CD28, and IL-27 (10 ng/ml) under Th1-polarizing conditions without IL-12 in the presence of anti-ICAM-1, anti-LFA-1, or control rat IgG (20 μg/ml each). Primed CD4⁺ T cells were expanded and restimulated with plate-coated anti-CD3 on day 6 for 24 h, and culture supernatants were analyzed for IFN-γ production in triplicate by ELISA. Data are shown as the mean ± SD. *C*, Induction of Th1 differentiation by ligation of LFA-1 with ICAM-1-Fc fusion protein. Naive CD4⁺ T cells were primed with plate-coated anti-CD3 and anti-CD28 under Th1-polarizing conditions without IL-12 in the presence of plate-coated ICAM-1-Fc fusion protein (0.01, 0.1, and 1 ng/ml). Primed CD4⁺ T cells were expanded, restimulated, and analyzed for IFN-γ production as described in *B*. Similar results were obtained in three independent experiments.

(Fig. 4C). These mAbs efficiently inhibited IL-27-induced Th1 differentiation, and combined treatment with these mAbs slightly, but additionally, inhibited it. These results suggest that IL-27 induces Th1 differentiation from naive CD4⁺ T cells stimulated with soluble anti-CD3 and irradiated T/NK cell-depleted spleen cells as APCs under Th1-polarizing conditions, but without IL-12, and that the rapid up-regulation of ICAM-1 expression on CD4⁺ T cells by IL-27 plays an important role in IL-27-induced Th1 differentiation.

T-bet is not essential for IL-27-induced rapid up-regulation of ICAM-1 expression and Th1 differentiation

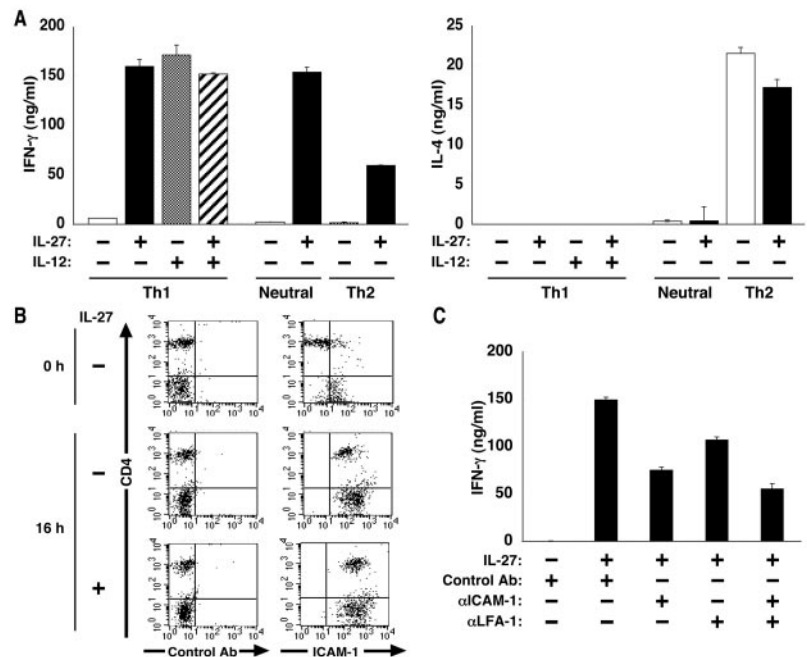
Because IL-27 induces the expression of T-bet, we next examined the requirement for T-bet in IL-27-induced Th1 differentiation using T-bet-deficient mice. We first confirmed the absence of T-bet expression in T-bet-deficient naive CD4⁺ T cells primed under various conditions (Fig. 5A). Surprisingly, IL-27 still efficiently induced Th1 differentiation in T-bet-deficient naive CD4⁺ T cells, whereas IL-12 failed to induce it (Fig. 5B). Although IL-27 appears to enhance Th2 differentiation in T-bet-deficient naive CD4⁺ T cells, this tendency was not always observed in repeated experiments. Even in T-bet-deficient naive CD4⁺ T cells, IL-27 rapidly and markedly enhanced ICAM-1 expression as in wild-type naive CD4⁺ T cells (Fig. 5C). Moreover, blocking mAbs against ICAM-1 and LFA-1 efficiently inhibited IL-27-induced Th1 dif-

ferentiation, and combined treatment with these mAbs slightly, but additionally, inhibited it (Fig. 5D) as in wild-type naive CD4⁺ T cells (Fig. 3B). These results suggest that T-bet is not essential for IL-27-induced rapid up-regulation of ICAM-1 expression and Th1 differentiation.

STAT1 is required for IL-27-induced rapid up-regulation of ICAM-1 expression and Th1 differentiation

We then investigated the role of STAT1 in IL-27-induced Th1 differentiation using STAT1^{-/-} and STAT1^{+/-} mice. In STAT1^{-/-} naive CD4⁺ T cells stimulated with plate-coated anti-CD3 and anti-CD28, induction of T-bet expression was almost abolished even in the presence of IL-27, IL-12, or both, although efficient induction of T-bet expression by IL-27 was observed in STAT1^{+/-} naive CD4⁺ T cells (Fig. 6A). Moreover, the absence of STAT1 markedly inhibited the Th1 differentiation induced by IL-27 as well as IL-12 (Fig. 6B). In contrast, IL-27 appeared to inhibit the basal level of Th1 differentiation induced in the presence and the absence of IL-12 from STAT1^{-/-} naive CD4⁺ T cells. This might imply the possibility of an inhibitory effect of IL-27 on the Th1 differentiation in STAT1^{-/-} naive CD4⁺ T cells, although additional studies are necessary to elucidate this. Consistent with the inability of IL-27 to induce Th1 differentiation, IL-27-induced rapid up-regulation of ICAM-1 expression was almost

FIGURE 4. IL-27 induces Th1 differentiation from naive CD4⁺ T cells stimulated with soluble anti-CD3 and spleen APCs in the absence of IL-12, and rapid up-regulation of ICAM-1 expression by IL-27 plays an important role in IL-27-induced Th1 differentiation. **A**, Induction of Th1 differentiation from naive CD4⁺ T cells stimulated with soluble anti-CD3 and spleen APCs by IL-27. Wild-type (BALB/c) naive CD4⁺ T cells were primed with soluble anti-CD3 and irradiated T/NK-depleted spleen cells in the presence and the absence of IL-27 (10 ng/ml) under Th1-polarizing conditions with and without IL-12 (10 ng/ml) and under neutral and Th2-polarizing conditions. Primed CD4⁺ T cells were expanded and restimulated with plate-coated anti-CD3 on day 6 for 24 h, and culture supernatants were analyzed for IFN- γ and IL-4 production in triplicate by ELISA. Data are shown as the mean \pm SD. **B**, Rapid up-regulation of ICAM-1 expression on naive CD4⁺ T by IL-27. Naive CD4⁺ T cells were primed with soluble anti-CD3 and nonirradiated T/NK-depleted spleen cells in the presence or the absence of IL-27 (10 ng/ml) under Th1-polarizing conditions without IL-12. After culture for 16 h, cells were analyzed for cell surface expression of ICAM-1 and LFA-1 by FACS using anti-ICAM-1, anti-LFA-1, and control rat IgG, followed by FITC-anti-rat IgG and PE-anti-CD4. **C**, Inhibition of IL-27-induced Th1 differentiation by anti-ICAM-1 and anti-LFA-1. Naive CD4⁺ T cells were primed with soluble anti-CD3, irradiated T/NK-depleted spleen cells, and IL-27 (10 ng/ml) under Th1-polarizing conditions without IL-12 in the presence of anti-ICAM-1, anti-LFA-1, or control rat IgG (20 μ g/ml each). Primed CD4⁺ T cells were expanded, restimulated, and analyzed for IFN- γ production as described in **A**. Similar results were obtained in two independent experiments.



completely suppressed on STAT1^{-/-} naive CD4⁺ T cells, but not on STAT1^{+/-} counterparts (Fig. 6C). These results suggest that STAT1 is required for IL-27-induced rapid up-regulation of ICAM-1 expression and Th1 differentiation.

STAT1 directly mediates IL-27-induced transcriptional up-regulation of ICAM-1 gene expression

It was previously demonstrated that STAT1 activated by IFN- γ binds to GAS located in the 5'-flanking region of the *ICAM-1* gene and confers IFN- γ responsiveness (21). To define the mechanism by which IL-27 induces up-regulation of ICAM-1 expression, we performed a reporter assay and EMSA. A plasmid construct containing 1.0 kb of the 5'-flanking region of the mouse *ICAM-1* gene fused upstream from a luciferase reporter gene and its mutated construct in the GAS (wild-type, TTTCCCGGAAA, to mutated, GACCTCTAGA, at nucleotide -63 to -53) were transiently transfected into T cell hybridoma (68-41) cells, which express both WSX-1 and gp130 (data not shown), stimulated with IL-27 for 6 h, and subjected to a reporter assay (Fig. 7A). The luciferase activity of cells transfected with the wild-type construct was greatly increased in response to IL-27 in a dose-dependent manner. In contrast, the luciferase activity of cells transfected with the mutated construct in GAS failed to increase in response to IL-27. Thus, GAS is important for IL-27-induced transcriptional activation of the *ICAM-1* gene. Then EMSA with nuclear protein extracts prepared from naive CD4⁺ T cells stimulated with plate-coated anti-CD3 and anti-CD28 in the presence or the absence of IL-27 for 15 h was conducted (Fig. 7B). A much more intensified binding complex with the wild-type GAS oligonucleotide probe was detected in extracts prepared from naive CD4⁺ T cells stimulated in

the presence of IL-27 compared with those in the absence of IL-27. The presence of 100-fold molar excess amounts of unlabeled wild-type oligonucleotide, but not oligonucleotide mutated in the GAS, inhibited the binding completely. No such binding complex was observed when the oligonucleotide probe mutated in the GAS was used. Moreover, anti-STAT1, but not anti-STAT4 as control Ab, inhibited binding complex formation and formed a supershifted complex. These results suggest that STAT1 directly mediates IL-27-induced transcriptional up-regulation of *ICAM-1* gene expression.

Discussion

In the present study we have revealed that IL-27 can induce anti-tumor activity by augmenting type 1 cell-mediated immunity in vivo in IL-12-independent manner (Fig. 1). Consistent with the in vivo results, IL-27 can induce in vitro Th1 differentiation under Th1-polarizing conditions, but most prominently without IL-12 in a STAT4- and IFN- γ -independent mechanism, and that the presence of IL-12 dose dependently overrules the ability of IL-27 to induce Th1 differentiation (Fig. 2). Moreover, we have found that IL-27 but not IL-12 induces rapid and marked up-regulation of ICAM-1 expression on naive CD4⁺ T cells, which plays an important role in the IL-27-induced Th1 differentiation (Figs. 3 and 4), whereas unexpectedly T-bet is not required for it (Fig. 5). Instead, STAT1 plays a critical role in IL-27-induced rapid up-regulation of ICAM-1 expression and Th1 differentiation (Fig. 6). STAT1 itself, activated by IL-27, directly binds to the GAS in 5'-flanking region of *ICAM-1* gene and forms a binding complex, resulting in transcriptional activation of the *ICAM-1* gene (Fig. 7). Thus, the present results clearly delineate a difference between the

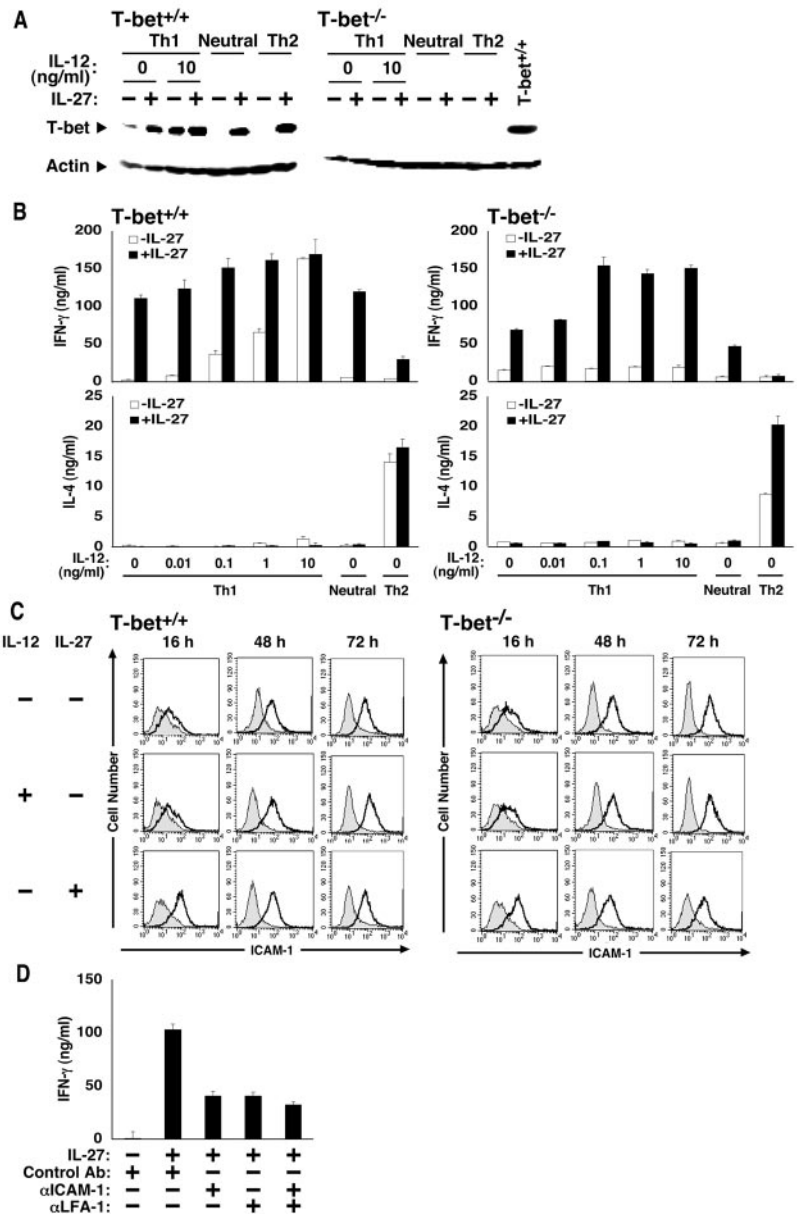


FIGURE 5. T-bet is not essential for IL-27-induced rapid up-regulation of ICAM-1 expression and Th1 differentiation. **A**, Absence of T-bet expression in T-bet-deficient naive CD4⁺ T cells. Wild-type (BALB/c) and T-bet-deficient naive CD4⁺ T cells were primed with plate-coated anti-CD3 and anti-CD28 in the presence and the absence of IL-12 (10 ng/ml) and neutral and Th2-polarizing conditions. After culture for 48 h, total cell lysates were prepared and subjected to Western blotting using anti-T-bet and anti-actin. **B**, T-bet is not essential for IL-27-induced Th1 differentiation. Primed CD4⁺ T cells, as described in **A**, with various concentrations of IL-12 were expanded and restimulated with plate-coated anti-CD3 on day 6 for 24 h, and culture supernatants were analyzed for IFN- γ and IL-4 production in triplicate by ELISA. Data are shown as the mean \pm SD. **C**, T-bet is not required for rapid up-regulation of ICAM-1 expression by IL-27. Wild-type and T-bet-deficient naive CD4⁺ T cells were primed with plate-coated anti-CD3 and anti-CD28 in the presence or the absence of IL-27 (10 ng/ml) under Th1-polarizing conditions with and without IL-12 (10 ng/ml). After culture for various times, cells were analyzed for cell surface expression of ICAM-1 by FACS using anti-ICAM-1 (solid line), and control rat IgG (plain line with shading). **D**, Inhibition of IL-27-induced Th1 differentiation by anti-ICAM-1 and anti-LFA-1 in T-bet-deficient naive CD4⁺ T cells. T-bet-deficient naive CD4⁺ T cells were primed with plate-coated anti-CD3, anti-CD28, and IL-27 (10 ng/ml) under Th1-polarizing conditions with and without IL-12 (10 ng/ml) in the presence of anti-ICAM-1, anti-LFA-1, or control rat IgG (20 μ g/ml each). Primed CD4⁺ T cells were expanded, restimulated, and analyzed for IFN- γ production as described in **B**. Similar results were obtained in two independent experiments.

mechanisms by which IL-27 and IL-12 induce Th1 differentiation. IL-27-induced Th1 differentiation is independent of STAT4, T-bet, and IFN- γ but is dependent on STAT1 and ICAM-1/LFA-1. In contrast, IL-12-induced Th1 differentiation is dependent on STAT4, T-bet, IFN- γ , and mostly STAT1 but is independent of ICAM-1/LFA-1. The inhibition of IL-27-induced Th1 differentiation by anti-ICAM-1 and anti-LFA-1 when naive CD4⁺ T cells were stimulated with soluble anti-CD3 and spleen APCs (Fig. 4C) appears to be less than when these cells were stimulated with plate-coated anti-CD3 and anti-CD28 (Fig. 3B). This difference might be ascribed to the other unknown effects of IL-27 on APCs in addition to the rapid up-regulation of ICAM-1 expression on naive CD4⁺ T cells. Moreover, because treatments with mAbs against ICAM-1 and LFA-1 failed to completely block the IL-27-induced Th1 differentiation, we cannot formally rule out the possibility that additional mechanisms may contribute to it.

ICAM-1 expressed on APCs binds to LFA-1 on Th cells during Ag presentation, and the ICAM-1/LFA-1 interaction has a pleiotropic effect in T cell recirculation, inflammation, and activation (31, 32). In particular, ICAM-1/LFA-1-mediated adhesion is a crit-

ical event for establishing and strengthening the physical contact between APCs and Th cells, leading to optimal Th cell activation (33, 34). In addition, several mouse studies revealed that the ICAM-1/LFA-1 interaction plays an important role in Th1 polarization (27, 28). In addition, ICAM-1 expressed on Th cells was shown to provide a costimulatory signal for T cell activation and Th1 cytokine production (29). Recently, it has been demonstrated that ligation of LFA-1 on human naive CD4⁺ T cells stimulated with anti-CD3/anti-CD28 and immobilized ICAM-1-Fc induces a marked shift toward Th1 development, accompanied by an increase in T-bet expression, but, reciprocally, a decrease in GATA-3 expression (30). However, the Th1 polarization by LFA-1 ligation is largely overruled by IL-12 in a dose-dependent manner. These properties are quite similar to the present findings in IL-27-induced Th1 differentiation, which is also overruled by IL-12 (Fig. 2B). Naive CD4⁺ T cells stimulated with plate-coated anti-CD3 and anti-CD28 in the presence of IL-27 were shown to rapidly and efficiently express both ICAM-1 and LFA-1 (Fig. 3A). Therefore, it is highly conceivable that not only does ICAM-1 act as a costimulatory molecule to ligate LFA-1 on CD4⁺ T cells, but

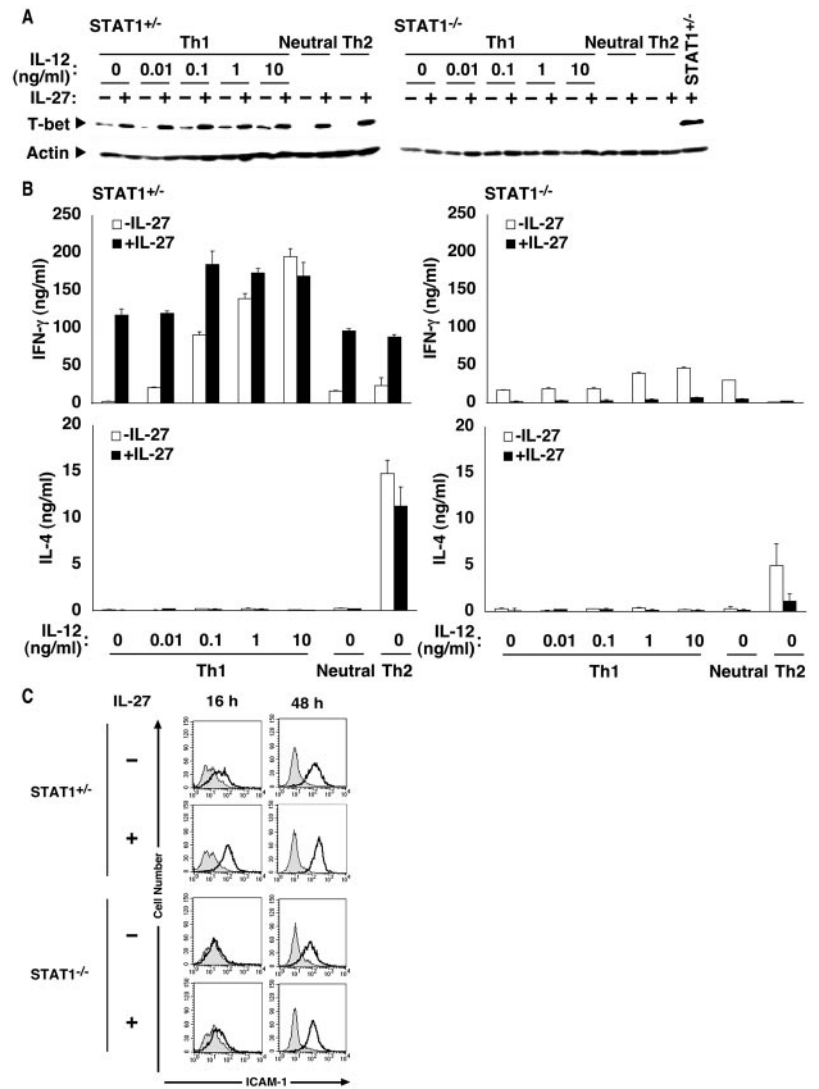


FIGURE 6. STAT1 is required for IL-27-induced rapid up-regulation of ICAM-1 expression and Th1 differentiation. **A**, Markedly reduced induction of T-bet expression in STAT1-deficient naive CD4⁺ T cells. STAT1^{+/-} and STAT1^{-/-} naive CD4⁺ T cells were primed with plate-coated anti-CD3 and anti-CD28 in the presence and the absence of IL-27 (10 ng/ml) under Th1-polarizing conditions with various concentrations of IL-12 and under neutral and Th2-polarizing conditions. After culture for 48 h, total cell lysates were prepared and subjected to Western blotting using anti-T-bet and anti-actin. **B**, STAT1 is required for IL-27-induced Th1 differentiation. Primed CD4⁺ T cells, as described in **A**, were expanded and restimulated with plate-coated anti-CD3 on day 6 for 24 h, and culture supernatants were analyzed for IFN-γ and IL-4 production in triplicate by ELISA. Data are shown as the mean ± SD. **C**, STAT1 is required for IL-27-induced rapid up-regulation of ICAM-1 expression. Wild-type and STAT1-deficient naive CD4⁺ T cells were primed with plate-coated anti-CD3 and anti-CD28 in the presence or the absence of IL-27 (10 ng/ml) under Th1-polarizing conditions without IL-12. After culture for various times, cells were analyzed for cell surface expression of ICAM-1 by FACS using anti-ICAM-1 (solid line) and control rat IgG (plain line with shading). Similar results were obtained in three independent experiments.

also signaling through ICAM-1 delivers a costimulatory signal to T cells, both of which result in augmentation of Th1 polarization (27–29). Generally, ICAM-1 expression is up-regulated on APCs by the interaction between APCs and Th cells. However, IL-27 appears to act much more efficiently on naive CD4⁺ T cells in the up-regulation of ICAM-1 expression than on APCs (Fig. 4B). Up-regulation of ICAM-1 expression on naive CD4⁺ T stimulated with plate-coated anti-CD3 and anti-CD28, even in the absence of IL-27, was also detected later (48 h after the stimulation), and IL-12 did not enhance it (Fig. 3A). Treatment with blocking mAbs against ICAM-1 and LFA-1 failed to affect the Th1 differentiation induced by IL-12 (Fig. 3B). These results suggest that the early rapid up-regulation of ICAM-1 expression on naive CD4⁺ T cells by IL-27 is important for the Th1 differentiation. However, the molecular mechanism by which the IL-27-induced rapid up-regulation of ICAM-1 expression leads to Th1 polarization in a T-bet-independent manner remains to be elucidated.

Early in the infection with a protozoan parasite, *L. major*, WSX-1-deficient mice are remarkably susceptible to the infection, showing impaired IFN-γ production and advanced lesion development (9, 10). Similarly, reduced production of IFN-γ is observed when WSX-1-deficient mice are challenged with an avirulent strain of mycobacterium (bacillus Calmette-Guérin) (9). However, defects in pathogen-induced IFN-γ production are transient, and as each

disease progresses, WSX-1-deficient mice generate Th1-type responses and control infection like wild-type mice (9, 10). It was also demonstrated that WSX-1-deficient mice can control replication of *T. gondii* parasites through the generation of robust Th1 responses as wild-type mice (5). Consistent with these in vivo results, it was demonstrated that WSX-deficient T cells are impaired in IFN-γ production when stimulated under weakly polarizing conditions in vitro, but they produce elevated levels of IFN-γ in a strongly polarizing environment (5, 8). Therefore, a key difference between infection with *L. major* or bacillus Calmette-Guérin and that with *T. gondii* is considered to be the induction level of IL-12, a principal mediator of strong type I immunity (35). *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 (36). It has been demonstrated recently that during the early stages of *L. major* infection, neutralization of IL-4 completely recovers the IFN-γ production in WSX-1-deficient mice and restores the ability to control parasite replication and promote the resolution of inflammatory lesions (10, 37). These results suggest that the requirement for WSX-1 signaling in Th1 differentiation is restricted to conditions in which IL-4 is produced. Because IL-4 is well known to inhibit IL-12 production and responsiveness (38, 39), neutralization of IL-4 induces favorable conditions for the generation of IL-12 activity. Taken together,

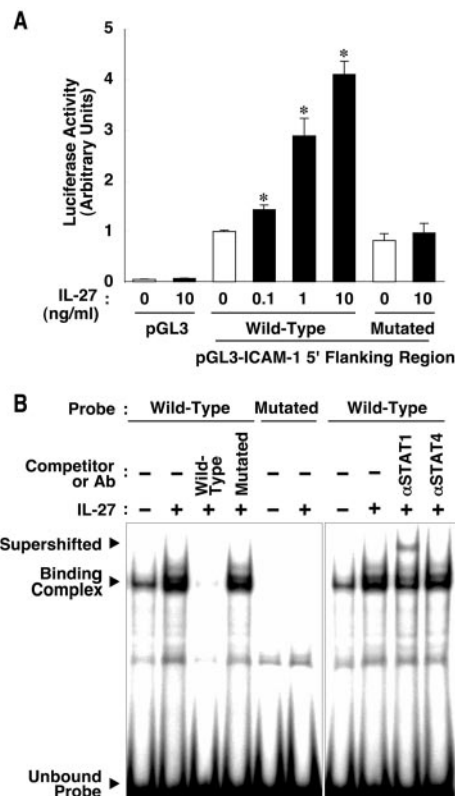


FIGURE 7. STAT1 directly mediates IL-27-induced transcriptional up-regulation of ICAM-1 gene expression. **A**, GAS in 5'-flanking region of ICAM-1 gene is important for IL-27-induced up-regulation of ICAM-1 expression. Reporter plasmid constructs containing the wild-type 5'-flanking region of ICAM-1 gene and its mutant in the GAS were transiently transfected into T cell hybridoma 68-41 cells and stimulated with IL-27. After 6 h, cell lysates were prepared and measured for luciferase activities. Data are shown as the mean \pm SD. **B**, STAT1 activated by IL-27 forms a binding complex with the GAS in the 5'-flanking region of ICAM-1 gene. Nuclear protein extracts prepared from naive CD4⁺ T cells activated with plate-coated anti-CD3 and anti-CD28 in the presence or the absence of IL-27 (10 ng/ml) under Th1-polarizing conditions without IL-12 for 15 h were mixed with radiolabeled oligonucleotide probe containing wild-type GAS in the 5'-flanking region of the ICAM-1 gene or its mutant under various conditions, as indicated in the figure, and subjected to EMSA. Similar results were obtained in two independent experiments.

these results suggest that under the conditions in which IL-12 production is limited, WSX-deficient naive CD4⁺ T cells are impaired in Th1 differentiation, but under the conditions in which IL-12 is produced abundantly, WSX-deficient naive CD4⁺ T cells efficiently differentiate into Th1 cells. This dichotomy agrees well with the present findings that the action of IL-27 on Th1 polarization is most prominent under Th1-polarizing conditions without IL-12 and that the presence of IL-12 dose dependently overrules the ability of IL-27 to induce Th1 differentiation, but thereafter IL-12 itself strongly induces Th1 differentiation.

Thus, the present study suggests that IL-27 efficiently induces Th1 differentiation under Th1-polarizing conditions, but without IL-12, and that the rapid up-regulation of ICAM-1 expression on naive CD4⁺ T cells is important for IL-27-induced Th1 differentiation. Considering that IL-27 is produced from macrophages and DCs earlier than IL-12 (1), IL-27 may play a pivotal role in the early efficient initiation of Th1 differentiation, but it is not necessary for the maintenance of Th1 responses once IL-12 is produced sufficiently.

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Disclosures

The authors have no financial conflict of interest.

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