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Augmentation of Effector CD8$^+$ T Cell Generation with Enhanced Granzyme B Expression by IL-27[1]

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IL-27 is a novel IL-12 family member that plays a role in the early regulation of Th1 initiation. We have recently demonstrated that IL-27 has a potent antitumor activity, which is mainly mediated through CD8$^+$ T cells, and also has an adjuvant activity to induce epitope-specific CTL in vivo. In this study, we further investigated the in vitro effect of IL-27 on CD8$^+$ T cells of mouse spleen cells. In a manner similar to CD4$^+$ T cells, IL-27 activated STAT1, -2, -3, -4, and -5, and augmented the expression of T-bet, IL-12Rβ2, and granzyme B, and slightly that of perforin in naive CD8$^+$ T cells stimulated with anti-CD3. IL-27 induced synergistic IFN-γ production with IL-12 and proliferation of naive CD8$^+$ T cells. Moreover, IL-27 enhanced proliferation of CD4$^+$ T cell-depleted spleen cells stimulated by allogeneic spleen cells and augmented the generation of CTL. In STAT1-deficient naive CD8$^+$ T cells, IL-27-induced proliferation was not reduced, but synergistic IFN-γ production with IL-12 was diminished with decreased expression of T-bet, IL-12Rβ2, granzyme B, and perforin. In T-bet-deficient naive CD8$^+$ T cells, IL-27-induced proliferation was hardly reduced, but synergistic IFN-γ production with IL-12 was diminished with decreased expression of IL-12Rβ2, granzyme B, and perforin. However, IL-27 still augmented the generation of CTL from T-bet-deficient CD4$^+$ T cell-depleted spleen cells stimulated by allogeneic spleen cells with increased granzyme B expression. These results suggest that IL-27 directly acts on naive CD8$^+$ T cells in T-bet-dependent and -independent manners and augments generation of CTL with enhanced granzyme B expression. *The Journal of Immunology,* **2005, 175: 1686–1693.**

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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). IL-27 is produced by activated APCs; is able to induce proliferation and T-bet expression in naive, but not memory CD4$^+$ T cells; and synergizes with IL-12 in IFN-γ production (1, 2). Recently, it has been demonstrated that the orphan cytokine receptor WSX-1/T cell cytokine receptor, which is homologous to the IL-12Rβ2 subunit, and gp130 constitute a functional signal-transducing receptor for IL-27 (3). IL-27 activates STAT1 and STAT3 (2, 4, 5), presumably through each subunit, WSX-1/T cell cytokine receptor and gp130, respectively, in naive CD4$^+$ T cells. STAT1 plays a critical role in IL-27-induced T-bet and subsequent IL-12Rβ2 expression, while STAT3 is considered to be important for IL-27-induced proliferation (6).

Effector CD8$^+$ CTLs play a pivotal role in successful clearance of viruses, intracellular microorganisms, and tumors. Generation of effector CD8$^+$ CTLs is a complex process that is triggered through Ag-specific TCR-CD3 complex involving Ag-specific selection, expansion, and differentiation (7). It has recently been demonstrated that a member of T-box family transcription factor, T-bet, known to be responsible for controlling lineage commitment in Th1 cells (8), controls the generation of effector CD8$^+$ CTLs as well (9). T-bet is required for differentiation of naive CD8$^+$ T cells into effector CTLs by Ag-specific stimuli (9), but not by Ag-nonspecific stimuli such as plate-coated anti-CD3 and anti-CD28 (10). T-bet-deficient CD8$^+$ T cells proliferate and expand normally, but are impaired in killing capacity and secrete low levels of IFN-γ, whereas IL-2, IL-4, and IL-10 production is elevated (9). Therefore, T-bet is a key transcription factor in the generation of type 1 immunity in both Th and CTLs.

We have recently evaluated the antitumor activity of IL-27 against a murine tumor model of colon carcinoma, colon 26 (C26),[3] by transducing with single-chain IL-27 cDNA, and found that IL-27 has a potent ability to induce tumor-specific antitumor activity, which is mainly mediated through CD8$^+$ T cells with enhanced CTL activity (11). Moreover, we have evaluated the adjuvant activity of IL-27 expression plasmid in the prime-boost immunization consisting of an hepatitis C virus (HCV) core expression plasmid and a recombinant adenovirus-expressing HCV core, and elucidated that IL-27 has a potent adjuvant activity for the induction of HCV-specific CTLs (12). These two studies clearly indicate that IL-27 augments CTL activity in vivo, whereas little is known on the molecular mechanism underlying the augmentation of CTL activity by IL-27. To clarify the molecular mechanism, we have investigated the in vitro effect of IL-27 on CD8$^+$ T cells. In the present study, we show that IL-27 directly acts on naive CD8$^+$ T cells in T-bet-dependent and -independent manners and augments generation of CTL with enhanced granzyme B expression.

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[3] Abbreviations used in this paper: C26, colon 26; Eomes, Eomesodermin; HCV, hepatitis C virus; HPRT, hypoxanthine phosphoribosyltransferase; MFI, mean fluorescence intensity; pY, phosphotyrosine.

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Materials and Methods

Cell culture and mice

Colon carcinoma (C26) was cultured in RPMI 1640 medium supplemented with 10% FBS. Naive $CD4^+$ and $CD8^+$ T, EL-4, and A20.2J cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 50 μM 2-ME. HEK293-F cells were purchased from Invitrogen Life Technologies and cultured in the serum-free medium (FreeStyle 293 Expression Medium; Invitrogen Life Technologies). IL-12-responsive mouse Th1 cell clone, 2D6, was kindly provided by H. Fujiwara (Osaka University, Osaka, Japan) and maintained in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-ME, and mouse rIL-12 (250 pg/ml). BALB/c and C57BL/6 mice were purchased from Japan SLC. STAT4-deficient mice (13), IL-12 p40-deficient mice (14), and T-bet-deficient mice (10) of BALB/c background were purchased from The Jackson Laboratory. STAT1-deficient mice (15) of 129/Sv background and wild-type 129/Sv mice were purchased from Taconic Farms. All animal experiments were performed in accordance with our institutional guidelines.

Reagents

Anti-FLAG (M2) and anti-actin were purchased from Sigma-Aldrich. Anti-CD3 (145-2C11), anti-IL-2 (S4B6), and anti-CD4 (GK1.5) were from American Type Culture Collection. Mouse rIL-4, anti-IL-12Rβ2 (HAM10B9), and anti-CD28 (37.51) were from BD Biosciences. Anti-STAT1, -3, -4, -5, and -6, and anti-T-bet (4B10) were from Santa Cruz Biotechnology. Anti-STAT2 and anti-phosphotyrosine (pY)-STAT2 were from Upstate Biotechnology. Anti-pY-STAT1, -3, -5, and -6 were from Cell Signaling Technology. Anti-pY-STAT4 was from Zymed Laboratories. Anti-granzyme B and anti-perforin (KM585) were from Lab Vision and Kyowa Medex, respectively. Mouse rIL-12 was from R&D Systems. Human rIL-2 and mouse rIFN-γ were kindly 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) by using Fugene 6 (Roche Molecular Biochemicals) and selected with Geneticin (G418), as described before (11). 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. Cytotoxicity activity of spleen cells of mice inoculated with C26 transfectants was determined against parental C26 tumor cells as target cells after restimulation in vitro with parental C26 for 5 days, as described before (11).

Preparation of purified rIL-27 protein

rIL-27 was prepared as a soluble tagged fusion protein by flexibly linking EBV-induced gene 3 to p28 using HEK293-F cells, as described before (16).

Preparation of naive $CD4^+$ and $CD8^+$ 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-CD8α or anti-CD4 (for negative isolation of $CD4^+$ or $CD8^+$ T cells, respectively), anti-B220, anti-Mac-1, anti-Ter119, 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 ($CD4^+$ or $CD8^+$ 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 $CD4^+$ or $CD8^+$ T cells ($CD62L^+$ cells >99%).

Western blotting

Cells were lysed in a lysis buffer containing protease inhibitors, and resultant cell lysates were separated on a SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membrane (Millipore), as described previously (17). 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. The intensity of each band was measured using densitometer and evaluated using NIH Image Analysis software.

RT-PCR

Total RNA was extracted by using a guanidine thiocyanate procedure; cDNA was prepared using oligo(dT) primer and SuperScript RT (Invitrogen Life Technologies); and RT-PCR was performed using *Taq*DNA polymerase, as described (18). Cycle conditions were 25–30 cycles consisting of 94°C for 40 s, 60°C for 20 s, and 72°C for 40 s. Primers used for hypoxanthine phosphoribosyltransferase (HPRT) were described (19). Following primers were also used: T-bet sense primer, 5′-GATCGTCCTGCAGTCTCTCC-3′; T-bet antisense primer, 5′-AACTGTGTTCCCGAGGTGTC-3′; IL-12Rβ2 sense primer, 5′-ACTGTCAGAGAGCCAAGGAG-3′; IL-12Rβ2 antisense primer, 5′-GTGCCCACCGTGATGATAG-3′, granzyme B sense primer, 5′-ACAGAAGGATCGGGAGTGTG-3′; granzyme B antisense primer, 5′-GAAGCCAGTCTTTGCAGTCC-3′; perforin sense primer, 5′-GATGTGAACCCTAGGCCAGA-3′; perforin antisense primer, 5′-AAAGAGGTGGCCATTTTGTG-3′; Eomesodermin (Eomes) sense primer, 5′-GGCAAAGCGGACAATAACAT-3′; and Eomes antisense primer, 5′-GACCTCCAGGGACAATCTGA-3′.

IFN-γ production and proliferation assays

For measurement of IFN-γ production, purified naive $CD8^+$ T cells (2×10^5 cells/200 μl) were stimulated with plate-coated anti-CD3 (6 μg/ml) in the absence of rIL-27 and/or rIL-12 and anti-IL-2 (100 μg/ml) for 72 h, and culture supernatants were collected and assayed for IFN-γ production by ELISA, as described (20). For measurement of proliferation, purified naive $CD8^+$ T cells (2×10^5 cells/200 μl) were stimulated with plate-coated anti-CD3 (6 μg/ml) in the presence of rIL-27 and/or rIL-12 and anti-IL-2 (100 μg/ml) for 72 h and pulsed with [^{3}H]thymidine for last 24 h.

Mixed leukocyte reaction

Spleen cells from BALB/c (H-2^d) mice were depleted of erythrocytes and incubated with anti-CD4 (GK1.5), followed by incubation with anti-rat IgG magnetic beads (Miltenyi Biotec). Resultant spleen cells were then passed through a magnetic cell sorting column (Miltenyi Biotec), and the negative fraction was collected ($CD4^+$ T cells <5%). The $CD4^+$ T cell-depleted spleen cells (1×10^6 cells/200 μl) were stimulated in vitro by coculture with spleen cells (4×10^5 cells/200 μl) from C57BL/6 (H-2^b) mice irradiated with 20 Gy in the presence of various amounts of rIL-27 and anti-IL-2 (30 μg/ml) or rIL-2 (25 U/ml) as positive control for 4 days and pulsed with [^{3}H]thymidine for last 24 h.

Cytotoxicity assay

$CD4^+$ T cell-depleted spleen cells (1×10^7 cells/1 ml) from BALB/c (H-2^d) mice were stimulated in vitro by coculture with spleen cells (4×10^6 cells/1 ml) from C57BL/6 (H-2^b) mice irradiated with 20 Gy in the presence of various amounts of rIL-27 and anti-IL-2 (30 μg/ml) or rIL-2 (25 U/ml) as positive control for 5 days. Viable cells were isolated from responder cells by centrifugation over Lympholite-M (Cedarlane Laboratories) and used as effector cells in a standard ^{51}Cr release assay. As target cells, EL-4 (H-2^b) or A20.2J (H-2^d) cells (1×10^6 cells) were labeled by incubating in 100 μCi of ^{51}Cr for 1 h at 37°C and washing three times. Labeled target cells (1×10^4 cells/200 μl/well) and serial dilutions of effector cells were incubated in RPMI 1640 containing 10% FBS in 96-well U-bottom plate at 37°C for 4 h. Supernatants were then analyzed in a scintillation counter. The percentage of lysis was determined for each triplicate experiment as: (experimental count − spontaneous count) × 100/(maximal count − spontaneous count). Results were expressed as the percentage of specific lysis.

Results

Induction of antitumor effect with enhanced CTL activity by IL-27 in IL-12-independent manner

We have recently evaluated the antitumor activity of IL-27 against a murine tumor model of colon carcinoma, C26, by transducing with single-chain IL-27 cDNA, and found that IL-27 has a potent ability to induce tumor-specific antitumor activity, which is mainly mediated through $CD8^+$ T cells with enhanced CTL activity (11). In this study (11), STAT4 was demonstrated not to be essential for the induction of antitumor activity by IL-27, because IL-27 still exhibits inhibition of tumor growth even in STAT4-deficient mice, as shown in Fig. 1*A*. First of all, we further examined whether IL-27 induces antitumor activity in IL-12 p40-deficient mice and enhances CTL activity in STAT4-deficient and IL-12 p40-deficient mice. C26-vector and C26-IL-27 were injected s.c. into these mice, and tumor volume was monitored. IL-27 still showed antitumor activity even in IL-12 p40-deficient mice as in wild-type and

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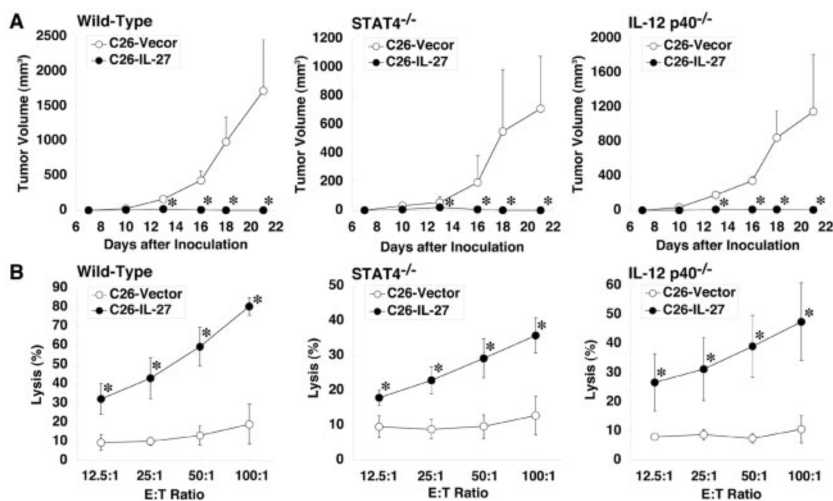

FIGURE 1. Induction of antitumor effect with enhanced CTL activity by IL-27 in IL-12-independent manner. *A*, Inhibition of tumor growth by IL-27 even in STAT4- or IL-12 p40-deficient mice. Wild-type BALB/c, STAT4-deficient, and IL-12 p40-deficient mice ($n = 3–5$) were injected s.c. with C26-vector or C26-IL-27, and tumor volume was monitored. Data are shown as the mean ± SD. *, Indicates $p < 0.05$, compared with C26-vector. *B*, Enhanced CTL activity against tumor by IL-27 even in STAT4- or IL-12 p40-deficient mice. Spleen cells of mice ($n = 3–5$) 3 wk after inoculation with C26-vector or C26-IL-27 were restimulated in vitro with irradiated parental C26 for 5 days, and CTL activity of resultant effector cells was measured against ^{51}Cr-labeled parental C26 target in a standard ^{51}Cr release assay. Data are shown as the mean ± SD. *, Indicates $p < 0.05$, compared with C26-vector. Similar results were obtained in two independent experiments.

STAT4-deficient mice (Fig. 1*A*). In addition, IL-27 less, but significantly enhanced CTL activity against tumor cells in STAT4-deficient and IL-12 p40-deficient mice as compared with that in wild-type mice (Fig. 1*B*). These results suggest that IL-12 is not essential for induction of antitumor effect and CTL activity by IL-27, and imply that IL-27 may directly act on CD8$^+$ T cells to generate tumor-specific effector CTL. Therefore, in the present study, we investigated on the direct effect of IL-27 on CD8$^+$ T cells.

Activation of STAT1, -2, -3, -4, and -5 in naive CD8$^+$ T cells by IL-27

As signaling molecules, JAKs/STATs are critically important for various cytokines to exert their biological responses. We (6) and others (2, 4, 5) previously reported that IL-27 activates STAT1, -2, -3, -4, and -5 in CD4$^+$ T cells. Therefore, we first investigated whether IL-27 activates similar STAT molecules in CD8$^+$ T cells. Naive CD8$^+$ T cells, which were purified from spleen cells of wild-type C57BL/6 mice and activated with plate-coated anti-CD3 for 16 h, were stimulated with IL-27, and their cell lysates were prepared and subjected to Western blotting with various anti-pY-STAT and total STAT Abs (Fig. 2*A*). IL-27 activated STAT1, -2, -3, -4, and -5 in naive CD8$^+$ T cells as in naive CD4$^+$ T cell.

Induction of T-bet, IL-12Rβ2, granzyme B, and perforin expression in naive CD8$^+$ T cells by IL-27

In CD4$^+$ T cells, IL-27 induces the expression of T-bet and subsequent IL-12Rβ2 (2), which is a key Th1 commitment step in which naive Th precursor cells commence differentiation into Th1 cells (21). We then examined whether IL-27 induces the expression of these molecules and also cytolytic proteins, granzyme B, and perforin, which are functionally important in effector CD8$^+$ T cells. Naive CD8$^+$ T cells were stimulated with plate-coated anti-CD3 in the presence and absence of IL-27, and RT-PCR (Fig. 2*B*), Western blotting (Fig. 2*C*), and FACS (Fig. 2*D*) analyses were performed to detect the expression of each molecule at both mRNA and protein levels. IL-27 greatly enhanced the expression of T-bet and IL-12Rβ2 in naive CD8$^+$ T cells activated with plate-coated anti-CD3. Mean fluorescence intensity (MFI) for IL-12Rβ2 and control expression in the absence of IL-27 was 100.5 ± 43.3 and 94.6 ± 43.2, respectively ($p > 0.05$, $n = 5$), and that in the presence of IL-27 was 244.6 ± 109.9 and 146.3 ± 95.7, respectively ($p < 0.01$, $n = 5$). In addition, IL-27 markedly augmented the expression of granzyme B, one of the effector molecules that are critically important in the induction of CTL activity. IL-27 also, but slightly increased the expression of perforin, another effector molecule. These results suggest that IL-27 induces the expression of T-bet, IL-12Rβ2, granzyme B, and perforin in naive CD8$^+$ T cells.

Induction of synergistic IFN-γ production with IL-12 and proliferation in naive CD8$^+$ T cells by IL-27

IL-27 synergistically enhances the IFN-γ production with IL-12 in naive CD4$^+$ T cells activated with plate-coated anti-CD3 and augments their proliferation (1, 2). Therefore, we next examined whether IL-27 similarly induces IFN-γ production and proliferation in naive CD8$^+$ T cells. Naive CD8$^+$ T cells prepared from wild-type spleen cells were stimulated with plate-coated anti-CD3 and various amounts of IL-27. Although IL-27 alone minimally increased IFN-γ production in naive CD8$^+$ T cells activated with plate-coated anti-CD3, IL-27 strongly enhanced the IFN-γ production in the presence of IL-12 (Fig. 2*E*). In addition, IL-27, but not IL-12, greatly augmented the proliferation of naive CD8$^+$ T cells (Fig. 2*F*) in a manner similar to that of naive CD4$^+$ T cells (6). These results suggest that IL-27 induces synergistic IFN-γ production with IL-12 and proliferation in naive CD8$^+$ T cells as in naive CD4$^+$ T cells.

Augmentation of generation of CTL by IL-27

Next, we investigated the effect of IL-27 on the proliferation in MLR. Responder CD4$^+$ T cell-depleted spleen cells from BALB/c mice were cultured with irradiated stimulator spleen cells from C57BL/6 mice in the presence of various amounts of IL-27 and anti-IL-2 or IL-2 as positive control for 4 days and [^{3}H]thymidine incorporation was measured for last 24 h. IL-2 strongly increased the proliferation in the MLR, while IL-27 less efficiently, but significantly increased the proliferation in a dose-dependent manner (Fig. 3*A*). Then we determined whether IL-27 can develop the cytotoxic activity after 5-day coculture in the MLR. IL-27 significantly augmented allospecific CTL activity against EL-4 (H-2^b) in a dose-dependent manner as IL-2 did (Fig. 3*B*). These responder cells exhibited much less lysis against the syngeneic tumor target, A20 (H-2^d) (<10% specific lysis; data not shown). These results suggest that IL-27 induces the development of CD8$^+$ T cells into an allospecific CTL population.

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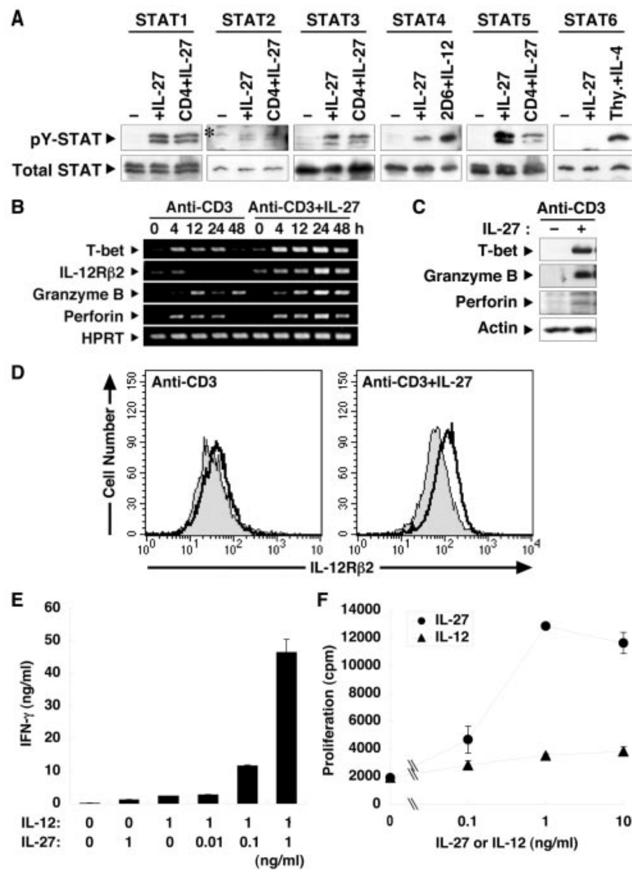

FIGURE 2. IL-27 directly acts on naive $CD8^+$ T cells in a manner similar to naive $CD4^+$ T cells. *A*, Activation of STAT1, -2, -3, -4, and -5 in naive $CD8^+$ T cells by IL-27. Purified naive $CD8^+$ T cells from wild-type C57BL/6 mice were stimulated by plate-coated anti-CD3 in the presence of anti-IL-2 for 16 h. After washing, these cells were stimulated with IL-27 (30 ng/ml) for 10 min. Total cell lysates were then prepared and subjected to Western blotting with anti-pY-STATs. For verification of the same protein expression level, each blot was reprobed with antitotal STATs. Cell lysates prepared from naive $CD4^+$ T cells stimulated with IL-27, from 2D6 cells stimulated with IL-12, and from thymocytes (Thy.) stimulated with IL-4 were used as positive controls for pY-STAT1, -2, -3, and -5, for pY-STAT4, and for pY-STAT6, respectively. *, Indicates a nonspecific band. Similar results were obtained in three independent experiments. *B–D*, Induction of T-bet, IL-12Rβ2, granzyme B, and perforin expression in naive $CD8^+$ T cells by IL-27. Purified naive $CD8^+$ T cells from wild-type mice were stimulated with plate-coated anti-CD3 and IL-27 (5 ng/ml) in the presence of anti-IL-2. After various time points, total RNA was prepared and RT-PCR was performed to detect mRNA expression of T-bet, IL-12Rβ2, granzyme B, and HPRT. After 48 h, total cell lysate was also prepared, and Western blotting was performed to detect the expression of T-bet, granzyme B, perforin, and actin at protein level. After 72 h, stimulated cells were analyzed for cell surface expression of IL-12Rβ2 (solid line) or isotype-matched control (plain line with shaded) by FACS. Similar results were obtained in at least three independent experiments. *E* and *F*, Induction of synergistic IFN-γ production with IL-12 and proliferation in naive $CD8^+$ T cells by IL-27. Purified naive $CD8^+$ T cells were stimulated with plate-coated anti-CD3, IL-27 (0.01, 0.1, and 1 ng/ml), and/or IL-12 (1 ng/ml) in the presence of anti-IL-2. After 72 h, culture supernatants were collected and assayed for IFN-γ production by ELISA in triplicate. Data are shown as the mean ± SD. Purified naive $CD8^+$ T cells from wild-type mice were stimulated with plate-coated anti-CD3 and IL-27 or IL-12 (0.1, 1, and 10 ng/ml) in the presence of anti-IL-2 for 72 h and pulsed with [^{3}H]thymidine for last 24 h. [^{3}H]Thymidine incorporation was measured in triplicate. Data are shown as the mean ± SD. Similar results were obtained in three independent experiments.

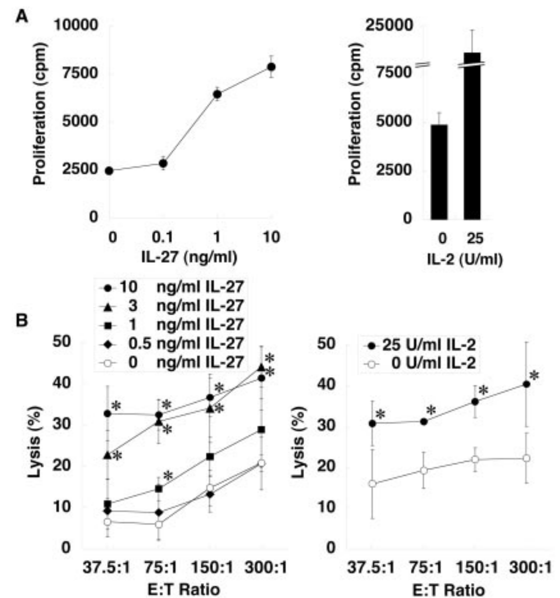

FIGURE 3. Augmentation of generation of CTL by IL-27. *A*, $CD4^+$ T cell-depleted spleen cells from BALB/c (H-2^d) mice were stimulated in vitro by coculture with irradiated spleen cells from C57BL/6 (H-2^b) mice in the presence of various amounts of IL-27 and anti-IL-2 or IL-2 as positive control for 4 days and pulsed with [^{3}H]thymidine for last 24 h. [^{3}H]Thymidine incorporation was measured in triplicate. Data are shown as the mean ± SD. *B*, $CD4^+$ T cell-depleted spleen cells from BALB/c mice were stimulated in vitro by coculture with irradiated spleen cells from C57BL/6 mice in the presence of various amounts of IL-27 and anti-IL-2 or IL-2 as positive control for 5 days and used as effector cells in a standard ^{51}Cr release assay. Data are shown as the mean ± SD. *, Indicates $p < 0.05$, compared with 0 ng/ml IL-27 or 0 U/ml IL-2. Similar results were obtained in three independent experiments.

A critical role for STAT1 in IL-27-induced T-bet, IL-12Rβ2, granzyme B, and perforin expression, and synergistic IFN-γ production with IL-12, but not in proliferation in naive $CD8^+$ T cells

We have demonstrated recently that STAT1 plays an indispensable role in IL-27-mediated T-bet and subsequent IL-12Rβ2 expression, but not in proliferation in naive $CD4^+$ T cells (6). Therefore, we next examined the role for STAT1 in IL-27-mediated immune responses in naive $CD8^+$ T cells using STAT1-deficient mice. We first confirmed the absence of STAT1 expression in STAT1-deficient naive $CD8^+$ T cells by Western blotting (Fig. 4*B*). IL-27-induced expression of T-bet, granzyme B, perforin, and IL-12Rβ2 was greatly diminished (Fig. 4, *A–C*). MFI for IL-12Rβ2 and control expression in the absence of IL-27 in wild-type naive $CD8^+$ T cells was 110.6 ± 63.8 and 129.5 ± 73.4, respectively ($p > 0.05$, $n = 4$), and that in the presence of IL-27 was 227.7 ± 54.8 and 174.0 ± 65.4, respectively ($p < 0.05$, $n = 4$). In contrast, MFI for IL-12Rβ2 and control expression in the absence of IL-27 in STAT1-deficient naive $CD8^+$ T cells was 66.4 ± 42.4 and 83.7 ± 51.1, respectively ($p > 0.05$, $n = 4$), and that in the presence of IL-27 was 96.9 ± 61.7 and 117.6 ± 85.0, respectively ($p > 0.05$, $n = 4$). IL-27-induced synergistic IFN-γ production with IL-12 was also reduced (Fig. 4*D*). In contrast, the proliferation induced by IL-27 in STAT1-deficient naive $CD8^+$ T cells activated with plate-coated anti-CD3 was similar or more augmented as compared with that in STAT1-deficient naive $CD8^+$ T cells (Fig. 4*E*). These results suggest that STAT1 plays a critical role in IL-27-mediated T-bet, IL-12Rβ2, granzyme B, and perforin expression,

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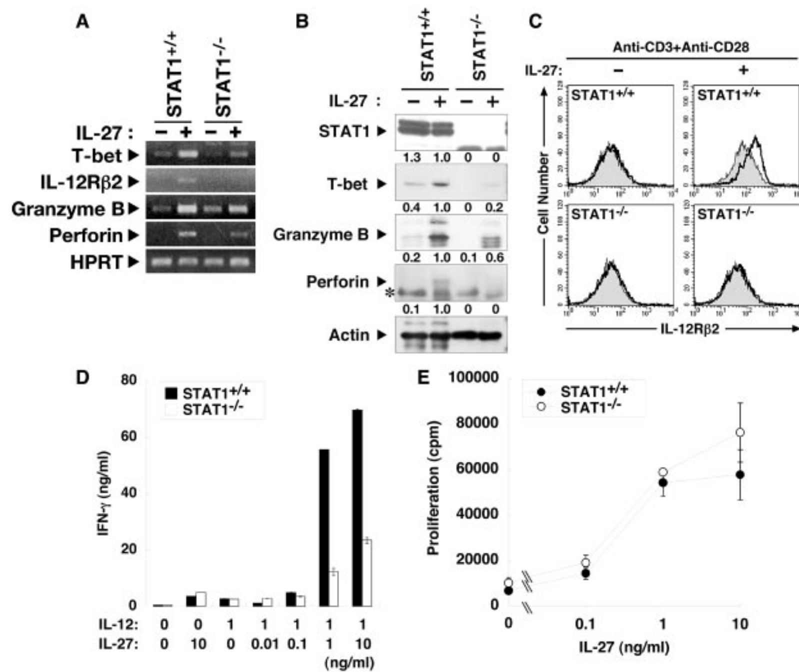

FIGURE 4. A critical role for STAT1 in IL-27-induced T-bet, IL-12Rβ2, granzyme B, and perforin expression, and synergistic IFN-γ production with IL-12, but not in proliferation in naive CD8$^+$ T cells. *A*, Purified naive CD8$^+$ T cells from wild-type and STAT1-deficient mice were stimulated with plate-coated anti-CD3, anti-CD28 (0.5 μg/ml), and IL-27 (5 ng/ml) in the presence of anti-IL-2 for 48 h, and total RNA was prepared and subjected to RT-PCR to detect the expression of T-bet, IL-12Rβ2, granzyme B, perforin, and HPRT at mRNA level. *B*, Total cell lysate was also prepared and subjected to Western blotting to detect the expression of T-bet, granzyme B, perforin, and actin at protein level. *, Indicates a nonspecific band. The densitometric intensity of each band was normalized to actin, and intensities relative to the wild-type cells stimulated with IL-27 are shown under each panel. *C*, These stimulated cells were analyzed for cell surface expression of IL-12Rβ2 (solid line) or its irrelevant isotype-matched control (plain line with shaded) by FACS. *D*, Purified naive CD8$^+$ T cells were stimulated with plate-coated anti-CD3, IL-27 (0.01, 0.1, 1, and 10 ng/ml), and/or IL-12 (1 ng/ml) in the presence of anti-IL-2. After 72 h, culture supernatants were collected and assayed for IFN-γ production by ELISA in triplicate. Data are shown as the mean $\pm$ SD. *E*, Purified naive CD8$^+$ T cells were stimulated with plate-coated anti-CD3 and IL-27 (0.1, 1, and 10 ng/ml) in the presence of anti-IL-2 for 72 h and pulsed with [^{3}H]thymidine for last 24 h. [^{3}H]Thymidine incorporation was measured in triplicate. Data are shown as the mean $\pm$ SD. Similar results were obtained in at least three independent experiments.

and synergistic IFN-γ production with IL-12, but not in proliferation in naive CD8$^+$ T cells.

An important role for T-bet in IL-27-induced IL-12Rβ2, granzyme B, and perforin expression, and synergistic IFN-γ production with IL-12, but not in proliferation in naive CD8$^+$ T cells

Recently, it has been demonstrated that T-bet-deficient CD8$^+$ T cells proliferate and expand normally after antigenic stimulation, but secrete low levels of IFN-γ, whereas IL-4 and IL-10 production is elevated (9). Therefore, we next examined the role for T-bet in IL-27-mediated immune responses in naive CD8$^+$ T cells using T-bet-deficient mice. We first confirmed the absence of T-bet expression in the T-bet-deficient naive CD8$^+$ T cells by RT-PCR and Western blotting (Fig. 5, *A* and *B*). IL-27-induced expression of granzyme B, perforin, and IL-12Rβ2 was diminished (Fig. 5, *A–C*). MFI for IL-12Rβ2 and control expression in the absence of IL-27 in wild-type naive CD8$^+$ T cells was 83.8 $\pm$ 44.9 and 84.0 $\pm$ 36.4, respectively ($p > 0.05$, $n = 4$), and that in the presence of IL-27 was 152.1 $\pm$ 101.6 and 102.6 $\pm$ 80.3, respectively ($p < 0.05$, $n = 4$). In contrast, MFI for IL-12Rβ2 and control expression in the absence of IL-27 in T-bet-deficient naive CD8$^+$ T cells was 104.7 $\pm$ 67.1 and 104.4 $\pm$ 64.9, respectively ($p > 0.05$, $n = 4$), and that in the presence of IL-27 was 120.8 $\pm$ 91.4 and 116.8 $\pm$ 82.6, respectively ($p > 0.05$, $n = 4$). IL-27-induced synergistic IFN-γ production with IL-12 was also reduced (Fig. 5*D*). In contrast, the proliferation induced by IL-27 in T-bet-deficient naive CD8$^+$ T cells activated with plate-coated anti-CD3 was almost similar to that in wild-type naive CD8$^+$ T cells (Fig. 5*E*). These results suggest that T-bet plays an important role in IL-27-mediated IL-12Rβ2, granzyme B, and perforin expression and synergistic IFN-γ production with IL-12, but not in proliferation in naive CD8$^+$ T cells.

A dispensable role for T-bet in IL-27-induced augmentation of generation of CTL

T-bet has been demonstrated to be required for the differentiation of naive CD8$^+$ T cells into effector CTLs under Ag-specific stimulation conditions (9). In addition, another T-box family member, Eomes, has been demonstrated recently to play a critical role in the generation of effector CD8$^+$ CTLs, including the induction of IFN-γ, perforin, and granzyme B expression (22). Therefore, we finally investigated the role for T-bet in IL-27-mediated augmentation of CTL activity using T-bet-deficient mice. Responder CD4$^+$ T cell-depleted spleen cells from wild-type BALB/c or T-bet-deficient mice were cultured with irradiated stimulator spleen cells from C57BL/6 mice in the presence of various amounts of IL-27 and anti-IL-2 or IL-2 as positive control. We first examined the expression of T-bet, granzyme B, perforin, and HPRT or actin in resultant responder cells (Fig. 6, *A* and *B*). T-bet expression was greatly enhanced in the presence of IL-27 in wild-type responder cells as in the presence of IL-2, while, as expected, no T-bet expression was detected in T-bet-deficient responder cells, regardless of the presence or absence of these cytokines. IL-27 greatly augmented the expression of Eomes and granzyme B in wild-type responder cells as IL-2 did, while the similar augmentation was

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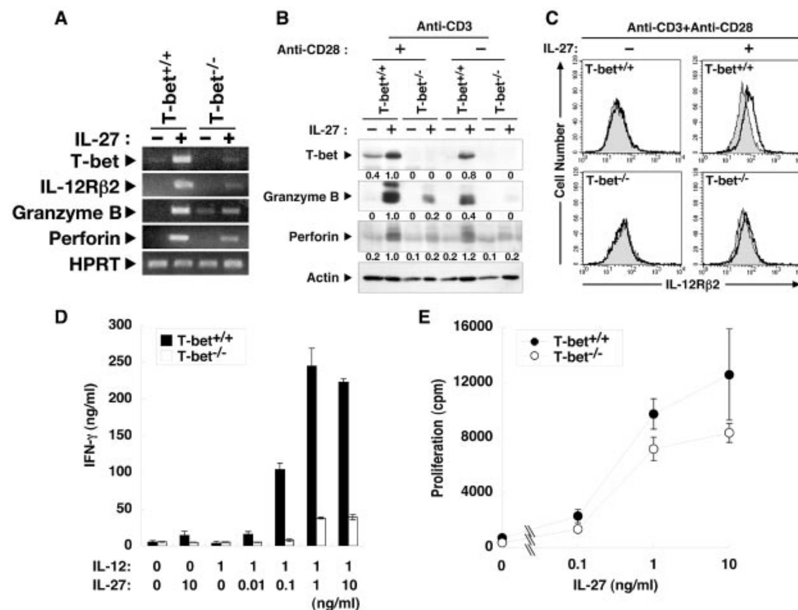

FIGURE 5. An important role for T-bet in IL-27-induced IL-12Rβ2, granzyme B, and perforin expression, and synergistic IFN-γ production with IL-12, but not in proliferation in naive CD8$^+$ T cells. *A*, Purified naive CD8$^+$ T cells from wild-type and T-bet-deficient mice were stimulated with plate-coated anti-CD3, anti-CD28 (0.5 μg/ml), and IL-27 (5 ng/ml) in the presence of anti-IL-2 for 48 h, and total RNA was prepared and subjected to RT-PCR to detect the expression of T-bet, IL-12Rβ2, granzyme B, perforin, and HPRT at mRNA level. *B*, Total cell lysate was also prepared and subjected to Western blotting to detect the expression of T-bet, granzyme B, perforin, and actin at protein level. The densitometric intensity of each band was normalized to actin, and intensities relative to the wild-type cells stimulated with IL-27 and anti-CD28 are shown under each panel. *C*, These stimulated cells were analyzed for cell surface expression of IL-12Rβ2 (solid line) or its irrelevant isotype-matched control (plain line with shaded) by FACS. *D*, Purified naive CD8$^+$ T cells were stimulated with plate-coated anti-CD3, IL-27 (0.01, 0.1, 1, and 10 ng/ml), and/or IL-12 (1 ng/ml) in the presence of anti-IL-2. After 72 h, culture supernatants were collected and assayed for IFN-γ production by ELISA in triplicate. Data are shown as the mean ± SD. *E*, Purified naive CD8$^+$ T cells were stimulated with plate-coated anti-CD3 and IL-27 (0.1, 1, and 10 ng/ml) in the presence of anti-IL-2 for 72 h and pulsed with [^{3}H]thymidine for last 24 h. [^{3}H]Thymidine incorporation was measured in triplicate. Data are shown as the mean ± SD. Similar results were obtained in at least three independent experiments.

still observed in T-bet-deficient responder cells as by IL-2. Perforin expression was marginally induced by IL-27 in wild-type responder cells, but less in T-bet-deficient responder cells. IL-2 more efficiently induced perforin expression even in T-bet-deficient responder cells. We then investigated whether or not T-bet is required for IL-27-induced augmentation of CTL generation (Fig. 6*C*). Responder CD4$^+$ T cell-depleted spleen cells from wild-type BALB/c or T-bet-deficient mice were cultured with irradiated stimulator spleen cells from C57BL/6 mice in the presence of various amounts of IL-27 and anti-IL-2 or IL-2 as positive control for 5 days and were used as effector cells to determine allospecific CTL activity against target cell EL-4. Consistent with the increased expression of granzyme B even in T-bet-deficient responder cells (Fig. 6, *A* and *B*), IL-27 still augmented the generation of CTL in T-bet-deficient responder cells (Fig. 6*C*) as IL-2 did (data not shown). These results suggest that T-bet is not essential for IL-27-induced augmentation of allospecific CTL generation with enhanced granzyme B expression, and presumably imply that IL-27-induced augmentation of Eomes expression may compensate for the absence of T-bet.

Discussion

Recently, we have found that IL-27 has a potent ability to induce tumor-specific antitumor activity, which is mainly mediated through CD8$^+$ T cells with enhanced CTL activity (11), and also that IL-27 has a potent adjuvant activity for the induction of HCV-specific CTLs (12). Consistent with these two studies indicating that IL-27 augments CTL activity in vivo, we have shown in this study that IL-27 directly acts on naive CD8$^+$ T cells in a manner similar to CD4$^+$ T cells and augments generation of CTL with enhanced expression of granzyme B. In naive CD8$^+$ T cells stimulated with anti-CD3, IL-27 activated STAT1, -2, -3, -4, and -5; augmented the expression of T-bet, IL-12Rβ2, and granzyme B, and slightly that of perforin; and induced synergistic IFN-γ production with IL-12 and proliferation. These actions by IL-27, except proliferation, were highly dependent on STAT1 and T-bet. However, because the IL-27-induced augmentation of granzyme B and perforin expression in these deficient CD8$^+$ T cells was not completely abolished, we cannot formally rule out the possibility that other molecules such as STAT3 and Eomes may also contribute to the augmentation. As a matter of fact, one of the other cytokines using gp130 and STAT3 as one of their receptor subunits and signaling molecules, IL-6, was previously demonstrated to have an ability to augment CTL generation as well (23, 24). Moreover, IL-27 enhanced proliferation of CD4$^+$ T cell-depleted spleen cells stimulated with allogeneic spleen cells and augmented the generation of allospecific CTL, whose actions were hardly dependent on T-bet. Thus, IL-27 directly acts on naive CD8$^+$ T cells in T-bet-dependent and -independent manners and augments generation of CTL.

It has been demonstrated recently that a member of T-box family transcription factor, T-bet, known to be responsible for controlling lineage commitment in Th1 cells (8), also controls the generation of effector CD8$^+$ CTLs (9). T-bet is required for differentiation of naive CD8$^+$ T cells into effector CTLs by Ag-specific stimuli (9), but not by Ag-nonspecific stimuli, such as plate-coated anti-CD3/anti-CD28 (10). In addition, another T-box family member, Eomes, has been demonstrated recently to play a critical role in the generation of effector CD8$^+$ CTLs, including the induction of IFN-γ, perforin, and granzyme B expression (22).

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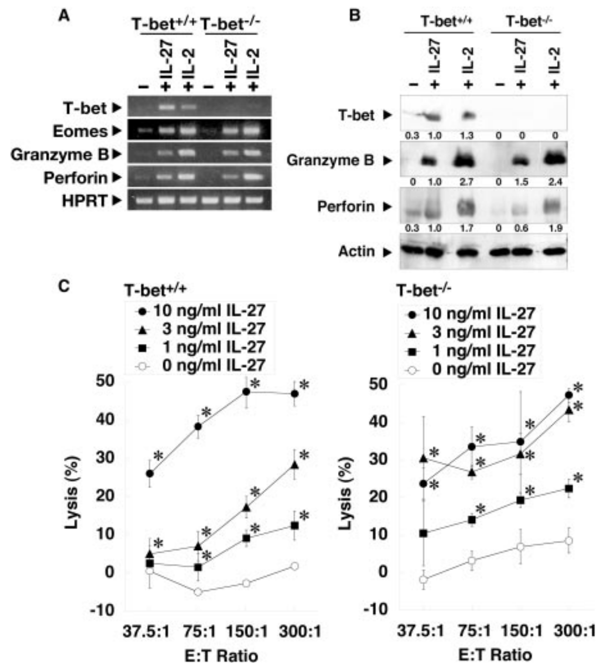

FIGURE 6. A dispensable role for T-bet in IL-27-induced augmentation of generation of CTL. *A*, $CD4^+$ T cell-depleted spleen cells from wild-type BALB/c (H-2^d) or T-bet-deficient mice were stimulated in vitro by coculture with irradiated spleen cells from C57BL/6 (H-2^b) mice in the presence of various amounts of IL-27 and anti-IL-2 or IL-2 as positive control for 4 days. Resultant responder cells were determined for expression of T-bet, Eomes, granzyme B, perforin, and HPRT by RT-PCR. *B*, Resultant responder cells obtained after culture for 5 days, as described above, were also determined for expression of T-bet, granzyme B, perforin, and actin by Western blotting. The densitometric intensity of each band was normalized to actin, and intensities relative to the wild-type cells stimulated with IL-27 are shown under each panel. *C*, $CD4^+$ T cell-depleted spleen cells from wild-type BALB/c or T-bet-deficient mice were stimulated in vitro by coculture with irradiated spleen cells from C57BL/6 mice in the presence of various amounts of IL-27 and anti-IL-2 for 5 days and used as effector cells in a standard ^{51}Cr release assay. Data are shown as the mean ± SD. *, Indicates $p < 0.05$, compared with wild-type naive $CD8^+$ T cells. Similar results were obtained in three independent experiments.

Thus, these two T-box family members, T-bet and Eomes, appear to cooperatively control the generation of effector functions in $CD8^+$ T cells. However, because Eomes appears not to be involved in up-regulation of IL-12Rβ2 expression unlike T-bet, Th1 differentiation is predominantly regulated by T-bet (22). Induction of granzyme B expression and augmentation of allospecific CTL generation by IL-27 was still observed even in T-bet-deficient responder $CD4^+$ T cell-depleted spleen cells stimulated with allogeneic spleen cells. These results suggest that other molecule(s) such as, presumably Eomes, could play a role in the augmentation of granzyme B expression and CTL generation by IL-27. Indeed, we observed the augmentation of Eomes mRNA expression by IL-27 in the responder cells regardless of the presence or absence of T-bet (Fig. 6*A*). The role of Eomes in IL-27-induced augmentation of granzyme B expression and CTL generation is currently under investigation. Alternatively, these results may also imply the possibility that T-bet is required for differentiation of naive $CD8^+$ T cells into effector CTLs by Ag-specific stimuli, but not by allogeneic stimuli. This is because the stimuli used in the previous study (10) are not only Ag nonspecific, but also allogeneic.

IL-27 augments proliferation in naive, but not memory $CD4^+$ T cells, induces expression of T-bet and resultant IL-12Rβ2, and synergizes with IL-12 in IFN-γ production (1, 2). Similarly, the present study has revealed that IL-27 induces expression of T-bet and resultant IL-12Rβ2, synergizes with IL-12 in IFN-γ production, and augments proliferation in naive $CD8^+$ T cells. Moreover, IL-27 enhances granzyme B expression in MLR and generation of allospecific CTL mostly in a T-bet-independent manner. We have demonstrated recently that IL-27 induces T-bet expression and IgG2a, but not IgG1, class switching in B cells in a STAT1-dependent, but IFN-γ-independent manner, and that IL-27 rather inhibits IL-4-induced IgG1 class switching (16). The IL-27-induced IgG2a class switching is highly dependent on T-bet in response to T-independent stimuli such as LPS, while it is much less dependent on T-bet in response to T-dependent stimuli, such as anti-CD40. Although anti-inflammatory property of IL-27 was also demonstrated in recent studies using WSX-1-deficient mice (25–27), IL-27 thus plays roles by directly acting on $CD4^+$ T cells, $CD8^+$ T cells, and B cells, to not only induce the initiation of Th1 differentiation and IgG2a class switching, but also augment generation of CTL in T-bet-dependent and -independent manners, resulting in enhanced type 1 cell-mediated immunity.

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Disclosures

The authors have no financial conflict of interest.

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