Transcription Factor Early Growth Response 3 Is Associated with the TGF-β1 Expression and the Regulatory Activity of CD4-Positive T Cells In Vivo

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TGF-β1 is an important anti-inflammatory cytokine, and several regulatory T cell (Treg) subsets including $CD4^+CD25^+Foxp3^+$ Tregs and Th3 cells have been reported to exert regulatory activity via the production of TGF-β1. However, it has not yet been elucidated which transcription factor is involved in TGF-β1 transcription. Early growth response 3 (Egr-3) is a zinc-finger transcription factor that creates and maintains T cell anergy. In this study, we found that Egr-3 induces the expression of TGF-β1 in both murine and human $CD4^+$ T cells. Egr-3 overexpression in murine $CD4^+$ T cells induced the production of TGF-β1 and enhanced the phosphorylation of STAT3, which is associated with TGF-β1 transcription. Moreover, Egr-3 conferred Ag-specific regulatory activity on murine $CD4^+$ T cells. In collagen-induced arthritis and delayed-type hypersensitivity model mice, Egr-3–transduced $CD4^+$ T cells exhibited significant regulatory activity in vivo. In particular, the suppression of delayed-type hypersensitivity depended on TGF-β1. In human tonsils, we found that $CD4^+CD25^-CD45RO^-$lymphocyte activation gene 3 $(LAG3)^-$ T cells express membrane-bound TGF-β1 in an EGR3-dependent manner. Gene-expression analysis revealed that $CD4^+CD25^-CD45RO^-LAG3^-$ T cells are quite different from conventional $CD4^+CD25^+Foxp3^+$ Tregs. Intriguingly, the $CD4^+CD25^-CD45RO^-LAG3^-$ T cells suppressed graft-versus-host disease in immunodeficient mice transplanted with human PBMCs. Our results suggest that Egr-3 is a transcription factor associated with TGF-β1 expression and in vivo regulatory activity in both mice and humans. *The Journal of Immunology*, **2013, 191: 2351–2359.**

Autoreactive $CD4^+$ T cells that escape central tolerance are an essential component of autoimmune disease. Suppressing autoreactive $CD4^+$ T cells and establishing peripheral tolerance is necessary for controlling autoimmune disease. There are several mechanisms for suppressing autoreactive T cells including anergy, activation-induced cell death, and active suppression by regulatory T cells (Tregs). Tregs strongly suppress the activity of effector T cells. There are various Treg subsets, and interest in Tregs has mainly focused on $CD4^+CD25^+Foxp3^+$ Tregs. Mice and humans without normal $CD4^+CD25^+Foxp3^+$ T cells develop multiorgan autoimmune inflammatory disease because they fail to regulate the activity of effector T cells (1, 2). With regard to the mode of effector T cell suppression by Tregs, several mechanisms have been proposed, including cell–cell contact, inhibitory cytokine release, cytolysis using granzyme B, and dendritic cell targeting (3). Among these, the production of inhibitory cytokines, for example IL-10, TGF-β1, and IL-35, is regarded as one of the principal mechanisms of effector T cell suppression by Tregs.

TGF-β1 is an inhibitory cytokine that is important for peripheral immune homeostasis because it has powerful antiproliferative and antiapoptotic effects (4, 5). TGF-β1 knockout mice develop early onset autoimmune disease, resulting in 100% lethality by 4 wk of age (6). Moreover, TGF-β1 plays an essential role in the differentiation of $CD4^+$ T cells. In vitro stimulation of naive T cells in the presence of TGF-β1 leads to the induction of Foxp3 (7, 8); however, treatment with a combination of TGF-β1 and IL-6 results in the induction of Th17 cells (9, 10). TGF-β1 also inhibits Th1 differentiation (11). Although the mechanism by which TGF-β1 suppresses immune reactions is not fully understood, its induction of $CD4^+CD25^+Foxp3^+$ Tregs and its inhibition of Th1 differentiation might contribute to its regulatory activity.

The significance of TGF-β1 as an inhibitory cytokine for peripheral immune homeostasis is well understood, and there are several reports about the source of TGF-β1 secretion. Th3 cells (12) and $CD4^+CD25^-$latency-associated peptide $(LAP)^+$ T cells (13) are reported to secrete TGF-β1; however, the regulation of TGF-β1 secretion is not fully understood. It was reported that STAT3 positively regulates TGF-β1 promoter activity and enhances TGF-β1 production (14, 15). Han et al. (16) reported that the binding of CD69 maintains the expression of membrane-bound TGF-β1 on $CD4^+CD25^-CD69^+$ T cells via ERK activa-

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Abbreviations used in this article: bCII, bovine type II collagen; CIA, collagen-induced arthritis; CIIT, bovine type II collagen-specific TCR; CKO, conditional knockout; DTH, delayed-type hypersensitivity; Egr-3, early growth response 3; ES, embryonic stem; GVHD, graft-versus-host disease; LAG3, lymphocyte activation gene 3; NOG, NOD/Shi-*scid* IL-2Rγ^{null}; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling; Treg, regulatory T cell.

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tion. Meanwhile, no transcription factors that regulate TGF-β1 secretion have been discovered.

It is worth noting that most T cell populations with regulatory activity are anergic. In this study, we examined the link between anergy-related molecules and regulatory activity. The early growth response (Egr) family members Egr-2 and Egr-3 are zinc-finger transcription factors that are reported to be associated with T cell anergy (17, 18). They display increased expression in anergic T cells and suppress the expression of IL-2. Moreover, T cells from Egr-3–deficient mice are resistant to anergy induction. TCR-induced Egr-1 and NGFI-A–binding protein 2 enhance T cell function, and Egr-2 and Egr-3 inhibit T cell function via the suppression of Egr-1 and NGFI-A–binding protein 2 expression (19). We have previously reported that Egr-2 is specifically expressed in IL-10–producing $CD4^+CD25^-$lymphocyte activation gene 3 $(LAG3)^+$ Tregs and that Egr-2 confers IL-10 and LAG3 expression and in vivo regulatory activity on $CD4^+$ T cells (20).

Recently, the emerging role of Egr-2 and Egr-3 in regulating T cell activation is extensively investigated. CD2-specific Egr-2–deficient (CD2-$Egr2^{-/-}$) mice develop systemic autoimmunity in later life (21), and B and T cell responses to Ag receptor stimulation in vitro were unchanged in the mice. Li et al. (22) reported the findings of mice with defects in both Egr-2 and Egr-3 in T and B cells (CD2-$Egr2^{-/-}Egr3^{-/-}$). Although $Egr\text{-}3^{-/-}$ mice do not develop any autoimmune symptoms, the CD2-$Egr\text{-}2^{-/-}Egr\text{-}3^{-/-}$ mice develop severe systemic autoimmune syndrome in their early life, with excessive accumulation of serum proinflammatory cytokines. In CD2-$Egr\text{-}2^{-/-}Egr\text{-}3^{-/-}$ mice, expression of suppressor of cytokine signaling (SOCS) 1 and SOCS3, inhibitors of STAT1 and STAT3, is increased, and Egr-2 was found to directly enhance promoter activity of both SOCS1 and SOCS3. Thus, Egr-3 may compensate for the function of Egr-2 in Egr-2 conditional knockout (CKO) mice because their phenotype is milder than CD2-$Egr2^{-/-}$ $Egr3^{-/-}$ mice. Although $Egr\text{-}3^{-/-}$ mice do not develop any autoimmune symptoms, Egr-2 may possibly compensate for the function of Egr-3. Therefore, functional analysis focused on Egr-3 is required for the understanding of autoimmunity. In this study, we focused on Egr-3, another Egr family member, and examined whether Egr-3 is associated with the anti-inflammatory cytokine production and regulatory activity of $CD4^+$ T cells.

In our experiment, contrary to Egr-2, Egr-3 induced the expression of TGF-β1 in $CD4^+$ T cells. Egr-3 conferred in vivo Ag-specific regulatory activity on murine $CD4^+$ T cells in a TGF-β1–dependent manner. In humans, we found that tonsillar $CD4^+CD25^-CD45RO^-$ $LAG3^-$ T cells showed EGR3-dependent membrane-bound TGF-β1 expression. Intriguingly, $CD4^+CD25^-CD45RO^-LAG3^-$ T cells suppressed graft-versus-host disease (GVHD) in immunodeficient mice transplanted with PBMCs. Our results suggest that Egr-3 is a transcription factor that is associated with TGF-β1 expression and in vivo regulatory activity in both mice and humans.

Materials and Methods

Animals

C57BL/6, BALB/c, and DBA/1J mice were purchased from Japan SLC (Shizuoka, Japan). OVATCR-transgenic DO11.10, C57BL/6-IL-$10^{-/-}$, and STAT3-flox/flox mice were obtained from The Jackson Laboratory (Bar Harbor, ME). NOD/Shi-*scid* IL-$2R\gamma^{null}$ (NOG) mice were obtained from CIEA Japan (Tokyo, Japan). All animal experiments were conducted in accordance with the institutional and national guidelines.

Generation of Egr-3 CKO mice

Targeting vector was made to insert loxp and neomycin sequences in the second exon sequence. Embryonic stem (ES) cells from C57BL/6 mice were transfected with the targeting construct, and ES genotyping was screened using PCR and Southern blotting. Positive ES cells were injected into blastocyst, and chimera mice were generated. The T cell–specific Egr-3 KO mice were generated by crossing the CD4-Cre mice with Egr-3 flox/flox mice.

Reagents, Abs, and media

The following reagents were purchased from BD Pharmingen: purified anti-mouse CD3ε mAb (145-2C11), purified anti-mouse CD28 mAb (37.51), biotin-conjugated anti-mouse CD8a mAb (53-6.7), biotin-conjugated anti-mouse CD11b mAb (HL3), biotin-conjugated anti-mouse CD19 mAb (1D3), Fc block (anti-mouse CD16/CD32 mAb), Alexa Fluor 647 anti-mouse CD4 mAb (RM4-5), PE anti-mouse CD4 mAb (H129.19), APC rat anti-mouse IgG1 (X56), and Alexa Fluor 647 anti–phospho-STAT3 (Y705) mAb (4/P-Stat3). The following reagents were purchased from eBioscience: human FcγR-binding inhibitor, PE-Cy7 anti-human CD25 mAb (BC96), and PE anti-human CD45RO mAb (UCHL1). The following reagents were purchased from R&D Systems: anti-human CD3ε mAb (UCHT1), anti-human TGF-β1 mAb (9016), anti–TGF-β1, -β2, -β3 mAb (1D11), recombinant mouse IL-2, and recombinant mouse IL-6. ATTO488 anti-human LAG3 mAb (17B4) was purchased from Alexis Enzo Life Sciences. PE anti-human CD45 (HI30) and PerCP/Cy5.5 anti-human CD4 mAb (RPA-T4) were purchased from BioLegend. Streptavidin-conjugated microbeads were purchased from Miltenyi Biotec. The culture medium used in all experiments, except the TGF-β1 cytokine assays, was RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME (all purchased from Life Technologies). For the TGF-β1 cytokine assays, splenocytes were cultured in X-VIVO 20 serum-free medium (BioWhittaker) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Plat-E was grown in DMEM (Sigma-Aldrich) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

FACS analysis and cell sorting

The murine infectant cells were stained with Alexa Fluor 647 anti-mouse CD4 mAb after Fc-blocking (anti-mouse CD16/CD32 mAb) and sorted according to the intensity of their GFP expression. The human tonsillar cells were stained with PerCP/Cy5.5 anti-human CD4 mAb, PE-Cy7 anti-human CD25 mAb, PE anti-human CD45RO mAb, ATTO488 anti-human LAG3 mAb after Fc-blocking (human FcγR-binding inhibitor), $CD4^+CD25^-$ $CD45RO^-LAG3^-$, $CD4^+CD25^-CD45RO^+LAG3^-$, and $CD4^+CD25^+$ $LAG3^-$ T cells were sorted. For the staining of membrane-bound TGF-β1, 2×10^5 $CD4^+CD25^-CD45RO^-LAG3^-$ T cells or $CD4^+CD25^-CD45RO^+$ $LAG3^-$ T cells were sorted and stimulated for 5 d on 96-well microplates that had been coated with 1 μg/ml anti-human CD3 mAb and stained with anti-human TGF-β1 mAb and APC rat anti-mouse IgG1. To check the engraftment of PBMC-transferred NOG mice, splenocytes of NOG mice were stained with PE anti-human CD45 after Fc-blocking. The FACS analysis and cell sorting were performed with EPICS ELITE (Beckman Coulter), FACSVantage SE (BD Biosciences), and FACSAriaII (BD Biosciences).

RNA isolation, cDNA synthesis, and quantitative real-time PCR

The RNA of the cells was extracted using the RNeasy Micro Kit (Qiagen) and then reverse-transcribed to cDNA with random primers (Invitrogen) and Superscript III (Invitrogen), according to the manufacturer's protocol. To determine the cellular expression of each protein, quantitative real-time PCR analysis was performed using an iCycler (Bio-Rad). The PCR mixture consisted of 25 μl SYBR Green Master Mix (Qiagen), 15 pM forward and reverse primers, and the cDNA samples in a total volume of 50 μl. We calculated the quantitative PCR data with the Δ threshold cycle method, and relative RNA abundance was determined based on control GAPDH abundance. For murine cells, the real-time PCR primer pairs were as follows: mouse *egr3* sense, 5′-CAACGACATGGGCTCCATTC-3′ and antisense, 5′-GGCCTTGATGGTCTCCAGTG-3′; mouse *lag3* sense, 5′-TTGCTTCTGGGACTGCTTTG-3′ and antisense, 5′-GCCACTGTCTG-GTTGATGTTG-3′; mouse *il10* sense, 5′-GGTTGCCAAGCCTTATCG-GA-3′, antisense, 5′-ACCTGCTCCACTGCCTTGCT-3′; and mouse *gapdh* sense, 5′-AGAGGGAAATCGTGCGTGAC-3′ and antisense, 5′-CAA-TAGTGATGACCTGGCCGT-3′. For human cells, the real-time PCR primer pairs were as follows: human EGR3 sense, 5′-GGAGCAAAT-GAAATGTTGGTG-3′ and antisense, 5′-AGGAAAACCTATGGGGAA-TG-3′; human TGFB1 sense, 5′-AGCGACTCGCCAGAGTGGTTA-3′ and antisense, 5′-GCAGTGTGTTATCCCTGCTGTCA-3′; human IFNG sense, 5′-CCAACGCAAAGCAATACATGA-3′ and antisense, 5′-CCTT-TTTCGCTTCCCTGTTTTA-3′; human *IL10* sense, 5′-GAGATGCCTT-CAGCAGAGTGAAGA-3′ and antisense, 5′-AGGCTTGGCAACCCAG-

GTAAC-3′; human FOXP3 sense, 5′-GAAACAGCACATTCCCAGAG-TTC-3′ and antisense, 5′-ATGGCCCAGCGGATGAG-3′; human EGR2 sense, 5′-GCACCAGCTGTCTGACAACATCTAC-3′ and antisense, 5′-AGCAAAGCTGCTGGGATATGG-3′; human GAPDH sense, 5′-GCTC-TCCAGAACATCATCCCTGCC-3′ and antisense, 5′-CGTTGTCATACC-AGGAAATGAGCTT-3′.

Retroviral gene transduction

cDNA for murine Egr-2 and Egr-3 were isolated from a murine T lymphocyte cDNA library according to the nucleotide sequences reported in the National Center for Biotechnology Information database (Egr-2:NM_010118 and Egr-3:NM_018781). Each full-length fragment was inserted into pMIG and designated as pMIG–Egr-2 or pMIG–Egr-3. For the bovine type II collagen-specific TCR (CIIT) (23) construct, we constructed the pMX-CIIT TCR (pMX-CIITα-internal ribosome entry site-CIITβ) vector (23). For the OVA-specific TCR (DO11.10) construct, we used the previously constructed vectors pMX-DOTAE and pMX-DOTBE (I-A^d restricted, $OVA_{323–339}$-specific TCR) (24). Plat-E (25) packaging cells were transfected with a retrovirus vector using the FuGENE 6 transfection reagent (Roche), and retroviral supernatants were harvested at 48 h after the transfection. Retroviral gene transduction was performed as described previously (24, 26). Total splenocytes were cultured for 48 h in the presence of Con A (10 μg/ml) and recombinant murine IL-2 (50 ng/ml). The viral supernatant was preloaded onto each well of the CH296 (RetroNectin; Takara-Bio)–coated 24-well plate, and the plate was spun at 1200 × *g* for 3 h at room temperature. This procedure was repeated a total of three times. The viral supernatant was removed, and stimulated splenocytes were plated onto each well (1×10^6 cells/well). Cells were cultured for 48 h to allow infection to occur.

Cytokine immunoassays

$CD4^+$ GFP-positive cells were sorted from infectants, and 1×10^5 cells were stimulated on 96-well microplates that had been coated with anti-CD3 mAb or anti-CD3/anti-CD28 mAb (each 10 μg/ml). The supernatant was collected 48 h after the stimulation, and the IL-10 concentration was measured with sandwich ELISAs using a BD OptEIA Mouse IL-10 set (BD Pharmingen), according to the manufacturer's protocol. The TGF-β1 concentration in the supernatant of 1×10^5 infectant cells stimulated for 72 h was measured with a Quantikine Mouse/Rat/Porcine/Canine TGF-β1 Immunoassay (R&D Systems). The samples were acidified by adding hydrogen chloride for 10 min and then neutralized with sodium hydroxide. An automatic microplate reader (Bio-Rad 550; Bio-Rad) was used to measure the OD of the samples.

p-STAT3 and p-STAT1 intracellular staining

Splenocytes from C57BL/6 or IL-10 knockout mice were gene transduced retrovirally. $CD4^+$ T cells were enriched using a MACS Cell Separation Kit (Miltenyi Biotec), and 1×10^6 cells were stimulated on 24-well plates that had been coated with anti-CD3/anti-CD28 mAb (each 10 μg/ml). Three days later, the cells were stained with PE anti-mouse CD4 mAb after Fc-blocking, lysed, and fixed in a single step using Lyse/Fix Buffer (BD Biosciences) for 10 min at 37°C. The cells were then permeabilized in Perm Buffer III (BD Biosciences) for 30 min on ice, before being washed twice in Stain Buffer (BD Biosciences) and stained with Alexa Fluor 647 anti–phospho-STAT3 (Y705) mAb or phospho-STAT1 (Y701) mAb for 30 min at room temperature. To produce a positive control of pSTAT3 staining (27), 1×10^6 splenocytes were stimulated on 24-well plates in the presence of 100 ng/ml recombinant mouse IL-6 for 15 min.

Collagen-induced arthritis

Collagen-induced arthritis (CIA) was induced as described previously (27, 28). DBA/1J mice were s.c. immunized with 100 μg bovine type II collagen (bCII) (Chondrex) emulsified with CFA, which was intradermally injected at the base of the tail on day 1. On day 20, DBA/1J splenocytes transduced with pMIG, pMIG–Egr-3, pMIG plus pMX-CIIT, or pMIG–Egr-3 plus pMX-CIIT were CD4 enriched with a MACS kit, and 1×10^7 cells were i.v. injected into each bCII-immunized mouse. On day 21, the mice were given a booster intradermal injection of 100 μg bCII emulsified with IFA. The arthritis score for each mouse was determined by assessing the degree of erythema, swelling, or ankylosis in each paw, as described previously (29). The clinical arthritis score of each mouse was defined as the sum of the scores for all four paws.

Pathological analysis of CIA mice

The tarsal joints of sacrificed CIA mice were embedded in paraffin wax after 10% formaldehyde fixation and decalcification. The sections were then stained with H&E. The synovial tissues were graded for mononuclear cell infiltration and pannus invasion, as described previously (30).

Delayed-type hypersensitivity

On day 1, BALB/c mice were s.c. immunized with 200 μg OVA (Sigma-Aldrich) emulsified with CFA at the base of the tail. On day 7, BALB/c splenocytes transduced with pMIG, pMIG–Egr-3, pMIG plus pMX-DOTAE, and pMX-DOTBE (DO11.10) or pMIG–Egr-3 plus pMX-DOTAE and pMX-DOTBE (DO11.10) were CD4 enriched with a MACS kit, and 5×10^6 cells were i.v. injected into each OVA-immunized mouse. On day 9, the thickness of both hind footpads was measured with an electrical micrometer caliper (Mitsutoyo), and then the mice were s.c. immunized with 100 μg OVA in 100 μl PBS at their right hind footpad. As a control, 100 μl PBS were also s.c. injected into the left hind footpad. Twenty-four hours after the reimmunization procedure, the thickness of each injected footpad was measured. Results were expressed as: specific footpad swelling = (24 h measurement − 0 h measurement) for experimental footpad − (24 h measurement − 0 h measurement) for control footpad. To examine the effect of neutralizing TGF-β1, 75 μg anti–TGF-β1, -2, and -3 Ab was i.p. injected on the same day as the gene-transduced cells. In this neutralizing experiment, 1×10^7 $CD4^+$ T cells transduced with pMIG or pMIG–Egr-3 were adoptively transferred into each OVA-immunized mouse. Seventy-five micrograms mouse IgG1 (R&D Systems) was i.p. injected into the negative control mice.

Isolation of human tonsillar cells and PBMCs

Human tonsils and peripheral blood were obtained from patients undergoing routine tonsillectomy at The University of Tokyo Hospital or Showa General Hospital. All patients or their parents gave informed consent. The use of the tissue samples was approved by the institutional review boards of both The University of Tokyo and Showa General Hospital. The tonsil samples were cut into pieces, and the lymphoid cells in the tissue were pushed through a sieve using a plastic syringe plunger. A cell suspension was obtained by rinsing the sieve with HBSS and slowly layered over Ficoll-Paque PLUS (GE Healthcare). Tonsillar mononuclear cells were isolated by density centrifugation at 1800 rpm for 20 min. Human peripheral blood was drawn using a heparin-coated syringe and slowly layered over Ficoll-Paque PLUS. PBMCs were isolated by density centrifugation at 1000 rpm for 30 min.

Small interfering RNA

Accell human EGR3 small interfering RNA (siRNA) and control siRNA were purchased from Thermo Scientific. According to the manufacturer's manual, 1×10^5 human T cells were mixed with Accell siRNA and Accell siRNA delivery media in the presence of 1 μg/ml anti-human CD3 mAb. Membrane-bound TGF-β1 expression was analyzed 24 h later, and mRNA analysis was performed 48 h later.

Induction of GVHD in NOG mice

Xenogenic GVHD was induced as reported previously (31). Human PBMCs and tonsillar mononuclear cells were isolated by density centrifugation and washed in PBS. NOG mice were irradiated with 2.5 Gy on the day before the cell transfer. Then, 5×10^6 PBMCs were transferred into the irradiated NOG mice through the tail vein, and 2×10^5 of tonsillar $CD4^+$ T cells were simultaneously cotransferred. Body weight was measured daily.

Pathological analysis of NOG mice

Liver, lungs, and kidneys from mice transplanted with human PBMC were fixed with 4% paraformaldehyde and embedded in paraffin. Sections were placed on glass slides and deparaffinized before being subjected to Ag retrieval and autoclaving (120°C, 10 min). After being blocked with fat-free milk, the sections were incubated with anti-human CD45 mAbs (DakoCytomation) overnight at 4°C and then were serially incubated with HRP-labeled anti-mouse Ig. The signals were amplified using the CSAII catalyzed signal amplification system (DakoCytomation). For color development, these sections were incubated with 0.02% 3,3′-diaminobenzidine substrate solution containing 0.006% H_2O_2. The immunostained sections were counterstained with hematoxylin to allow visualization of the nuclei. The immunohistochemical analysis was performed by the Biopathology Institute (Oita, Japan). Perivascular infiltration in the lung of human cells was measured according to the method of a previous report (32). ImageJ software (National Institutes of Health) was used to quantify the density of anti-human CD45 Ab-labeled mononuclear cells within perivascular infiltrates.

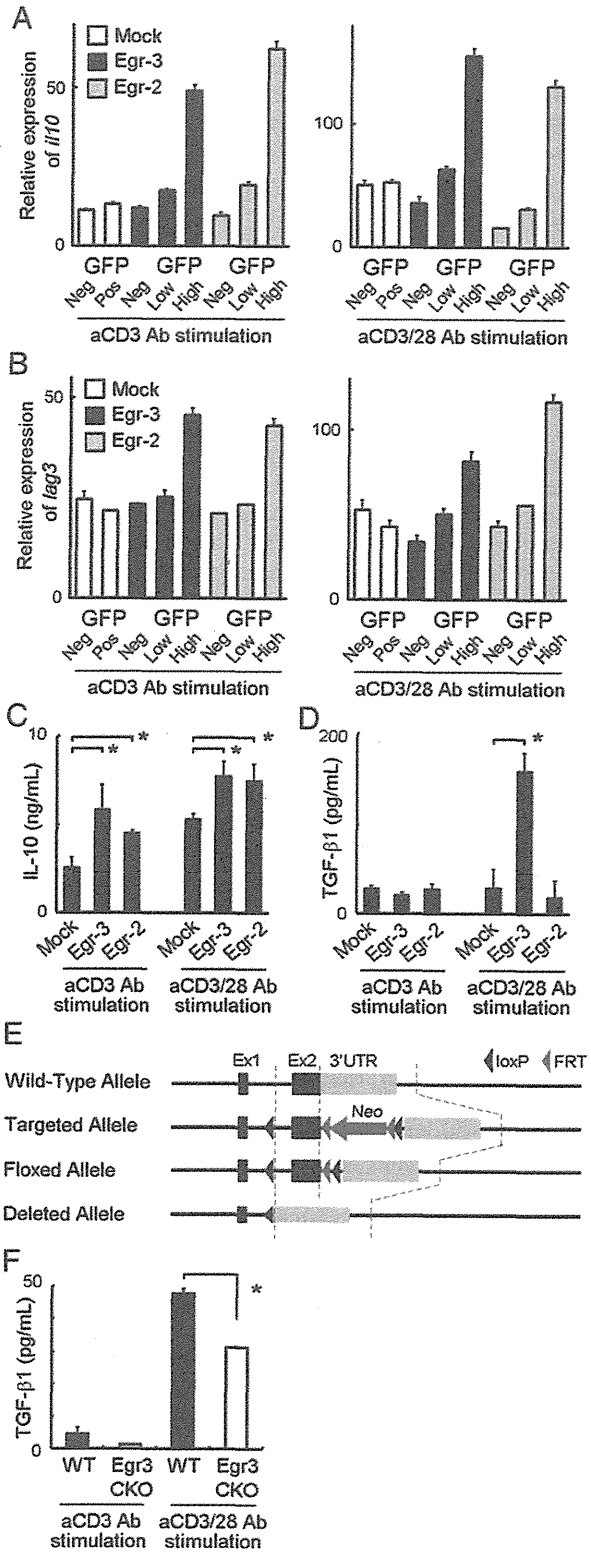

FIGURE 1. Egr-3 increases the mRNA expression of IL-10 and LAG3 and specifically induces TGF-β1 secretion. (**A** and **B**) Egr-2 or Egr-3 was transduced into mice splenocytes and then was stimulated with anti-CD3 mAb, or anti-CD3/anti-CD28 mAb. Forty-eight hours later, $CD4^+$ T cells were sorted according to the intensity of their GFP expression. Quantitative PCR was used to determine the relative mRNA expression levels of *il10* (A) and *lag3* (B) compared with *gapdh*. The results are shown as the means of three independent experiments. (**C** and **D**) Egr-2– or Egr-3–transduced $CD4^+$ cells were sorted and stimulated with anti-CD3 mAb alone or anti-CD3/anti-CD28 mAb. ELISA of IL-10 (C) and TGF-β1 (D) in the supernatant fluid of gene-transduced T cells are presented. (**E**) Schematic illustration for the generation of Egr-3 flox/flox mice and $CD4^+$ T cell–specific deletion of Egr-3 by mating with CD4-Cre mice. (**F**) Egr-3–deficient or wild-type (WT) $CD4^+$ T cells were stimulated with anti-CD3 mAb alone or anti-CD3/anti-CD28 mAb. ELISA of TGF-β1 in the supernatant fluid is presented. The results are shown as the means of three independent experiments. All error bars represent SD. $*p < 0.01$. Neg, Negative; Pos, positive; UTR, untranslated region.

Statistical analysis

Data are expressed as means ± SD. All results were obtained from at least three independent experiments. Statistical significance was determined by unpaired Student *t* tests, and differences among groups were assessed with the Bonferroni/Dunn test.

Results

Egr-3 increases the mRNA expression of IL-10 and LAG3 and specifically induces TGF-β1 secretion

We investigated whether Egr-3 expression in murine $CD4^+$ T cells induces the expression of suppressive cytokines or molecules. After 48-h stimulation with anti-CD3 mAb alone or anti-CD3/anti-CD28 mAb, Egr-2– or Egr-3–transduced $CD4^+$ T cells were sorted according to the intensity of their GFP expression. Similar to our previous observation that Egr-2 induces LAG3 and IL-10 expression in $CD4^+$ T cells (20), quantitative PCR showed that Egr-3 induced the mRNA expression of IL-10 (Fig. 1A) and LAG3 (Fig. 1B) in a dose-dependent manner.

We then investigated the cytokine production of Egr-2– or Egr-3–transduced $CD4^+$ T cells. A significant amount of IL-10 was detected in the supernatants of both the Egr-2– and Egr-3–transduced $CD4^+$ T cells in the presence of TCR stimulation (Fig. 1C), although no significant IL-10 production was detected in the absence of TCR stimulation (data not shown). Notably, only the Egr-3–transduced $CD4^+$ T cells produced a significantly higher amount of TGF-β1 than the controls in response to anti-CD3/anti-CD28 mAb stimulation. CD28 stimulation appears to be important for TGF-β1 production because stimulation with anti-CD3 mAb alone did not induce enhanced TGF-β1 production in Egr-3–transduced $CD4^+$ T cells (Fig. 1D).

To further address the relationship between Egr-3 and TGF-β1 production, we generated Egr-3 flox/flox mice (Fig. 1E) and crossed them with CD4-Cre mice to obtain mice lacking Egr-3 specifically in T cells. In the Egr-3 CKO mice, TGF-β1 production under anti-CD3 and CD28 Ab stimulation was significantly decreased (Fig. 1F).

Egr-3 enhances the phosphorylation of STAT3

It was reported that STAT3 positively regulates TGF-β1 promoter activity and enhances TGF-β1 production (14, 15). Intracellular staining revealed significantly enhanced STAT3 phosphorylation in the Egr-3–transduced $CD4^+$ cells compared with the GFP-negative cells (Fig. 2A). As described above, Egr-3–transduced $CD4^+$ T cells produce a significant amount of IL-10, and there is a possibility that the IL-10 produced by $CD4^+$ T cells affects the phosphorylation of STAT3 in an autocrine manner. To exclude the effects of IL-10 produced by $CD4^+$ T cells, we transduced Egr-3 into $CD4^+$ T cells from IL-10 knockout mice. The IL-10–deficient $CD4^+$ T cells transduced with Egr-3 still showed enhanced STAT3 phosphorylation (Fig. 2B). Therefore, Egr-3 induces the phosphorylation of STAT3 in an IL-10–independent manner. In contrast, the phosphorylation of STAT1 was not affected by Egr-3

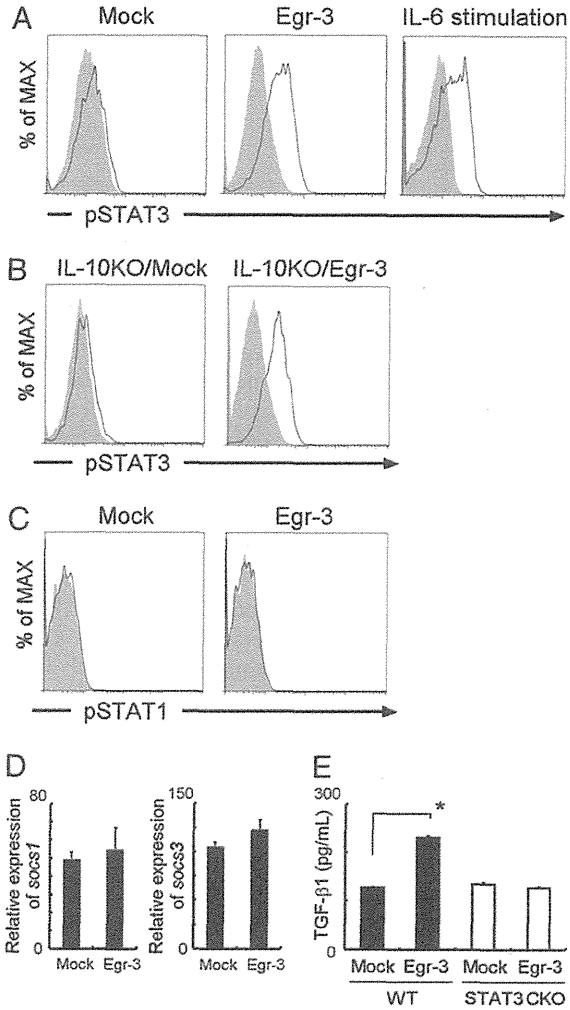

FIGURE 2. Egr-3 enhances STAT3 phosphorylation. (**A**) Gene-transduced $CD4^+$ T cells were stimulated with anti-CD3/anti-CD28 mAb for 3 d and intracellularly stained with Ab to p-STAT3. In the *left and middle panels*, the open histograms represent $CD4^+GFP^+$ T cells, and the filled histograms represent $CD4^+GFP^-$ T cells. As a positive control, $CD4^+$ T cells stimulated with IL-6 (100 ng/ml) for 15 min were also analyzed. In the *right panel*, the open histogram represents $CD4^+$ T cells with IL-6 stimulation, and the filled histogram represents $CD4^+$ T cells without IL-6 stimulation. (**B**) $CD4^+$ T cells from IL-10 knockout (KO) mice were gene-transduced, stimulated with anti-CD3/anti-CD28 mAb, and stained with an Ab to p-STAT3. The open histograms represent $CD4^+GFP^+$ T cells, and the filled histograms represent $CD4^+GFP^-$ T cells. (**C**) Gene-transduced $CD4^+$ T cells were stimulated with anti-CD3/anti-CD28 mAb for 3 d and intracellularly stained with Ab to p-STAT1. The open histograms represent $CD4^+GFP^+$ T cells, and the filled histograms represent $CD4^+GFP^-$ T cells. Representative data from three independent experiments are shown. (**D**) Mock or Egr-3 was transduced into mice splenocytes, and GFP-positive T cells were sorted. Relative expression of *socs1* and *socs3* were compared with *gapdh*. (**E**) $CD4^+$ T cell from STAT3 CKO mice or control mice were stimulated with anti-CD3 mAb alone or anti-CD3/anti-CD28 mAb. ELISA of TGF-β1 in the supernatant fluid is presented. The results are shown as the means of three independent experiments. All error bars represent SD. $*p < 0.01$. WT, Wild-type.

overexpression, suggesting the specificity of the effect of Egr-3 expression (Fig. 2C).

To investigate how Egr-3 regulates STAT3 activation, we explored whether Egr-3 alters SOCS1 and SOCS3 expressions (33). SOCS1 and SOCS3 are the negative regulators for STAT1 and STAT3, and Egr-2 directly induces SOCS1 and SOCS3 expression (22). We observed no evidence that the forced expression of Egr-3 modifies SOCS1 and SOCS3 expressions (Fig. 2D). However, STAT3 was critically required for Egr-3–mediated TGF-β1 induction, because Egr-3 overexpression failed to induce TGF-β1 production in STAT3-deficient $CD4^+$ T cells (Fig. 2E).

Egr-3 confers the suppression of Ag-specific T cell response in vivo

Taking these findings together, Egr-3–transduced $CD4^+$ cells produce the suppressive cytokines IL-10 and TGF-β1 under signaling via TCR and CD28. These findings suggest that Egr-3 expression in $CD4^+$ T cells is associated with immune regulatory function. Therefore, we used a CIA mouse model to investigate the effect of Egr-3 on Ag-specific immune reactions in vivo. The arthritis score and incidence rate revealed that the Egr-3–transduced $CD4^+$ T cells significantly ameliorated arthritis compared with empty vector (Mock) –transduced $CD4^+$ T cells. Moreover, Egr-3 and bCII-specific TCR (34) cotransduced $CD4^+$ T cells suppressed arthritis more significantly than Egr-3–transduced $CD4^+$ T cells (Fig. 3A, 3B). The pathological score results agreed with the arthritis score results (Fig. 3C). The above findings indicate that Egr-3 confers in vivo regulatory activity on $CD4^+$ T cells in an Ag-specific manner.

Egr-3 suppresses Ag-specific T cell responses via TGF-β1

We further analyzed the in vivo regulatory activity of Egr-3 in delayed-type hypersensitivity (DTH). BALB/c mice were immunized with OVA protein and then i.v. administered Egr-3–transduced $CD4^+$ T cells. Egr-3 and OVA-specific TCR DO11.10 cotransduced $CD4^+$ T cells or DO11.10-transduced $CD4^+$ T cells were also injected into each group of mice, and then their footpads were reinjected with OVA protein. As expected, the DO11.10-transduced $CD4^+$ T cells enhanced footpad swelling. In contrast, the Egr-3–transduced $CD4^+$ T cells significantly suppressed the DTH response compared with Mock-transduced $CD4^+$ T cells. Moreover, the Egr-3 and DO11.10 cotransduced $CD4^+$ T cells displayed superior regulatory activity to the Egr-3–transduced $CD4^+$ T cells (Fig. 4A). This result was consistent with the result obtained in the CIA mouse model experiment.

As Egr-3 expression was found to be associated with TGF-β1 production, the contribution of TGF-β1 to the regulatory activity of Egr-3–transduced $CD4^+$ T cells was examined. The effect of TGF-β1 neutralization was analyzed in BALB/c DTH model mice administered Mock- or Egr-3–transduced $CD4^+$ T cells. In this experiment, twice as many gene-transduced $CD4^+$ T cells were adoptively transferred as in the experiment shown in Fig. 4A. Intriguingly, TGF-β1 neutralization almost totally abrogated the suppressive effect of Egr-3–transduced $CD4^+$ T cells on the DTH response (Fig. 4B). This indicated that the in vivo regulatory activity of Egr-3–transduced $CD4^+$ T cells is dependent on TGF-β1.

Human $CD4^+CD25^-CD45RO^-LAG3^-$ T cells express high levels of EGR3 and TGF-β1

In T cells, Egr-3 is induced by TCR engagement (17). We examined Egr-3–expressing murine $CD4^+$ T cells to identify TGF-β1–producing T cell populations with regulatory activity, but no specific signal of Egr-3 expression could be detected in murine $CD4^+$ T cells. Although we identified Egr-2–expressing $CD4^+CD25^-LAG3^+$ T cells in mice (20), these $CD4^+CD25^-LAG3^+$ T cells did not show increased *egr3* expression compared with naive $CD4^+$ T cells.

We then examined human $CD4^+$ T cell subsets. Cell sorting and quantitative PCR revealed that $CD4^+CD25^-CD45RO^-LAG3^-$

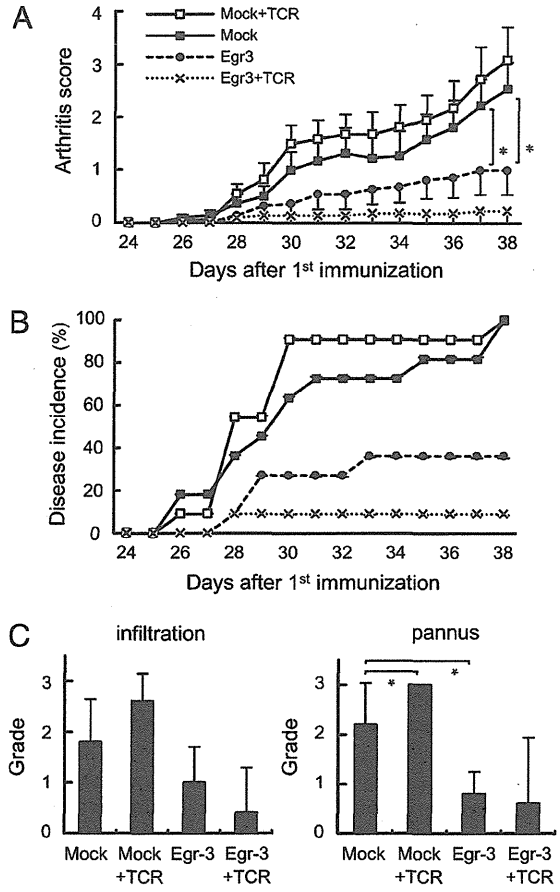

FIGURE 3. Egr-3 confers the suppression of Ag-specific T cell responses in vivo. (**A**) Arthritis scores of CIA mice that were transferred with gene-transduced $CD4^+$ T cells. TCR indicates transduction of the TCR gene specific to bCII. Mice were transferred with $CD4^+$ T cells transduced with Mock, Egr-3, Mock+TCR, or Egr-3+TCR genes 20 d after first immunization. On day 21, the mice were given a booster immunization. n = 11/group. $*p < 0.05$. (**B**) Arthritis incidence rate of CIA mice transferred with gene-transduced $CD4^+$ T cells (same mice as in A). n = 11/group. (**C**) Pathological arthritis score of each CIA mouse group. n = 5/group. All error bars represent SD. $*p < 0.01$.

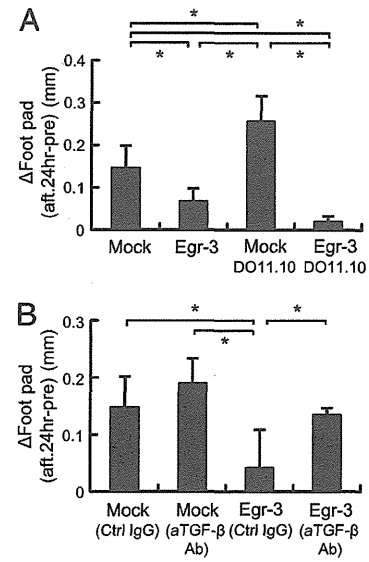

FIGURE 4. Egr-3 suppresses Ag-specific T cell responses via TGF-β1. BALB/c mice were immunized with OVA protein on day 1, and then gene-transduced $CD4^+$ T cells were i.v. injected on day 7. On day 9, their left footpad was reimmunized with OVA, and PBS was s.c. injected into the right footpad as a negative control. The DTH reaction in the footpad was determined 24 h after the second immunization. (**A**) Mice were transferred with 5×10^6 $CD4^+$ T cells transduced with Mock, Egr-3, Mock plus DO11.10, or Egr-3 plus DO11.10 genes. The DTH reaction was evaluated in each group, and differences among groups were assessed. n = 6/group. (**B**) Mice were transferred with 1×10^7 $CD4^+$ T cells transduced with Mock or Egr-3 genes. On the same day as the gene-transduced cell treatment, anti–TGF-β neutralizing Ab (aTGF-β Ab) or isotype control (Ctrl) IgG was i.p. injected. The DTH reaction was evaluated in each group, and differences among groups were assessed. n = 6/group. All error bars represent SD. $*p < 0.05$.

T cells from a certain patient's tonsil expressed significantly high amounts of *EGR3* and *TGFB1* (Fig. 5A, 5B). In contrast, this population did not express high amounts of IL-10. These features were shared among different individuals (Fig. 5C).

Although *EGR3*-expressing $CD4^+CD25^-CD45RO^-LAG3^-$ T cells did not produce significant amounts of IL-10 or TGF-β1 after TCR stimulation (data not shown), FACS analysis revealed significant expression of membrane-bound TGF-β1 on $CD4^+CD25^-CD45RO^-LAG3^-$ T cells (Fig. 5D). As the amount of membrane-bound TGF-β1 was diminished by the siRNA for the human *EGR3* gene, we consider the TGF-β1 expression of human $CD4^+CD25^-CD45RO^-LAG3^-$ tonsillar T cells to be *EGR3* dependent (Fig. 5E).

Human $CD4^+CD25^-CD45RO^-LAG3^-$ T cells suppress the GVHD reactions of humanized mice

We examined the regulatory activity of $CD4^+CD25^-CD45RO^-LAG3^-$ T cells in vivo using a xenogenic GVHD model (i.e., severely immunodeficient NOG mice). In these mice, GVHD was induced by the transfusion of human PBMC, and severe body weight loss was observed after the development of GVHD (31).

Human PBMCs with or without tonsillar $CD4^+$ T cells from the same individual were administered to the NOG mice. Appropriate engraftment was observed in each humanized NOG mouse (Fig. 6A). Although the NOG mice treated with memory phenotype $CD4^+CD25^-CD45RO^+LAG3^-$ T cells showed no improvement in their body weight loss, $CD4^+CD25^-CD45RO^-LAG3^-$ T cells ameliorated the body weight loss of the NOG mice (Fig. 6B). This indicated that tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells suppress xenogenic GVHD immune reactions. Pathological analysis of the lungs of humanized NOG mice revealed that tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells suppressed the infiltration of human $CD45^+$ cells (Fig. 6C). These observations indicated that tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells have the ability to control immune reactions in vivo.

To confirm the effect of EGR3 in tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells, we knocked down *EGR3* gene using siRNA. Tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells were transfected with *EGR3* siRNA and then injected into irradiated NOG mice with PBMC. *EGR3* siRNA-transfected tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells showed significantly decreased suppressive effect on xenogenic GVHD (Fig. 6D).

Discussion

We analyzed the function of the transcription factor Egr-3, which had been reported to be associated with T cell anergy and the suppression of T cell activation (17). We found that Egr-3 increases the expression of IL-10 and LAG3 in vitro in the presence of TCR stimulation. This is similar to the function of Egr-2, which controls $CD4^+CD25^-LAG3^+$ Tregs (20). However, TGF-β1 produc-

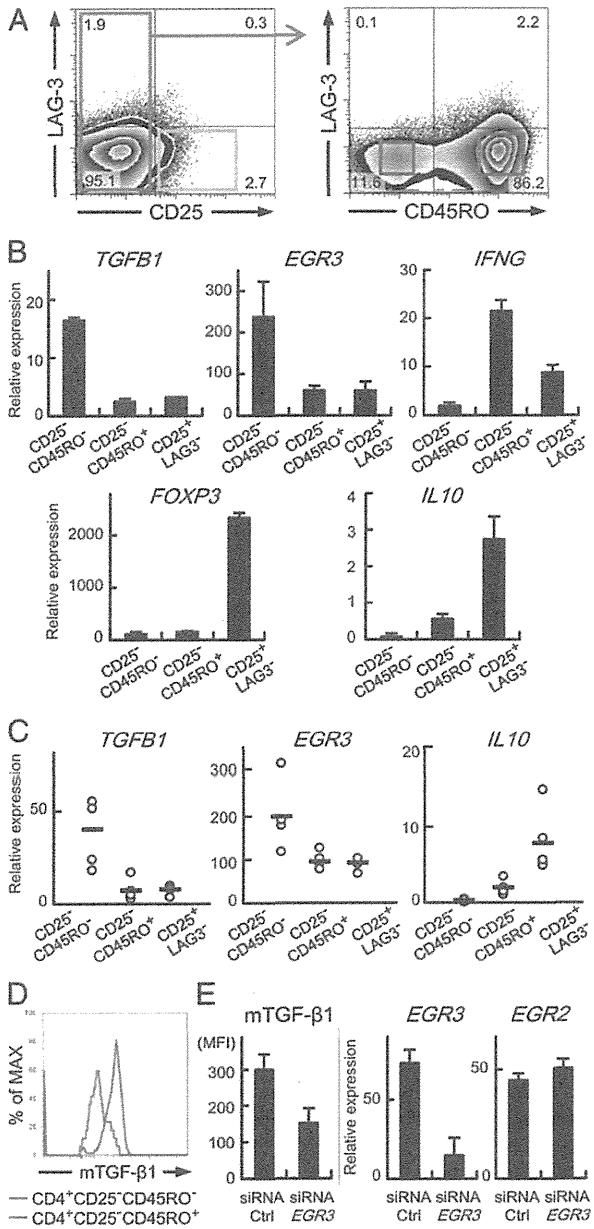

FIGURE 5. $CD4^+CD25^-CD45RO^-LAG3^-$ T cells express high levels of EGR3 and TGF-β1. (**A**) Representative FACS profile of human tonsillar lymphocytes. CD25 and LAG3 expression in CD4 gated tonsillar T cells (*left panel*). CD45RO and LAG3 expression in $CD4^+CD25^-$ gated tonsillar T cells (*right panel*). $CD4^+CD25^-CD45RO^-LAG3^-$ T cells (green), $CD4^+CD25^-CD45RO^+LAG3^-$ T cells (red), and $CD4^+CD25^+LAG3^-$ T cells (orange) were sorted according to the presented gates. (**B**) Relative expressions of human *TGFB1*, *EGR3*, *IFNG*, *FOXP3*, and *IL10* were compared with *GAPDH* in human tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$, $CD4^+CD25^-CD45RO^+LAG3^-$, and $CD4^+CD25^+LAG3^-$ T cells taken from a certain patient. The results are shown as the mean of three independent experiments. (**C**) Quantitative PCR of human *TGFB1*, *EGR3*, and *IL10* expression in each tonsillar T cell group from five different individuals. The open circles represent individuals, and the bars represent the mean of five individuals. (**D**) FACS analysis of human tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ and $CD4^+CD25^-CD45RO^+LAG3^-$ T cells for membrane-bound TGF-β1. T cells were stimulated with anti-CD3 Ab for 5 d. (**E**) Mean fluorescence intensity (MFI) of the membrane-bound TGF-β1 of tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells treated with the negative control siRNA (siRNA Ctrl) or siRNA for human *EGR3* (siRNA *EGR3*) (*left panel*). Relative *EGR3* and *EGR2* expressions of tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells were compared with GAPDH after treatment with the negative control siRNA or siRNA for human *EGR3* (*middle and right panels*). All error bars represent SD.

tion was observed in Egr-3–transduced $CD4^+$ T cells, but not in Egr-2–transduced $CD4^+$ T cells. This suggests that Egr-3 confers different specific features on $CD4^+$ T cells from Egr-2. Although we examined Egr-3–expressing murine $CD4^+$ T cells to identify TGF-β1–producing T cell populations with regulatory activity, no specific signal of Egr-3 expression could be detected in murine $CD4^+$ T cells. Phospho-STAT3 was reported to interact with the promoter regions of both TGF-β1 and IL-10 (14, 15), and the enhancement of STAT3 phosphorylation by Egr-3 is supposed to be associated with TGF-β1 production.

From our analysis of in vivo immune reaction models including CIA and DTH mouse models, we confirmed that the transcription factor Egr-3 confers regulatory activity on $CD4^+$ T cells. In DTH, the suppression was mediated via the production of TGF-β1. This result is consistent with the findings of previous reports that anergic T cells are associated with regulatory activity. The in vivo regulatory activities of Egr-3– and TGF-β1–expressing T cells were also confirmed in humans from the suppression of GVHD by tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells.

In humans, several regulatory $CD4^+$ T cell populations have been reported to exist, including $CD4^+CD25^+Foxp3^+$ Tregs and type 1 Tregs (35–37). Tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells are clearly different from $CD4^+CD25^+Foxp3^+$ Tregs because tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells lack CD25 and *FOXP3*. Moreover, tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells do not produce IL-10, which is a characteristic of conventional type 1 Tregs. Tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells display similar TGF-β1 expression to Th3 cells (12); however, tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells do not secrete the soluble form of TGF-β1. Therefore, this cell group could be a new Treg population that contributes to peripheral tolerance. Although several human Treg populations other than $CD4^+CD25^+$ Tregs have been reported, most of these populations produce IL-10 (37, 38), and tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells are unique in that they express TGF-β1, but not IL-10.

It is well known that TGF-β1 contributes to the suppressive function of $CD4^+CD25^+$ Tregs (39), and it was reported that $CD4^+CD25^+$ Tregs express persistently high levels of TGF-β1 on their cell surface (40). However, in this study the tonsillar $CD4^+CD25^+LAG3^-$ T cells did not display increased *EGR3* expression compared with the tonsillar $CD4^+CD25^-CD45RO^+LAG3^-$ T cells, which did not demonstrate regulatory activity in the GVHD model. As a limited fraction of $CD4^+CD25^+$ Tregs were found to express the membranous form of TGF-β1 in an airway inflammation model (41), our results do not exclude the possibility that Egr-3 contributes to cell-surface TGF-β1 expression in a subpopulation of $CD4^+CD25^+$ Tregs.

In our results, the enhancement of TGF-β1 production and STAT3 phosphorylation were observed in Egr-3–transduced $CD4^+$ T cells stimulated with both anti-CD3 and CD28 Abs. This observation was consistent with the previous report that TGF-β1 production was induced by the stimulation with anti-CD3 and anti-CD28 Abs in human $CD4^+CD25^-$ T cells (42). Moreover, costimulation of CD28 induces STAT3 phosphorylation in $CD4^+CD25^-$ T cells (43). We think STAT3 plays an important role, because enhanced phosphorylation of STAT3 in Egr-3–transduced cells was demonstrated under CD28 costimulation (Fig. 2A). The enhanced phosphorylation of STAT3 was specific because STAT1 showed no

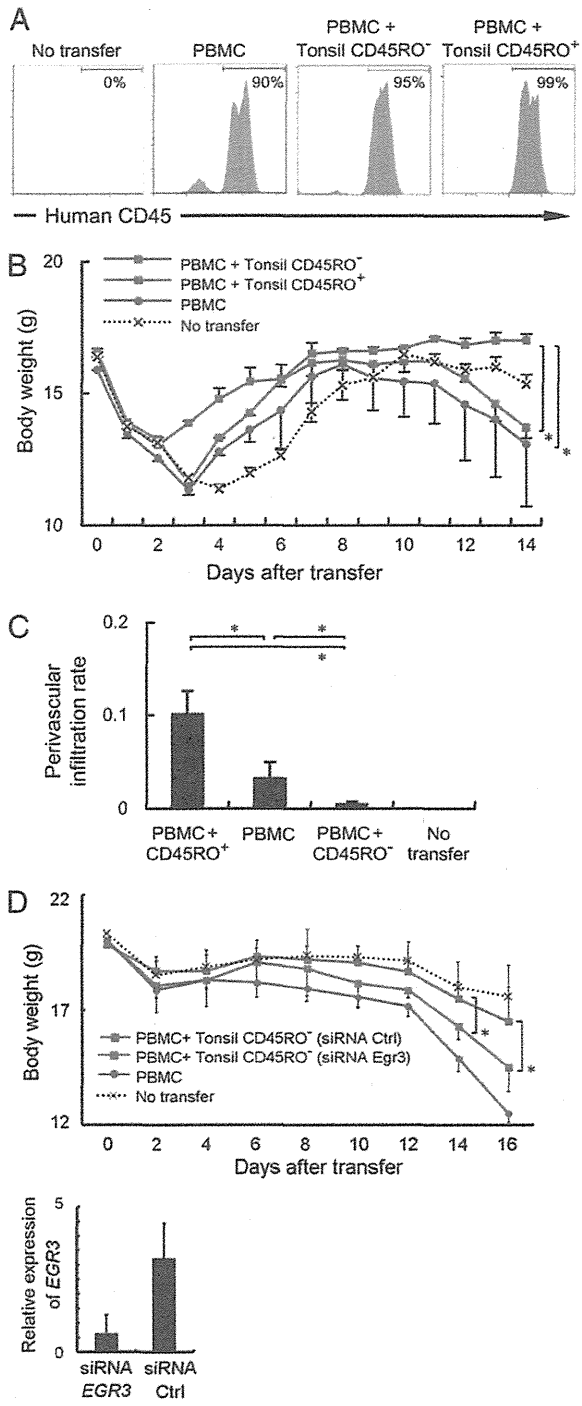

FIGURE 6. $CD4^+CD25^-CD45RO^-LAG3^-$ T cells suppress GVHD in humanized mice. (**A**) Representative FACS analysis of the splenocytes of PBMC-transferred NOG mice. The whole lymphocytes were gated and stained with anti-human CD45 Ab. The human CD45 positivity rate representing the engraftment rate is depicted. The results are shown as the mean of three independent experiments. (**B**) Body weight change of NOG mice that had been administered human PBMC with or without human tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells (Tonsil $CD45RO^-$) or $CD4^+CD25^-CD45RO^+LAG3^-$ T cells (Tonsil $CD45RO^+$) from the same individuals. n = 3/group. Three independent experiments yielded similar results and one representative experiment is shown. (**C**) Perivascular infiltration rate of cells stained with anti-human CD45 Ab in the lung perivascular area of xenogenic NOG mice. n = 4/group. (**D**) Total of 2×10^5 tonsillar $CD4^+CD25^-CD45RO^-$ T cells transfected with *EGR3* siRNA were injected into irradiated NOG mice with 5×10^6 PBMCs. Body weight change was observed. n = 3/group (*top panel*). Relative *EGR3* expressions of tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells were compared after treatment with the negative control siRNA (siRNA Ctrl) or siRNA for *EGR3* (*bottom panel*). All error bars represent SD. $^*p < 0.01$.

enhancement of phosphorylation (Fig. 2C). In addition, the importance of STAT3 was confirmed by the experiment showing that Egr-3 overexpression failed to induce TGF-β1 production in STAT3-deficient $CD4^+$ T cells (Fig. 2E). Although the precise mechanism for the enhancement of STAT3 phosphorylation by Egr-3 expression remains to be investigated, our experiments showed the indispensable linkage between Egr-3 and STAT3 for TGF-β1 production.

When T cells isolated from human tonsils were examined according to their CD45 expression, naive type tonsillar T cells ($CD4^+CD45RA^+$) were found to proliferate at a similar rate to memory type tonsillar T cells ($CD4^+CD45RO^+$) after rhinovirus stimulation (44). Continuous stimulation with an infectious agent might lead to the chronic activation of tonsillar T cells. As chronic Ag stimulation renders $CD4^+$ T cells anergic (45), tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells could acquire regulatory activity and their ability to express EGR3 during chronic Ag stimulation.

We found that Egr-3 expression enabled $CD4^+$ T cells to secrete TGF-β1 upon CD3/CD28 costimulation. In a previous study, it was reported that CTLA4 stimulation is required for TGF-β1 production by CD3/CD28-stimulated $CD4^+$ T cells (46). Therefore, Egr-3 expression could substitute for CTLA4 stimulation in TGF-β1 production by $CD4^+$ T cells. CTLA4 stimulation decreases the production of IL-2 and IFN-γ by CD3/CD28-stimulated $CD4^+$ T cells, and decreased production of IL-2 and IFN-γ has also been reported in Egr-3–transduced T cells (17). The mechanisms responsible for the similarities between Egr-3– and CTLA4-mediated signals should be investigated further.

In terms of the expression of IL-10 and LAG3, there is a discrepancy between human tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells and Egr-3–overexpressed murine cells. Although we could not clarify the reason, this difference might depend on the activation status of T cells. We think that our data do not exclude the possibility that $CD4^+CD25^-CD45RO^-LAG3^-$ T cells preferentially express IL-10 and LAG3 under some optimal costimulation.

Collectively, our data suggest that Egr-3 is a transcription factor that is associated with TGF-β1 expression and in vivo regulatory activity in both mice and humans. The adoptive transfer of tonsillar $CD4^+CD25^-CD45RO^-LAG3^-$ T cells and the modulation of EGR3 expression might aid the development of practical treatments for GVHD and other immune-mediated diseases.

Egr-3 is induced by costimulation free TCR signaling that leads to dominant NFAT activation (17). Therefore, repeated TCR stimulation without costimulation can be one candidate condition for in vitro induction of $CD4^+CD25^-CD45RO^-LAG3^-$ T cells. However, for the confirmation of in vitro induction, identification of a reliable surface marker for $CD4^+CD25^-CD45RO^-LAG3^-$ T cells is required.

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Disclosures

The authors have no financial conflicts of interest.

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Egr-2 transcription factor is required for Blimp-1-mediated IL-10 production in IL-27-stimulated CD4$^+$ T cells

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Interleukin-27 (IL-27) suppresses immune responses through inhibition of the development of IL-17 producing Th17 cells and induction of IL-10 production. We previously showed that forced expression of early growth response gene 2 (Egr-2), a transcription factor required for T-cell anergy induction, induces IL-10 and lymphocyte activation gene 3 expression and confers regulatory activity on CD4$^+$ T cells in vivo. Here, we evaluated the role of Egr-2 in IL-27-induced IL-10 production. Among various IL-10-inducing factors, only IL-27 induced high levels of Egr-2 and lymphocyte activation gene 3 expression. Intriguingly, IL-27 failed to induce IL-10 in Egr-2-deficient T cells. IL-27-mediated induction of *Prdm1* that codes B lymphocyte induced maturation protein-1, a transcriptional regulator important for IL-10 production in CD4$^+$ T cells, was also impaired in the absence of Egr-2. Although IL-27-mediated IL-10 induction was dependent on both STAT1 and STAT3, only STAT3 was required for IL-27-mediated Egr-2 induction. These results suggest that IL-27 signal transduction through Egr-2 and B lymphocyte induced maturation protein-1 plays an important role in IL-10 production. Furthermore, Egr-2-deficient CD4$^+$ T cells showed dysregulated production of IFN-γ and IL-17 in response to IL-27 stimulation. Therefore, Egr-2 may play key roles in controlling the balance between regulatory and effector cytokines.

Keywords: Blimp-1 · Egr-2 · IL-10 · IL-27 · inducible regulatory T (Treg) cells · Prdm1

See accompanying Commentary by Vasanthakumar and Kallies

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Introduction

Naïve $CD4^+$ T cells play central roles in immune regulation by differentiating into effector as well as Treg-cell subsets. Recently, a number of Treg-cell subsets, which are important for suppressing effector T cells, tissue inflammation, and autoimmunity, have also been identified. On one hand, $CD4^+CD25^+$ Treg cells, which express the transcription factor Foxp3, have a dominant function in immune suppression and the maintenance of immune homeostasis [1, 2]. On the other hand, other Treg cells, which arise in the periphery, such as Treg type I (Tr1) cells and Th3 cells produce the suppressive cytokines IL-10 and TGF-β1, and contribute to the suppression of immune responses in a Foxp3-independent manner [3, 4]. IL-10 is an anti-inflammatory cytokine which was initially described as a cytokine associated with Th2 cells that inhibits the production of IFN-γ by Th1 cells [5, 6]. A number of reports have revealed that IL-10 suppresses cytokine production and proliferation of T cells [7, 8] and inhibits the T-cell-stimulating capacity of APCs [9]. IL-10-deficient mice die with spontaneously developed inflammatory bowel disease [10].

Interleukin-27 (IL-27), a member of the IL-12/IL-23 heterodimeric family of cytokines produced by APCs, is composed of two chains, p28 and EBV-induced gene 3 [11]. IL-27 induces the expansion of Th1 cells by activating the STAT1-mediated T-bet pathway [12], but IL-27Rα-deficient mice developed severe EAE with enhanced Th17-cell responses [13]. The immunosuppressive effects of IL-27 depend on inhibition of the development of Th17 cells and induction of IL-10 production [14]. Recently, IL-27 has been identified as a differentiation factor for IL-10-producing Tr1 cells [15–17]. On the other hand, B lymphocyte induced maturation protein-1 (Blimp-1) (coded by *Prdm1* gene), a zinc finger-containing transcriptional regulator that is well known to be a regulator of plasma cell differentiation, is also important for IL-10 production in naïve $CD4^+$ T cells. Martins et al. [18, 19] reported that Blimp-1-deficient $CD4^+$ T cells proliferated more and produced excess IL-2 and IFN-γ, but reduced IL-10 after TCR stimulation.

Early growth response gene 2 (Egr-2) and Egr-3 have been reported to be transcription factors for TCR-induced negative regulatory program controlling Cbl-b expression [20]. We previously identified a Treg population expressing lymphocyte activation gene 3 (LAG-3) in a fraction of $CD4^+CD25^-CD45RB^{low}$ T cells and showed that forced expression of Egr-2 induces IL-10, LAG-3, and Blimp-1 expressions and confers regulatory activity in vivo on $CD4^+$ T cells [21]. We here describe that IL-27 induces Egr-2 and LAG-3 as well as IL-10 in $CD4^+$ T cells. Moreover, Egr-2-deficient $CD4^+$ T cells exhibited reduced expression of IL-10 and Blimp-1 and reciprocally enhanced secretion of IFN-γ and IL-17 in response to IL-27. Results from a LUC assay and ChIP assay show that Egr-2 binds to the promoter lesion of *Prdm1* to activate its transcription. These results indicate that IL-27 signal transduction through Egr-2 and Blimp-1 is required for IL-10 production in $CD4^+$ T cells and controls the balance between regulatory and inflammatory cytokines.

Results

IL-27 induces Egr-2, IL-10, and LAG-3 expression in naïve $CD4^+$ T cells

We previously reported that the forced expression of Egr-2 induces IL-10 production in $CD4^+$ T cells and confers the phenotype of $CD4^+CD25^-LAG3^+$ Treg cells [21]. First, we confirmed the moderate induction of intracellular Egr-2 in TCR-stiumulated $CD4^+$ T cells and observed that IL-10 production was restricted to cells expressing intracellular Egr-2 (Fig. 1A). Then, we explored the factor inducing Egr-2, which confers the phenotype of $CD4^+CD25^-LAG3^+$ Treg cells. Various IL-10-inducible cytokines, such as IL-27, TGF-β [22], IL-21 [23], and IL-10, were added to a co-culture of splenic $CD4^+$ T cells from TEα TCR transgenic mice expressing I-Eα-specific TCR [24] and B cells from B6 WT mice in the presence of $E\alpha_{52-68}$ peptides. In addition, the effect of the IL-10-inducible chemical substance zymosan was examined because it induces DCs to secrete abundant IL-10 in a TLR-2- and dectin-1-mediated activation of ERK/MAPK-dependent manner [25]. Notably, IL-27 predominantly induced both Egr-2 and LAG-3 mRNA expressions relative to the other cytokines and zymosan. IL-27 did not induce Foxp3 mRNA expression (Fig. 1B), which is compatible with previous reports [15] and the fact that $CD4^+CD25^-LAG3^+$ Treg cells hardly expressed Foxp3 protein [21]. When we added IL-27 to naïve $CD4^+$ T cells stimulated with plate-coated anti-CD3ε and anti-CD28 mAbs, Egr-2 protein was clearly detected by intracellular staining. This induction was abolished in Egr-2-deficient $CD4^+$ T cells cultured with IL-27 and also in IL-27Rα (WSX-1)-deficient $CD4^+$ T cells (Fig. 1C). Interestingly, LAG-3 was predominantly induced in B6 WT $CD4^+$ T cells expressing Egr-2, and IL-27 alone did not induce Egr-2 in the absence of TCR stimulation. IL-27 more efficiently induced $Egr2^+LAG3^+$ cells than the other IL-12 family cytokines, IL-12 and IL-23 (Fig. 1D). Although IL-2 is required for IL-27-induced IL-10 expression through Blimp-1 in $CD8^+$ T cells [26], IL-2 by itself could not induce $Egr2^+LAG3^+$ cells and showed no additive effect on IL-27-induced Egr-2 and LAG-3 expressions (Fig. 1D). No significant association was seen between the extent of cell division and the amount of Egr-2 expression, while Egr-2 induction was limited to proliferating cells (Fig. 1E).

IL-27-mediated induction of IL-10 and Blimp-1 is impaired in Egr-2 conditional KO (CKO) mice

Multiple observations support the idea that Blimp-1 regulates T-cell responsiveness by attenuating IL-2 production. IL-2 production in Blimp-1-deficient $CD4^+$ T cells is elevated by stimulation via TCR [18]. As IL-2 signaling induces Blimp-1 transcription, Blimp-1 makes a negative feedback loop for *Il2* transcription in T cells [19]. Recently, it was shown that Blimp-1 positively regulates IL-10 production in $CD4^+$ T cells [18, 27]. Blimp-1 is required for IL-10 production and high ICOS expression in

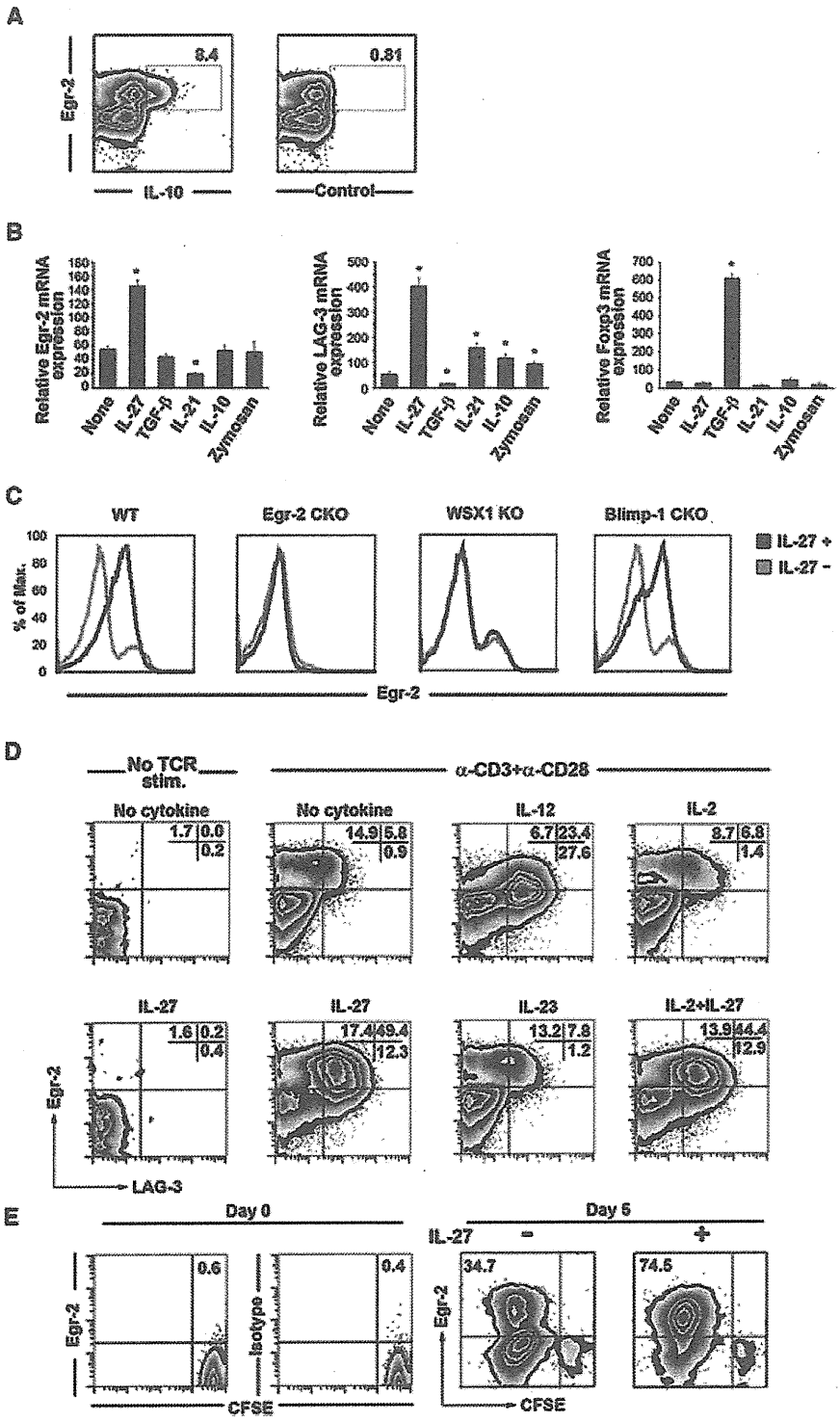

Figure 1. IL-27 induces the simultaneous expression of Egr-2 and LAG-3 in $CD4^+$ T cells. (A) Naïve $CD4^+$ T cells from C57BL/6 WT mice were cultured with anti-CD3/CD28 mAb. On day 5, Egr-2 and IL-10 expression were analyzed by intracellular staining. Data shown are representative of three experiments performed. (B) Naïve $CD4^+$ T cells from TEα TCR transgenic mice were cultured with B cells from WT mice with $E\alpha_{52-68}$ peptide in the presence of indicated factors. Expression of Egr-2, LAG-3, and Foxp3 was measured by quantitative RT-PCR. Data are presented as mean + SD ($n = 3$; replicate wells). Experiments were performed three times. (C) The induction of Egr-2 in $CD4^+$ T cells stimulated with IL-27. Naïve $CD4^+$ T cells from C57BL/6 WT, Egr-2 CKO, Blimp-1 CKO, and WSX-1 KO mice were cultured with anti-CD3/CD28 mAb in the presence or absence of IL-27. Egr-2 expression was detected by intracellular staining on day 5. Data shown are representative of five experiments performed. (D) $CD4^+$Egr-$2^+$$LAG3^+$ T-cell induction was analyzed. Naïve $CD4^+$ T cells from C57BL/6 WT mice were cultured with or without anti-CD3/CD28 mAb under the indicated conditions. Data shown are representative of two or three experiments performed. (E) Carboxyfluorescein diacetate succinimidyl diester labeled naïve $CD4^+$ T cells from C57BL/6 WT mice were stimulated as in (C). On day 5, T-cell proliferation and Egr-2 expression were determined by flow cytometry. Data shown are representative of five experiments performed. *$p < 0.05$; Student's t-test.

$CD4^+CD25^+Foxp3^+$ Treg cells [28]. Therefore, the role of Egr-2 and Blimp-1 in IL-27-induced IL-10 production was examined using naïve $CD4^+$ T cells from Egr-2 CKO ($Egr2^{fl/fl}$-CD4-Cre^+) and Blimp-1 CKO ($Prdm1^{fl/fl}$-CD4Cre^+) mice. Consistent with our previous observation that the forced expression of Egr-2 induced the high mRNA expression levels of Blimp-1 in $CD4^+$ T cells [21], Egr-2-induction by IL-27 was not affected in the absence of Blimp-1 (Fig. 1C). In $CD4^+$

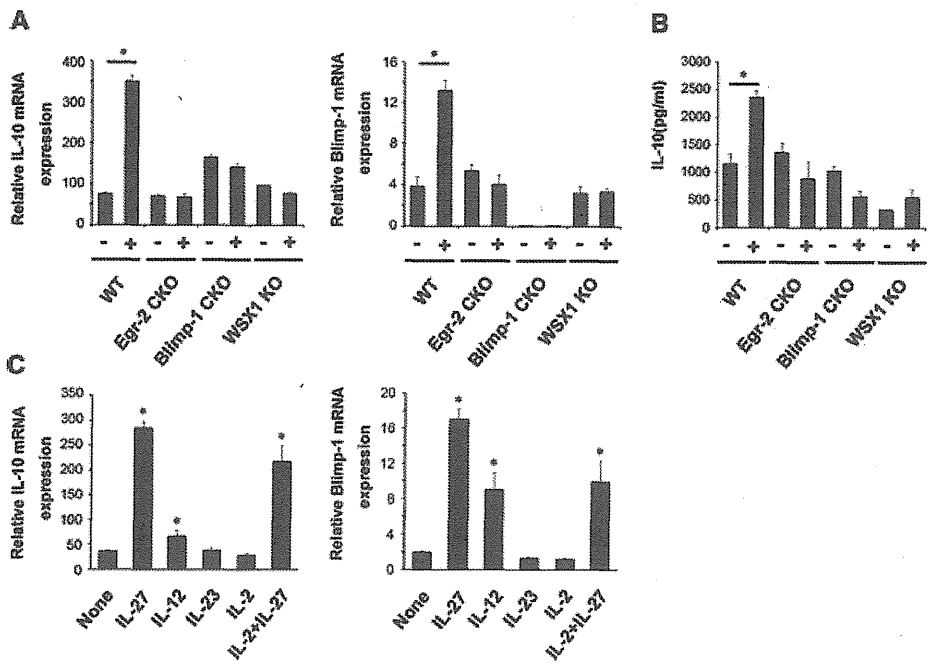

Figure 2. IL-27-mediated induction of IL-10 and Blimp-1. (A) Expression of IL-10 (left) and Blimp-1 (right) mRNA was measured by quantitative RT-PCR. Naïve CD4$^+$ T cells were isolated from C57BL/6 WT, Egr-2 CKO, Blimp-1 CKO, or WSX-1 KO mice and cultured with anti-CD3/CD28 mAb in the presence or absence of IL-27. Data are shown as mean + SD (n = 3; replicate wells). Experiments were performed four times. (B) IL-10 concentrations in culture supernatants of stimulated CD4$^+$ T cells from C57BL/6 WT, Egr-2 CKO, Blimp-1 CKO, or WSX-1 KO mice were measured by ELISA. Data are shown as mean + SD (n = 3; replicate wells). Experiments were performed three times. (C) Expression of IL-10 and Blimp-1 mRNA was measured by quantitative RT-PCR. Naïve CD4$^+$ T cells were isolated from C57BL/6 WT and cultured with anti-CD3/CD28 mAb under the indicated condition. Data are shown as mean + SD (n = 3; replicate wells). Experiments were performed two times. *p < 0.05; Student's t-test.

T cells both from Egr-2 CKO mice and Blimp-1 CKO mice, the induction of *Il10* transcription and IL-10 protein expression by IL-27 was impaired (Fig. 2A and B), and these inductions were not observed in CD4$^+$ T cells from WSX-1 KO mice (Fig. 2A and B). Moreover, Blimp-1 mRNA induction by IL-27 was also impaired in Egr-2-deficient CD4$^+$ T cells (Fig. 2A). This result suggested that Egr-2 is essential for IL-10 production via Blimp-1 expression in IL-27-stimulated CD4$^+$ T cells. When we analyzed the induction of IL-10 and Blimp-1 mRNA expressions by other IL-12 family cytokines, IL-12 showed only marginal induction of IL-10 and Blimp-1 mRNA expressions and IL-23 induced no up-regulation of IL-10 and Blimp-1 mRNA expressions (Fig. 2C). We also found that IL-2 had no additive effect on IL-27-induced IL-10 and Blimp-1 mRNA expressions in CD4$^+$ T cells (Fig. 2C).

Egr-2 directly binds to the promoter region of *Prdm1* and enhances its activity

Next, we investigated whether Egr-2 regulates Blimp-1 transcription. To address this possibility, we performed a LUC reporter assay. A pGL3-LUC vector subcloned with the promoter region from –1500 bp to the *Prdm1* transcription start site [29] was co-transfected with a pMIG-Egr-2 vector to 293T cells. As shown in Figure 3A, Egr-2 significantly enhanced the activity of the *Prdm1* promoter. Next, a ChIP assay was performed with antibodies against Egr-2 to investigate whether Egr-2 directly binds to the promoter region of Blimp-1 in CD4$^+$ T cells. Among four promoter regions examined (−3000 bp, −2000 bp, −1000 bp, and +1000 bp from its transcription site) of Blimp-1, only one region (−1000 bp) showed significant enrichment compared with control, indicating that Egr-2 specially binds to the Blimp-1 promoter, but not to *Lag3* and *Il10* promoters (Fig. 3B and Supporting Information Fig. 2A). Cretney et al. reported that Blimp-1 binds to intron 1 of the *Il10* locus and, together with IFN regulatory factor-4, directly regulates IL-10 expression in CD4$^+$CD25$^+$ Treg cells by the remodeling of active chromatin at the *Il10* locus [28]. Our observation suggested that IL-10 regulation with Blimp-1 was controlled by Egr-2.

IL-27-induced Egr-2 expression is dependent on STAT3

STAT1 and STAT3 have been shown to be crucial for IL-10 production from IL-27-stimulated naïve CD4$^+$ T cells [17]. We investigated the effect of STAT1 and STAT3 deficiencies on IL-27-induced Egr-2 expression. As shown in Figure 4A and B, Egr-2 induction by IL-27 in CD4$^+$ T cells was impaired by a STAT3 deficiency, but not by a STAT1 deficiency. When we analyzed the induction of *Il10* transcription and IL-10 protein expression by IL-27 in STAT1- and

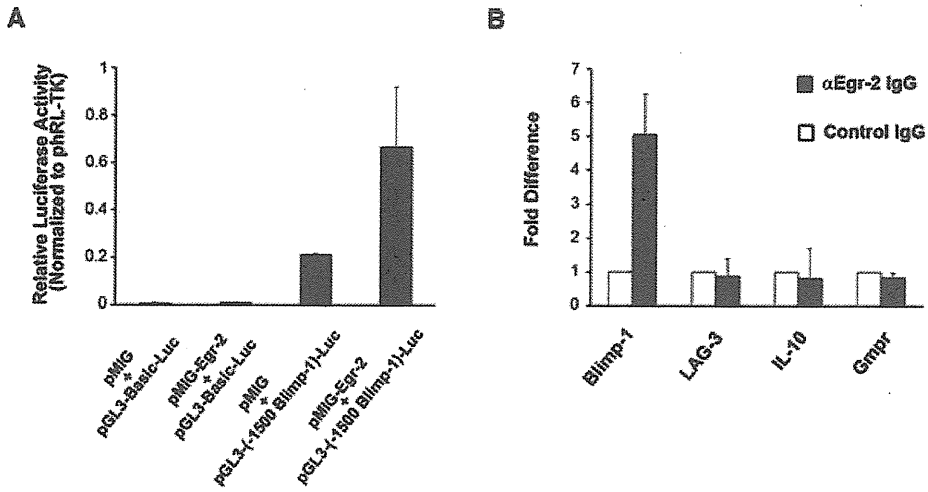

Figure 3. Blimp-1 promoter activity is directly regulated by Egr-2. (A) Egr-2 enhanced LUC activity regulated by the Blimp-1 promoter. 293T cells were co-transfected with pGL-3-(-1500 Blimp-1)-LUC vector or pGL-3-Basic-LUC control vector and a pMIG-Egr-2 vector or a pMIG mock vector. LUC activity was measured 24 h after transfection. Data are shown as mean + SD of duplicates, from one experiment representative of at least three performed. (B) ChIP-coupled quantitative PCR analysis of Egr-2 binding to promoter regions in mouse $CD4^+$ T cells. Normal IgG and anti-Egr-2 antibodies were used for IP assays. The guanosine monophosphate reductase locus was used as a negative control. The enrichment of Egr-2 binding to each promoter was determined. Mean + SD of triplicates done in one experiment representative of at least three performed are shown.

$STAT3$-deficient $CD4^+$ T cells, IL-10 protein induction by IL-27 was abolished both in STAT1 KO and in STAT3 CKO $CD4^+$ T cells, although IL-10 mRNA expression levels were slightly up-regulated by IL-27 in STAT1 KO $CD4^+$ T cells (Fig. 4C and D). These results suggest that IL-27-induced Egr-2 expression in $CD4^+$ T cells is mostly dependent on STAT3, although both STAT1 and STAT3 are important for IL-10 production by IL-27. Next, we investigated the effect of other STAT1 or STAT3 activating cytokines for Egr-2 induction. IL-6 and IFN-γ were selected as the representatives of cytokines activating STAT3- and STAT1-mediated pathways, respectively. As shown in Figure 4E, IL-6 induced Egr-2 expression as effectively as IL-27 in $CD4^+$ T cells, but IFN-γ did not. Interestingly, both IL-10 and Blimp-1 mRNA expressions were also elevated by IL-6, but expression levels seemed to be lower than those by IL-27 (Fig. 4F). IL-6 is a type I cytokine that shares structural homology and a receptor subunit, gp130, with IL-27 and has already been shown to induce IL-10 in $CD4^+$ T cells [17]. These results suggest that Egr-2 is important for IL-10 production mediated both by IL-27 and by IL-6 through the STAT3-dependent pathway.

Enhanced production of IFN-γ and IL-17 by IL-27 stimulation in Egr-2 CKO mice

To examine the role of Egr-2 in inflammatory cytokine production, we investigated the production of IFN-γ and IL-17 in response to IL-27 stimulation. It has already been shown that Egr-2-deficient $CD4^+$ T cells produce high amounts of IFN-γ and IL-17 after TCR stimulation [30]. As shown in Fig. 5, IFN-γ and IL-17 production from IL-27-stiumulated $CD4^+$ T cells was enhanced by an Egr-2 deficiency, which suggests that Egr-2 may also play an important role in controlling effector cytokine production.

Discussion

Recently, Tr1 cells, characterized by their high secretion of IL-10 and lack of Foxp3 expression, were induced by IL-27 [15–17, 31]. STAT1 and STAT3 have been shown to play an important role in the molecular mechanism of IL-10 production by IL-27 in $CD4^+$ T cells [17]. Although it is clear that STAT1-driven IL-10 production is independent of T-bet, the precise mechanism still remains unclear [17]. The underlying mechanism of IL-10 production through the activation of STAT3 is that the activation of STAT3 leads to the induction of transcription factor c-Maf [32], which is essential for IL-10 production induced by IL-27 [33]. Motomura et al. [34] have reported that transcription factor E4 promoter-binding protein 4 is important for IL-10 production from IL-27-stimulated $CD4^+$ T cells cultured under a Th1 skewing condition. E4 promoter-binding protein 4-deficient Th1 cells failed to produce IL-10 by IL-27 stimulation. It seems that IL-10 production from T cells is controlled by a complex pathway, depending on each subset or surrounding cytokine condition. In this study, we found that another transcription factor Egr-2 mediates IL-10 expression in IL-27-stimulated $CD4^+$ T cells via direct binding to the Blimp-1 promoter. Furthermore, we have shown that IL-27-induced Egr-2 expression in $CD4^+$ T cells is dependent on STAT3, but not on STAT1. Although Egr-2 may be less involved in STAT1- and T-bet-mediated pathways, which are required for IL-10 production, Egr-2 is associated with STAT3-mediated IL-10 production.

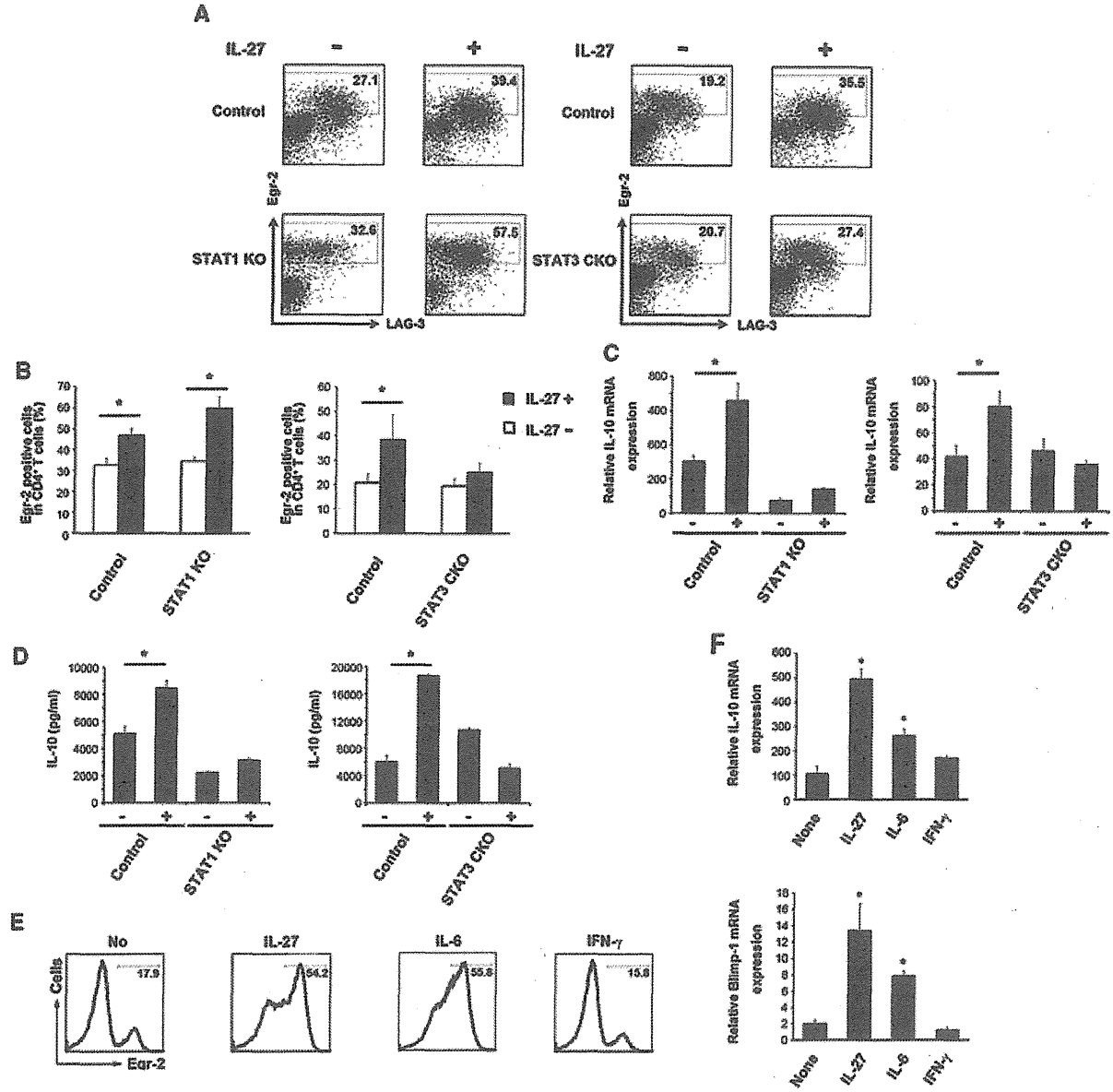

Figure 4. IL-27-induced Egr-2 expression in STAT1 and STAT3 deficiencies. (A) Naïve CD4$^+$ T cells from STAT1 KO, STAT3 CKO, and control mice were cultured with or without IL-27 in the presence of anti-CD3/CD28 mAb. Egr-2 expression was analyzed by flow cytometry on day 5. Plots are gated-on CD4$^+$ cells. Data shown are representative of three experiments performed. (B) Naïve CD4$^+$ T cells were cultured as shown in (A). The percentage of Egr-2-expressing cells in CD4$^+$ T cells was analyzed by flow cytometry on day 5. Data are shown as mean ± SD ($n = 3$; replicate experiments). (C) The expression of IL-10 mRNA was measured by quantitative RT-PCR. Naïve CD4$^+$ T cells were isolated from STAT1 KO, STAT3 CKO, or control mice and cultured with anti-CD3/CD28 mAb in the presence or absence of IL-27. Data are shown as mean ± SD ($n = 3$; replicate wells). Experiments were performed two times. (D) IL-10 concentrations in culture supernatants of stimulated naïve CD4$^+$ T cells as in (C) from STAT1 KO, STAT3 CKO, or each control mice were measured by ELISA. Data are shown as mean ± SD ($n = 3$; replicate wells). Experiments were performed three times. (E) Naïve CD4$^+$ T cells were cultured as shown in (A) under the indicated condition. Egr-2 expression was analyzed by intracellular staining. Data shown are representative of three experiments performed. (F) Expression of IL-10 and Blimp-1 mRNA were measured by quantitative RT-PCR. Naïve CD4$^+$ T cells were cultured as shown in (A) under the indicated condition. Data are shown as mean ± SD ($n = 3$; replicate wells). Experiments were performed two times. *$p < 0.05$; Student's t-test.

IL-27-induced IL-10 production has been considered to be important for gut immunity. In IL-27 receptor (WSX-1)-deficient mice, higher steady-state levels of Th17 cells were observed in the lamina propria and these mice were susceptible to high-dose dextran sulfate, a model of acute intestinal inflammation-induced colitis [35]. Similar to IL-10-deficient mice [36], WSX-1-deficient mice infected with *Toxoplasma gondii* develop a lethal CD4$^+$ T-cell-mediated response characterized by excessive production of proinflammatory cytokines and massive lymphocytic infiltrates in multiple organs [37]. WSX-1-deficient

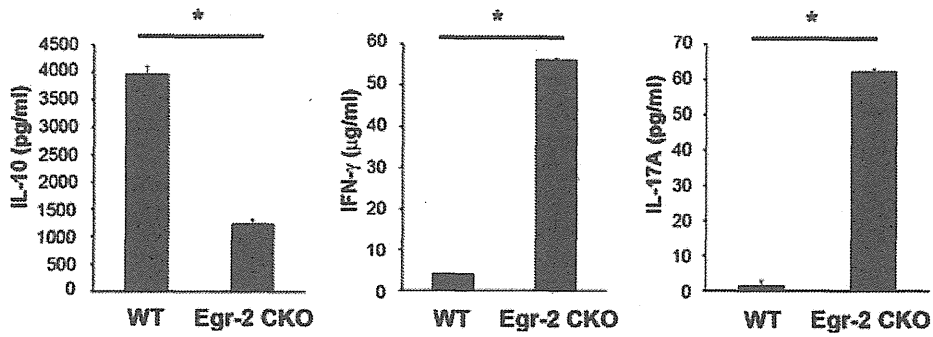

Figure 5. IL-10, IFN-γ, and IL-17 production in response to IL-27 stimulation. Naïve $CD4^+$ T cells from C57BL/6 WT and Egr-2 CKO mice were cultured with anti-CD3/CD28 mAb in the presence of IL-27. On day 5, IL-10, IFN-γ, and IL-17 concentrations in culture supernatants were measured by ELISA. Data are shown mean + SD ($n = 3$; replicate wells). Experiments were performed two times. *$p < 0.05$; Student's t-test.

$CD4^+$ T cells have been shown to be impaired in IL-10 production in $CD4^+$ T cells [17]. Although the $Foxp3^+$ Treg cell is one of the IL-10 producers, it has been shown that there are IL-10-producing T cells other than $Foxp3^+$ Treg cells in the intestine [38]. Moreover, CD4-specific IL-10-deficient mice have been shown to develop more severe colitis than $Foxp3^+$ Treg-specific IL-10-deficient mice [39], suggesting that Foxp3-negative, IL-10-producing T cells may be important for the maintenance of homeostasis in gut immunity. Egr-2-expressing $CD4^+CD25^-LAG3^+$ Treg cells are Foxp3-negative, IL-10-producing T cells and are enriched in Peyer's patch [21]. Our observation that IL-27 induces $CD4^+Egr2^+LAG3^+$ T cells may be associated with IL-27-mediated control of gut homeostasis; however, a more detailed investigation is required to elucidate the role of IL-27 in keeping intestinal homeostasis.

It has been well documented that stimulation of T cells through TCR in the absence of co-stimulation can result in long-term hyporesponsiveness to subsequent stimulation, which is termed anergy. It has been also reported that Egr-2 is required for the full induction of T-cell anergy [20, 40]. Egr-2 expression is rapidly induced within 6 h after TCR stimulation [41] and our results indicated that although IL-27-mediated Egr-2 induction was dependent on TCR stimulation, the TCR signal was not sufficient to support sustained Egr-2 expression. In addition to IL-27, another STAT3 activating cytokine, IL-6, also induced expressions of Egr-2, Blimp-1, and IL-10. This result was consistent with a previous report in which IL-6 induced STAT3-mediated production of IL-10 in $CD4^+$ T cells [17] and suggested that not only STAT1-STAT3 heterodimers in response to IL-27 stimulation but also STAT3 homodimers in response to IL-6 stimulation could induce Egr-2 expression. However, IL-27 induces Blimp-1 and IL-10 more efficiently than IL-6 and the involvement of STAT1 should be addressed further.

It is well known that IL-2 has paradoxical functions in T-cell homeostasis, acting as a T-cell growth factor and having a crucial function in the maintenance of self-tolerance. Sun et al. [26] reported that the effective induction of IL-10-producing $CD8^+$ CTLs by IL-27 requires the presence of IL-2, and that the IL-2-IL-27-mediated induction of IL-10 as well as the IL-27-mediated induction of IL-10 was Blimp-1 dependent. However, we observed that the addition of IL-2 did not up-regulate IL-10 and Blimp-1 mRNA induction levels by IL-27 in $CD4^+$ T cells. In addition, IL-2 showed no synergistic effect on IL-27-induced Egr-2 and LAG-3 expressions in our experiments. This result is consistent with the fact that increased Egr-2 level by Ag activation was not affected by the addition of IL-2 in peptide treatment-induced $CD4^+$ Treg cells [42]. These observations suggest that Blimp-1 is important for IL-27-induced IL-10 production both in $CD4^+$ and $CD8^+$ T cells, but the pathway leading to the activation of Blimp-1 is differently regulated between these cells.

Egr-2-expressing $CD4^+CD25^-LAG3^+$ Treg cells are anergic and have regulatory activities at least in part via IL-10 production. Because our results showed that Egr-2 is indispensable for the full production of IL-10 in $CD4^+$ T cells after IL-27 stimulation, Egr-2 could be one of the molecular links between anergy and IL-10 production in $CD4^+$ T cells. Further studies will be required to elucidate the relationship between the Egr-2-Blimp-1 pathway and other pathways, which have already been reported to contribute to IL-10 production by IL-27 stimulation.

We also found enhanced production of IFN-γ and IL-17 in Egr-2 CKO mice after IL-27 stimulation. Egr-2 CKO mice develop autoimmune disease characterized by the accumulation of IFN-γ and IL-17-producing $CD4^+$ T cells, and massive infiltration of T cells into multiple organs. The expressions of T-bet, a Th1 transcription factor, IL-6, IL-21, and IL-23, which can induce Th17 differentiation in $CD4^+$ T cells, were not altered in aged Egr-2 CKO mice [30]. Blimp-1 CKO mice develop severe colitis with age and Blimp-1-deficient $CD4^+$ T cells have been shown to produce more IFN-γ than WT after stimulation with PMA plus ionomycin or with TCR plus IL-2 [18]. Recently, Lin et al. [43] reported that NOD-background Blimp-1-deficient $CD4^+$ T cells exhibit significantly enhanced IL-17 production in a steady-state as well as in a Th17-polarizing condition. These observations indicate that increased IFN-γ and IL-17 production in IL-27-stimulated Egr-2-deficient $CD4^+$ T cells may be a direct consequence of reduced Egr-2-Blimp-1 signaling. Although Egr-2 CKO mice did not exhibit colitis, a single-nucleotide polymorphism in a locus at chromosome 10q21, which was identified by genome-wide analysis to have a strong relationship with Crohn's disease susceptibility, exists in a strong linkage disequilibrium region of Egr-2 [44, 45].

In summary, we have shown that Egr-2 mediates IL-27-induced IL-10 production through Blimp-1 transcription in $CD4^+$ T cells. Additionally, IFN-γ and IL-17 production by IL-27 was reciprocally regulated by Egr-2. Egr-2 may play a crucial role in maintaining the balance between regulatory and inflammatory cytokines. Our observation could contribute to the elucidation of the molecular regulation of IL-10 production in $CD4^+$ T cells.

Materials and methods

Mice

C57BL/6 mice and Prdm1-floxed mice were purchased from Japan SLC and The Jackson Laboratory, respectively. Blimp-1 CKO mice were generated by crossing Prdm1-floxed mice with CD4-Cre transgenic mice in which Cre-induced recombination was detected only in $CD4^+$ T cells. Egr-2 CKO mice were generated by crossing Egr-2-floxed mice [46] with CD4-Cre transgenic mice. TEα TCR transgenic mice were purchased from The Jackson Laboratory. WSX-1 deficient (WSX-1 KO) mice were prepared as described previously [47]. STAT1 KO mice were purchased from Taconic. STAT3 CKO mice ($STAT3^{fl/fl}$-CD4-Cre^+) were generated by crossing STAT3-floxed mice with CD4-Cre transgenic mice. CD4-Cre transgenic mice (line 4196), originally generated by Wilson and colleagues [48], were purchased from Taconic. All mice were used at 7–10 weeks of age. All animal experiments were conducted in accordance with Institutional and National Guidelines.

Reagents, antibodies, and media

The following reagents were purchased from BD Pharmingen: purified mAbs for CD3ε (145–2C11) and CD28 (37.51), Fc block (anti-CD16/32), FITC anti-CD45RB (16A), phycoerythrin (PE) anti-LAG-3 (C9B7W), PE anti-IgG2a (R35–95), PE anti-CD62L (MEL-14), allophycyanin-anti-CD25 (PC61), allophycyanin-Cy7 anti-CD25 (PC61), allophycyanin anti-CD4 (RM4–5), allophycyanin-Cy7 anti-CD4 (RM4–5), allophycyanin anti-LAG-3 (C9B7W), allophycyanin anti-IL-10 (JES5–16E3), allophycyanin anti-IgG2b (MPC-11), biotinylated mAb for CD8α (53–6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD25 (PC61), CD62L (MEL-14), Ter119 (TER119), and streptavidin (SA)- allophycyanin, SA-allophycyanin Cy7, SA-FITC. Qdot605 anti-CD4 (RM4–5) and SA-Qd605 were obtained from Invitrogen. Alexa Fluor 488 anti-LAG-3 (C9B7W) was obtained from AbD Serotec. PE anti-Egr-2 (erongr2) was obtained from e-Bioscience. Streptavidin-conjugated microbeads were purchased from Miltenyi Biotec. Recombinant murine IL-2, IL-10, IL-12, IL-21, and IL-27 were obtained from R&D Systems. Recombinant human TGF-β1 was purchased from R&D Systems. Recombinant murine IL-23 was obtained from Biolegend. Zymosan was obtained from Sigma. $E\alpha_{52-68}$ peptide was purchased from Takara (Otsu, Japan). T cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 μg/mL L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μM 2-mercaptoethanol (all purchased from Sigma).

In vitro T-cell differentiation

Naïve $CD4^+$ T cells ($CD4^+CD45RB^{hi}CD62L^{hi}CD25^-$) from C57BL/6 WT, Egr-2 CKO, or Blimp-1 CKO mice, WSX-1 KO mice, and STAT1 KO, or STAT3 CKO mice were isolated from their splenocytes. Briefly, single cell suspensions were first purified by negative selection with MACS (Miltenyi Biotec) using anti-CD8α mAb, anti-CD11b mAb, anti-CD11c mAb, anti-CD19 mAb, anti-CD25mAb, and anti-Ter119 mAb, and were then purified by positive selection with anti-CD62L microbeads. The purity of MACS sorted cells was >90%. Purified cells were cultured in flat-bottomed 24-well plates coated with anti-CD3ε (2 μg/mL) and anti-CD28 (2 μg/mL). Mouse IL-27 (25 ng/mL) was added at the start of culturing. To assess T-cell proliferation, purified naïve $CD4^+$ T cells were labeled with 1 μM carboxyfluorescein diacetate succinimidyl diester (Invitrogen) by incubation for 5 min at 37°C in the dark at a density of 2×10^6 cells/mL in RPMI medium. Other cytokines used were as follows: IL-2; 20 ng/mL, IL-6; 10 ng/mL, IL-12; 20 ng/mL, IL-23; 20 ng/mL and IFN-γ; 10 ng/mL.

In vitro response of TCR transgenic $CD4^+$ T cells to peptide

A total of 1×10^6 cells of $CD4^+$ T cells from $E\alpha_{52-68}$/$I\text{-}A^b$-specific transgenic mice were purified by positive selection with anti-CD4 microbeads and cultured with 5×10^5 cells of B cells from C57BL/6 WT mice in the presence of $E\alpha_{52-68}$ peptide (3 μM) in flat-bottomed 24-well plates. IL-27 (20 ng/mL), TGF-β1 (20 ng/mL), IL-21 (50 ng/mL), IL-10 (50 ng/mL), and zymosan (25 μg/mL) were added, respectively.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

$CD4^+$ T-cell RNA was prepared using an RNeasy Micro Kit (Qiagen). RNA was reverse-transcribed to cDNA with random primers (Invitrogen) and Superscript III (Invitrogen) in accordance with the manufacturer's protocol (Invitrogen). The cellular expression level of each gene was determined by quantitative real-time PCR analysis using an iCycler (Bio-Rad). The PCR mixture consisted of 25 μL of SYBR Green Master Mix (Qiagen), 15 pmol of forward and reverse primers, and cDNA samples in a total volume of 50 μL. Expression was normalized to the expression of β-actin. Specific primers for each indicated promoter were listed in Supporting Information Table 1.

Flow cytometry and cell sorting

Cultured T cells were harvested and stained using predetermined optimal concentrations of the respective antibodies. After Fc

blocking (antimouse CD16/CD32 mAb), prepared cells were stained with the indicated mAbs: Qdot605 anti-CD4, allophycocyanin anti-LAG-3, and SA-allophycocyanin Cy7. For intracellular anti-Egr-2 staining, cells were stained using the Foxp3 staining buffer set (e-Bioscience). For co-staining of Egr-2 and IL-10, cells were re-stimulated for 4 h at 37°C with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Sigma), ionomycin (500 ng/mL; Sigma), and for final 2 h with GolgiStop (1 μL/mL; BD Biosciences), followed by surface staining. Cells were then fixed with 2% paraformaldehyde for 10 min at room temperature and permeabilized with 0.5% saponin (Sigma) containing anti-Egr-2 and anti-IL-10 antibodies for 30 min at room temperature in the dark. Analysis and cell sorting of $CD4^+$ T cells were performed using FACSVantage with CellQuest (Becton Dickinson). Data were processed with FlowJo software. A full gating strategy was shown in Supporting Information Fig. 1.

ELISA

Cytokines in culture supernatants of $CD4^+$ T cells were analyzed using ELISA kits according to the manufacturer's instructions (Thermo Scientific and Biolegend).

LUC reporter assay

The Dual-Luciferase Reporter Assay System was used (Promega). 293T cells were cultured in 96-well plates and transfected with pGL-3-(-1500 Blimp-1) LUC reporter plasmids and phRL-(thymidine kinase) LUC control plasmids with either a pMIG vector or pMIG vector containing Egr-2 using Fugene6 (Roche). Cells were harvested 48 h later and LUC activity was assessed using MicroLumat Plus LB96V Luminometer (Berthold).

ChIP assay

Splenocytes from C57BL/6 mice were cultured for 24 h with anti-CD3 Ab (10 μg/mL) and $CD4^+$ T cells were then purified using the MACS system. The ChIP assay was carried out using a Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology). Briefly, $CD4^+$ T cells were fixed with formaldehyde and quenched with glycine. Crude nuclei were isolated and digested enzymatically using Micrococcal Nuclease and then sonicated to reduce chromatin DNA length to approximately 500 bp. Chromatin solutions was diluted in IP dilution buffer containing protease inhibitor and incubated with anti-Egr-2 Ab (Covance) or normal rabbit IgG. Cross-links were reversed by incubation overnight at 65°C, and immunoprecipitated chromatin (DNA) was purified by phenol-chloroform extraction and ethanol precipitation. Quantitative PCR analysis was performed using primers; corresponding sequences −3000 bp (assay position; −2399 bp), −2000 bp (assay position; −1294 bp), −1000 bp (assay position; −388 bp), and +1000 bp (assay position; +601 bp) from the transcription start site of Blimp-1, −3000 bp (assay position; −2386 bp), −2000 bp (assay position; −1388 bp), −1000 bp (assay position; −398 bp), and +1000 bp (assay position; +602 bp) from the transcription start site of LAG-3, and −1000 bp (assay position; −399 bp) and +1000 bp (assay position; +605 bp) from the transcription start site of IL10. Each primer was obtained from SA Bioscience. The promoter sequence of guanosine monophosphate reductase was used as a control. PCR products were subjected to gel electrophoresis to check the amplicon size (Supporting Information Fig. 2B).

Statistical analysis

Statistical analysis was performed using the Student's *t*-test. A *p*-value of <0.05 was considered to indicate a significant difference.

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Abbreviations: **Blimp-1**: B lymphocyte induced maturation protein-1 · **CKO**: conditional KO · **Egr-2**: early growth response gene 2 · **LAG-3**: lymphocyte activation gene 3 · **Tr1**: type I Treg

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