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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+CD25Foxp3+ 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+CD25Foxp3+ 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 its regulatory activity in both mice and humans. *The Journal of Immunology, 2013, 191: 000–000.

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utoactive CD4+ T cells that escape central tolerance are an essential component of autoimmune disease. Suppressing autoreactive CD4+ T cells and establishing peripher al 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. 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 its regulatory activity in both mice and humans. *The Journal of Immunology, 2013, 191: 000–000.

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Abbreviations used in this article: bCII, bovine type II collagen; CIA, collagen-induced arthritis; CIT, 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-2Rγnull; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling; Treg, regulatory T cells.

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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+ Tregs and that Egr-2 confers IL-10 and LAG3 expression and 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 a second exon sequence. Embryonic stem (ES) cells from C57BL/6 mice were used in all experiments, except the TGF-β1–producing 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+ Tregs and that Egr-2 confers IL-10 and LAG3 expression and vivo regulatory activity on CD4+ T cells (20).

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**Materials and Methods**

**Animals**

C57BL/6, BALB/c, and DBA/11 mice were purchased from Japan SLC (Shizuoka, Japan). OVATCR-transgenic D011.10, C57BL/6–IL-10−/−, and STAT3-flox/flox mice were obtained from The Jackson Laboratory (Bar Harbor, ME). NOD/Shi-scid IL-2Rγnull (NOG) mice were obtained from CLEA Japan (Tokyo, Japan). All animal experiments were conducted in accordance with the institutional and national guidelines.

**Generation of Egr-3 KO 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 CD45 mAb (53-6.7), biotin-conjugated anti-mouse CD11b mAb (HL3), biotin-conjugated anti-mouse CD19 mAb (ID3), Fe 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-phenoxy-STAT3 (Y705) mAb (4/P-Stat3). The following reagents were purchased from eBio-science: 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 (UCH1), 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 Alexa Enzo Life Sciences. PE anti-human CD45HI (3I0) 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–producing T cells, 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–producing T cells, the following reagents were used: 2 μM 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, 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/CD20 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 CD25 mAb, PE-human anti-human CD45RO mAb, ATTO488 anti-human LAG3 mAb (17B4) was purchased from Alexa Enzo Life Sciences. PE anti-human CD45HI (3I0) 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–producing T cells, was RPMI 1640 medium supplemented with 10% FCS, 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.

**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), 10 μM forward and reverse primer, and 50 μl 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′-CAAGCAGCATGGGCTCCATT-3′ and antisense, 5′-GGGCTCTTAATGTTCTCAGTG-3′; mouse gapdh sense, 5′-TGTCCTCGCGACTCGCTTTG-3′ and antisense, 5′-GCACGTGCG-TCTGATGTCTG-3′; mouse il10 sense, 5′-GGTTGCGCAAGCCTACGC-GA-3′, antisense, 5′-ACCCTCCTACCTCCTGTTCTG-3′; and mouse sodh sense, 5′-AGAGGGGAAAATCATGCGTGCAG-3′ and antisense, 5′-CAAATGTTGACTGCCGTCG-3′. For human cells, the real-time PCR primer pairs were as follows: human egr3 sense, 5′-GGGAGCAGATATGGGCTCCATT-3′ and antisense, 5′-GGGCTCTTAATGTTCTCAGTG-3′; human gapdh sense, 5′-TGTCCTCGCGACTCGCTTTG-3′ and antisense, 5′-GCACGTGCG-TCTGATGTCTG-3′; human sodh sense, 5′-GGTTGCGCAAGCCTACGC-GA-3′, antisense, 5′-ACCCTCCTACCTCCTGTTCTG-3′; and mouse sodh sense, 5′-AGAGGGGAAAATCATGCGTGCAG-3′ and antisense, 5′-CAAATGTTGACTGCCGTCG-3′. For human cells, the real-time PCR primer pairs were as follows: human egr3 sense, 5′-GGGAGCAGATATGGGCTCCATT-3′ and antisense, 5′-GGGCTCTTAATGTTCTCAGTG-3′; human gapdh sense, 5′-TGTCCTCGCGACTCGCTTTG-3′ and antisense, 5′-GCACGTGCG-TCTGATGTCTG-3′; mouse il10 sense, 5′-GGTTGCGCAAGCCTACGC-GA-3′, antisense, 5′-ACCCTCCTACCTCCTGTTCTG-3′; and mouse sodh sense, 5′-AGAGGGGAAAATCATGCGTGCAG-3′ and antisense, 5′-CAAATGTTGACTGCCGTCG-3′.
GTAC-3'- human FOXP3 sense, 5'-GAAACAGCACATTCCCGAG-TTCC-3' and antisense, 5'-ATGCCGACGGGTAGATGC-3'; human EGR2 sense, 5'-GCAACAGCTGTCGAACACATAC-T3'- and antisense, 5'-AGAAAGCTGCTGGATATTG-3'; human GAPDH sense, 5'-GCTC-TCCAGAACATCCTCCGGC-3' and antisense, 5'-CTTGTGTGATACCAGGAATGACTC-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 (CIT) (23) construct, we constructed the pMIX–CITR TCR (pMIX-CITr–internal ribosome entry site-CITR) vector (23). For the OVA-specific TCR (DO11.10) construct, we used the previously constructed vectors pMX–DOTAE and pMX–DOTBE (I-Ad restricted, OVA323–339–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 precleared, and stimulated splenocytes were plated onto each well (10^6 cells/well). This procedure was repeated a total of three times. The viral supernatant was removed, and stimulated splenocytes were plated onto each well (10^6 cells/well). Cells were cultured for 48 h to allow infection to occur.

Cytokine immunomassays
CD4+ GFP-positive cells were sorted from infectedants, and 1 x 10^5 cells were stimulated on 96-well microplates that had been coated with anti-CD3 antibody (eBioscience) or anti-CD28 antibody (BD Biosciences) (each 10 μg/ml). The supernatant was collected 48 h after the stimulation, and the IL-10 concentration was measured using a magnetic bead immunoassay kit (Miltenyi Biotec), and 10^6 splenocytes were stimulated on 24-well plates in the presence of 100 ng/ml recombinant mouse IL-6 for 15 min.

Delayed-type hypersensitivity
On day 1, BALB/c and C57BL/6 mice were s.c. immunized with 200 μg OVA (Sigma-Aldrich) emulsified with CFA at the base of the tail. On day 5, BALB/c splenocytes transfected with pMIG, pMIG–Egr-3, pMIG plus pMX–DOTAE, and pMX–DOTBE (DO11.10) were CD4+ enriched with a MACS kit, and CD4+ GFP-positive cells were sorted from infectants, and 10^6 cells were injected at the base of the tail on day 1. On day 20, DBA/1J splenocytes were injected into NOG mice through the tail vein, and 2 x 10^7 NOG mice were i.p. injected on the same day as the gene-transduced cells. In this neutralizing experiment, 1 x 10^7 CD4+ T cells transfected 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 tonsillec- tomy at The University of Tokyo Hospital or Showa General Hospital, respectively. 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 tonsillar 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 x 10^7 human T cells were mixed with Accell siRNA and Accell siRNA delivery media in the presence of 1 μg/ml 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 PB. NOG mice were irradiated with 2.5 Gy on the day before the cell transfer. Then, 5 x 10^7 PBMCs were transferred into the irradiated NOG mice through the tail vein, and 2 x 10^6 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 (DakoCytometry) overnight at 4°C and then were serially incubated with HRP-labeled anti-mouse Ig. The signals were amplified using the Envision system (DakoCytometry). For color development, these sections were incubated with 0.02% 3,3'-diaminobenzidine substrate solution containing 0.006% H₂O₂. 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.
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 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.
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 bHLH-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 reinfected 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−
Egr-3 promotes the production of TGF-β1 and Treg activity

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-
LYMPHOCYTES. CD25 and LAG3 expression in CD4+ T cells of tonsillar T cells. (Left panel) The mean of five individuals. The open circles represent individuals, and the bars represent the mean of five individuals. (Right panel) Representative FACS profile of tonsillar CD4+ T cells. (A) Quantitative PCR of human tonsillar CD4+CD25+LAG3+ T cells treated with the negative control siRNA or siRNA for human EGR3 (siRNA Ctrl) or siRNA for human EGR3 (siRNA EGR3). (Left panel). Relative EGR3 expression in each tonsillar T cell group from five different individuals. The expression of EGR3 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+CD25Foxp3+ 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+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 treatment with the negative control siRNA or siRNA for human EGR3 (middle and right panels). All error bars represent SD.

![Figure 5](http://www.jimmunol.org/)
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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**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 mean of three independent experiments. (B) Body weight change of NOG mice with 5×10⁶ PBMCs. Body weight change was observed. n = 3/group (top panel). Relative EGR3 expression 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.
Disclosures
The authors have no financial interests of conflict.

References


