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

Shuji Sumitomo, Keishi Fujio, Tomohisa Okamura, Kaoru Morita, Kazuyoshi Ishigaki, Keigo Suzukawa, Kaori Kanaya, Kenji Kondo, Tatsuya Yamasoba, Asayo Furukawa, Noboru Kitahara, Hirofumi Shoda, Mihoko Slibuya, Akiko Okamoto and Kazuhiko Yamamoto

_J Immunol_ 2013; 191:2351-2359; Prepublished online 31 July 2013; doi: 10.4049/jimmunol.1202106

http://www.jimmunol.org/content/191/5/2351
Transcription Factor Early Growth Response 3 Is Associated with the TGF-β1 Expression and the Regulatory Activity of CD4-Positive T Cells In Vivo

Shuji Sumitomo,* Keishi Fujio,* Tomohisa Okamura,* Kaoru Morita,* Kazuyoshi Ishigaki,* Keigo Suzukawa,† Kaori Kanaya,† Kenji Kondo,† Tatsuya Yamasoba,† Asayo Furukawa,‡ Noboru Kitahara,‡ Hirofumi Shoda,* Mihoko Shibuya,* Akiko Okamoto,* and Kazuhiko Yamamoto*†

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.

A putoreactive CD4+ T cells that escape central tolerance are an essential component of autoimmune disease. Supressing 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, cytolyis 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 autoimmunity 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-
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+ T lymphocyte activation gene 3 (LAG3) Tregs and that Egr-2 confers IL-10 and LAG3 expression in vivo and 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-Egr-2−/−) mice develop systemic autoimmune 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 both Egr-2 and Egr-3 in T and B cells (CD2-Egr2−/−Egr3−/−). Although Egr-3−/− mice do not develop any autoimmune symptoms, the CD2-Egr-2+ Egr-3−/− mice develop severe systemic autoimmune syndrome in their early life, with excessive accumulation of serum proinflammatory cytokines. In severe systemic autoimmune syndrome in their early life, mice transplanted with PBMCs. Our results suggest that Egr-3 is associated with the anti-inflammatory cytokine CD2-Egr2−/−Egr3−/− 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-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 autoimmune. 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/11 mice were purchased from Japan SLC (Shizuoka, Japan). OVA/TCR-transgenic DO11.10, C57BL/6−IL-10−/−, and STAT3−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). NOD/Shi−scid IL-2Rγ−/− (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 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 CD4 mAb (53-6.7), biotin-conjugated anti-mouse CD11b mAb (HL3), biotin-conjugated anti-mouse CD19 mAb (ID3), 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, bi2, β3 mAb (1D11), recombinant mouse IL-2, and recombinant mouse IL-6. ATT0488 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-Cy7 anti-human CD25 mAb, PE anti-human CD45RO mAb, ATT0488 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 × 10^6 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 and APC rat anti-mouse IgG1. To check the engrafment of PMBC-transferred NOG mice, spleenocytes 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), FACS Vantage SE (BD Biosciences), and FACS AriaII (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 μM forward and reverse primers, and the DNA 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 eg3 sense, 5′-CAAGCAGATGGGGGCCCTATTCT-C′′-C′′-3′ and antisense, 5′-GGCCTTGATTGCGTCTCATG-3′; mouse eg2 sense, 5′-TGGCTTCTGGOACTCCTTGTG-3′ and antisense, 5′-GCACTGTGTCGTTGACCTG-3′; mouse il10 sense, 5′-GGTTGCCAACGCTTACAGGA-3′ and antisense, 5′-ACCTGCTCCTACCTTGTTG-3′; mouse gapdh sense, 5′-AGAGGGGAATCGTGGCCTGAC-3′ and antisense, 5′-CAATACTGCCATCTGGGCTG-3′. For the human T cells, the real-time PCR primer pairs were as follows: human eg3 sense, 5′-GAGCAGAATTTGGAATGGTGGT-3′ and antisense, 5′-AGGAAACCTTAGTGGGTTG-3′; human TGFb1 sense, 5′-AGGCACTGCGACAGTTGCTA-3′ and antisense, 5′-GGAAGAGATTGGTGGACCTG-3′; human TGFb2 sense, 5′-CCACACCGAAGACGATAGCA-3′ and antisense, 5′-CCCTTTTCTGGCTTCCTTGTATG-3′; human IL10 sense, 5′-GAGATGCTCTTGAGCAGTGAAGA-3′ and antisense, 5′-AGGCTTGCCAAACCCAG.
GTAC-3; human FOXP3 sense, 5'-GAAACACGAGTCTCCAGAGTTCC-3' and antisense, 5'-ATGGCCACGCGTATGAGG-3'; human EGR2 sense, 5'-GGCAAGCTGTCTGACAACTAC-3' and antisense, 5'-AGCAAAAGTCTGGGATATGG-3'; human GAPDH sense, 5'-GCTCTCCAGAAGATCCTCCTGGC-3' and antisense, 5'-CTGTGTCATACCAGAATACTGGT-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), we constructed the pMX-CIT TCR (pMX-CITεe-internal ribosome entry site-CITββ) 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).

CD4+ GFP-positive cells were sorted from infectedants, and 1 × 105 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). Three days later, the cells were stained with PE–anti-mouse CD4 mAb after Fc-blocking, isoyed, 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 37˚C. To produce a positive control of pSTAT3 staining (27), 1 × 105 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 spleenocytes transduced with pMIG, pMIG–Egr-3, pMX plus pMX-CIT, or pMX–Egr-3 plus pMX-CIT were CD4 enriched with a MACS kit, and 1 × 105 cells were i.v. injected into each bCII-immunized mouse. On day 9, the thickness of both hind footpads was measured with an electrical micrometer caliper (Mitutoyo), 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 × 105 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 tonsillecctomy 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 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 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 × 105 cells were stimulated on 24-well plates 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 Biosciences), according to the manufacturer’s protocol. The TGF-β1 concentration in the supernatant of 1 × 105 infected 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 hydrochloric acid for 10 min and then neutralized with sodium hyoscyanate. An automatic microplate reader (Bio-Rad 550; Bio-Rad) was used to measure the OD of the samples.

Pathological analysis of CIA mice
The tarsal joints of sacrificed CIA mice were embedded in paraffin wax after fixation and paraffin sectioning. Sections were counterstained with hematoxylin to allow visualization of the nuclei. The immunohistochemical analysis was performed by the Bio-pathology 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.
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 reimplanted 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−
transduced CD4+ T cells (same mice as in gene, we consider the TGF-
EGR3
* 

tination. On day 21, the mice were given a booster immunization.
Mock, Egr-3, Mock+TCR, or Egr-3+TCR genes 20 d after first immuniza-
GVHD (31).
severe body weight loss was observed after the development of
GVHD was induced by the transfusion of human PBMC, and
(i.e., severely immunodeficient NOG mice). In these mice,

Human CD4+CD25
* 

T cells did not produce significant amounts of IL-10 or TGF-

We examined the regulatory activity of CD4+CD25
* 

Human CD4+CD25
* 

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
in vivo. (FIG. 3A, 3B). In contrast, this population did not express high amounts of IL-10. These features were shared among different individuals (Fig. 3C).

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. 3D). 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. 3E).

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 transfection of human PBMC, and severe body weight loss was observed after the development of GVHD (31).

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. *p < 0.05. (B) Mice were transferred with 1 × 10^6 CD4+ T cells transduced with Mock or Egr-3 genes. On the same day as the gene-transduced cell treatment, anti-TGF-β1 neutralizing Ab (aTGF-β1 Ab) or isotype control (Ctrl) IgG was i.p. injected. The DTH reaction was evaluated in each group, and differences among groups were assessed. *p < 0.05.

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 gated tonsillar T cells and IL10 in independent experiments. (taken from a certain patient. The results are shown as the mean of three GAPDH compared with expressions of human EGR3 and 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 treated 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.
Egr-3 PROMOTES THE PRODUCTION OF TGF-β1 AND Treg ACTIVITY

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 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.

Acknowledgments

We thank Dr. Munetaka Ushio, Dr. Naoya Egami, Dr. Shintaro Baba, Dr. Aki Inoue, Dr. Shu Kikuta, Dr. Atsushi Ochi, and Dr. Takuya Yasui for help with performing tonsillectomies. We also thank Kanako Sakashita and Kayoko Watada for excellent technical assistance.