＜研究成果の刊行に関する一覧表（岸本）＞

【書 籍】

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ
青江啓介	一部に骨形成を伴った胸膜中皮腫症例	岸本卓巳	アスベスト関連疾患早期発見・診断の手引き－中皮腫を正しく診断するために－(改訂版)	日本労務研究会	東京	2013	25-27
青江啓介	悪性胸膜中皮腫と鑑別困難な症例(滑膜肉腫の症例)	独立行政法人労働者健康福祉機構編	アスベスト関連疾患日常診療ガイド(増補改訂2版)	労働調査会	東京	2012	41-42

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Ⅲ.　研究成果の別刷

CD26-Mediated Induction of EGR2 and IL-10 as Potential Regulatory Mechanism for CD26 Costimulatory Pathway

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CD26 is associated with T cell signal transduction processes as a costimulatory molecule, and CD26$^+$ T cells have been suggested to be involved in the pathophysiology of diverse autoimmune diseases. Although the cellular and molecular mechanisms involved in CD26-mediated T cell activation have been extensively evaluated by our group and others, potential negative feedback mechanisms to regulate CD26-mediated activation still remain to be elucidated. In the present study, we examine the expression of inhibitory molecules induced via CD26-mediated costimulation. We show that coengagement of CD3 and CD26 induces preferential production of IL-10 in human CD4$^+$ T cells, mediated through NFAT and Raf-MEK-ERK pathways. A high level of early growth response 2 (EGR2) is also induced following CD26 costimulation, possibly via NFAT and AP-1–mediated signaling, and knockdown of EGR2 leads to decreased IL-10 production. Furthermore, CD3/CD26-stimulated CD4$^+$ T cells clearly suppress proliferative activity and effector cytokine production of bystander T cells in an IL-10–dependent manner. Taken together, our data suggest that robust CD26 costimulatory signaling induces preferential expression of EGR2 and IL-10 as a potential mechanism for regulating CD26-mediated activation. *The Journal of Immunology*, **2015, 194: 960–972.**

CD26 is a 110-kDa type II membrane-bound glycoprotein with dipeptidyl peptidase IV (DPPIV) activity in its extracellular domain (1–3). CD26 is associated with T cell signal transduction processes as a costimulatory molecule, as well as being a marker of T cell activation (4, 5). Whereas CD26 expression is increased following activation of resting T cells, CD4$^+$ CD26^{high} T cells respond maximally to recall Ags such as tetanus toxoid (6). Moreover, crosslinking of CD26 and CD3 with solid-phase immobilized mAbs can induce T cell costimulation and IL-2 production by CD26$^+$ T cells. Furthermore, high CD26 cell surface expression in CD4$^+$ T cells is correlated with the production of Th1 and Th17-type cytokines and high migratory activity (4, 7). In fact, patients with autoimmune diseases, such as multiple sclerosis (MS), Grave's disease, and rheumatoid arthritis, have been found to have increased numbers of CD4$^+$CD26$^+$ T cells in inflamed tissues as well as in their peripheral blood, with enhancement of CD26 expression correlating with the autoimmune disease severity (8–10). We have recently found that cytotoxic activity of CD8$^+$ T cells is also regulated via CD26-mediated costimulation, and that CD26$^+$ T cells are deeply involved in the pathophysiology of acute graft-versus-host disease (11, 12). These findings imply that CD26$^+$ T cells play an important role in the inflammatory process and subsequent tissue damage in such diseases.

Activation of T cells through costimulatory signals is essential for an effective Ag-specific immune response, whereas the suppressive control of excessive immune responses is also critical for maintaining self tolerance and preventing autoimmunity. CD28 is a representative T cell costimulatory molecule, required for optimal production of cytokines and proliferation, and CD28-deficient mice have markedly reduced responses to exogenous Ags (13, 14). Alternatively, CTLA-4 functions as a potent negative regulator of the T cell response, and CTLA-4–deficient mice develop massive lymphadenopathy, autoimmunity, and early death (15, 16). Both CD28 and CTLA-4 are members of the Ig supergene family and are able to bind CD80 and CD86 expressed on APCs. CTLA-4 is not expressed on naive T cells, but it is induced after activation. CTLA-4 interacts with CD80 and CD86 with a 50- to 100-fold higher binding avidity than CD28, leading to interference of CD28 signaling (17). In addition to the ligand competition, the cytoplasmic domain of CTLA-4 associates with phosphatases SHP2 and PP2A to negatively regulate T cell activation, and it also inhibits the formation of lipid rafts (18). We have identified caveolin-1 on APCs as a functional ligand for CD26, and the ligation of CD26 with caveolin-1 recruits a complex consisting of CD26, CARMA1, Bcl10, MALT1, and IκB kinase to lipid rafts, leading to NF-κB activation (19, 20). CD26 possesses DPPIV enzyme activity, able to cleave dipeptides from polypeptides with N-terminal penultimate proline or alanine. Molecules displaying DPPIV-like enzymatic activity and/or structural similarity to the DPPIV/CD26 have been grouped to a family of "dipeptidyl pep-

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Received for publication August 28, 2014. Accepted for publication November 19, 2014.

This work was supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to K. Ohnuma and C.M.) and the Ministry of Health, Labor and Welfare, Japan (to C.M.), as well as Foundation of Strategic Research Projects in Private Universities Grant-in-Aid S1311011 from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to C.M.). This work was also supported by Japan Society for the Promotion of Science KAKENHI Grant 26860760 (to R.H.) and Japan Society for the Promotion of Science Research Fellowships for Young Scientists (to R.H.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: Cav-Ig, fusion protein of the N-terminal domain of human caveolin-1 and human IgG1 Fc; CD26 sup, supernatant of CD3/CD26-stimulated T cells; CD28 sup, supernatant of CD3/CD28-stimulated T cells; CyA, cyclosporin A; DPPIV, dipeptidyl peptidase IV; EGR2, early growth response 2; IRF4, IFN regulatory factor 4; LAG3, lymphocyte-activation gene 3; LAP, latency-associated protein; MFI, mean fluorescence intensity; MS, multiple sclerosis; siRNA, small interfering RNA; Treg, regulatory T cell; Tr1, type 1 regulatory T cell.

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tidase IV activity and/or structure homologs (DASH)," including enzymatically active members such as fibroblast activation protein-α, quiescent cell proline dipeptidase/DPP-II/DPP7, DPP8, DPP9, and enzymatically inactive members such as DPP6 and DPP10 (21). However, a DASH family member that functions as a negative regulator of CD26-caveolin-1–mediated T cell activation has not yet been reported.

Active suppression by regulatory T cells (Tregs) is essential for the control of autoreactive cells. Tregs suppress immune responses through either direct cell–cell interactions or the release of inhibitory cytokines. $CD4^+CD25^{high}$ Tregs expressing FOXP3, a master regulatory gene for the suppressive activity, suppress immune cells mainly through soluble or membrane-bound TGF-β (22). Recently, IL-35 has been reported as a new inhibitory cytokine preferentially expressed by mouse $Foxp3^+$ Tregs, and it is required for their maximal suppressive activity (23). IL-35 is a member of the IL-12 heterodimeric cytokine family, composed of EBV-induced 3 and p35 (also known as IL-12α). Type 1 Tregs (Tr1s) are characterized by high-level production of IL-10 with regulatory activity (24). Lymphocyte-activation gene 3 (LAG3) and early growth response 2 (EGR2) have been recently reported as markers of Tr1-like cells (25). LAG3 binds to MHC class II molecules with higher affinity than CD4, leading to transduction of inhibitory signals for both T cells and APCs (26, 27). EGR2 is an essential transcription factor that regulates the T cell anergy program by directly upregulating diacylglycerol kinase α, and it also plays an important role in the induction of LAG3 and IL-10 expression (28).

Although the signaling events involved in CD26-mediated T cell activation or the cellular functions of CD26-expressing T cells have been studied extensively by our group and others, the potential negative feedback mechanism of CD26-mediated costimulation still remains to be elucidated. The present study focuses on the expression of inhibitory molecules induced in $CD4^+$ T cells following CD26 costimulation and the signaling pathways involved in this biological process.

Materials and Methods

Preparation of human T cells

Human PBMCs were collected from healthy adult volunteers after the documented informed consent and Institutional Review Board approval were obtained. This study has been performed according to the principles set out in the Declaration of Helsinki. For purification, the MACS cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) and MACS human $CD4^+$ T cell isolation kit (Miltenyi Biotec) were used. Purity of $CD4^+$ T cells was ≥97% as confirmed by FACSCalibur (BD Biosciences, San Jose, CA).

Abs and reagents

For T cell stimulation, anti-CD3 mAb (OKT3), anti-CD28 mAb (4B10), anti-CD26 mAb (1F7), and the fusion protein of the N-terminal domain of human caveolin-1 and human IgG1 Fc (Cav-Ig) developed in our laboratory were used (20). The following human-specific Abs were used for flow cytometry: FITC-labeled anti-LAG3 mAb (clone 17B4) and FITC-labeled mouse IgG1, κ isotype control (clone MOPC-21) were purchased from Enzo Life Sciences (Farmingdale, NY). PE-labeled anti–CTLA-4 mAb (clone L3D10) and PE-labeled mouse IgG_1, κ isotype control (clone MOPC-21) were purchased from BioLegend (San Diego, CA). PE-labeled anti–IL-10 mAb (clone JES3-9D7) and PE-labeled rat IgG1, κ isotype control (clone eBRG1) were purchased from eBioscience (San Diego, CA). PerCP-labeled anti–latency-associated protein (LAP) mAb (clone 27232) and PerCP-labeled mouse IgG1 isotype control (clone 11711) were purchased from R&D Systems (Minneapolis, MN). Alexa Fluor 647–labeled anti–IFN-γ mAb (clone B27), Alexa Fluor 647–labeled anti-FOXP3 mAb (clone 259D/C7), and Alexa Fluor 647–labeled mouse IgG1, κ isotype control (clone MOPC-21) were purchased from BD Biosciences. Alexa Fluor 647–labeled anti-CD26 mAb (clone 19) recently developed in our laboratory was used for the detection of CD26 even in the presence of another anti-CD26 mAb, 1F7 (29). For Western blot analysis, mouse anti-NFAT1 mAb (clone 639402), goat anti-NFAT2 polyclonal Ab, and rabbit anti–p-ERK1 (pT202/pY204)/ERK2 (pT185/pY187) polyclonal Ab were purchased from R&D Systems. Mouse anti–TATA-binding protein mAb (clone mAbcam 51841) and rabbit anti-EGR2 mAb (clone EPR4004) were purchased from Abcam (Cambridge, U.K.). Mouse anti–β-actin mAb (clone AC-15) was purchased from Sigma-Aldrich (St. Louis, MO). HRP-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG were purchased from GE Healthcare (Buckinghamshire, U.K.), and HRP-conjugated donkey anti-goat IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For neutralizing IL-10, anti–human IL-10 mAb (clone JES3-9D7), anti–human IL-10R mAb (clone 3F9), rat IgG1, κ isotype control (clone RTK2071), and rat IgG2a, κ isotype control (clone RTK2758) were purchased from BioLegend. Recombinant human IL-10 and the NFAT inhibitor cyclosporin A (CyA) were purchased from Sigma-Aldrich. The MEK1/2 inhibitor U0126 and the NF-κB activation inhibitor quinazoline were purchased from Merck Millipore (Billerica, MA).

Preparation of culture supernatant and measurement of cytokines

Purified $CD4^+$ T cells (1×10^5) were cultured in serum-free AIM-V medium (Invitrogen, Carlsbad, CA) in 96-well flat-bottom plates (Costar; Corning, Corning, NY), with stimulatory mAbs or Cav-Ig being bound in the wells beforehand at the following concentrations: 0.5 μg/ml OKT3 and/or 2, 5, 10, 20, or 50 μg/ml 4B10 or 1F7 or Cav-Ig. Cells were cultured in 5% CO_2 and 100% humidified incubator at 37°C for 48, 72, 96, or 120 h. After incubation, supernatants were collected and cytokine concentrations were examined using ELISA. BD OptEIA kits for human IL-2, IL-4, IL-5, IL-10, TNF-α, IFN-γ, or TGF-β1 were purchased from BD Biosciences, and the Ready-SET-Go! kits for human IL-17A or IL-21 were purchased from eBioscience. The absorbance at 450/570 nm was measured in a microplate reader (Bio-Rad, Hercules, CA), and data were analyzed with Microplate Manager 6 software (Bio-Rad).

Flow cytometry

Purified $CD4^+$ T cells (1×10^5) were incubated with plate-bound OKT3 (0.5 μg/ml) and/or 2, 5, 10, 20, or 50 μg/ml of 4B10 or 1F7 in 96-well flat bottom plates for 96 h, and cells were then collected and prepared for the analysis of cell surface expression of CTLA-4, LAG3, and LAP. Acquisition was performed using FACSCalibur, and data were analyzed with FlowJo software (Tree Star, Ashland, OR). For the analysis of intracellular FOXP3 and LAG3, a human FOXP3 buffer set (BD Biosciences) was used. For the staining of intracellular cytokines, cells were treated with GolgiStop (monensin) (BD Biosciences) for the last 5 h of culture in the presence or absence of 50 ng/ml PMA (Sigma-Aldrich) plus 1 μg/ml ionomycin (Merck Millipore) and then stained using BD Cytofix/Cytoperm Plus fixation/permeabilization kit (BD Biosciences) according to the manufacturer's instructions.

Preparation of lysates and Western blotting

To analyze nuclear expression of ERK or NFAT, purified $CD4^+$ T cells (1.5×10^6 cells/well, three wells per sample) were incubated with plate-bound OKT3 (0.5 μg/ml) and/or 4B10 (50 μg/ml) or 1F7 (50 μg/ml) in 24-well flat-bottom plates (Costar) for 0.5, 1, or 2 h. After incubation, cells were collected and nuclear extracts were prepared using EpiQuik nuclear extraction kit (Epigentek, Farmingdale, NY) supplemented with 2% protease inhibitor mixture (Sigma-Aldrich) and 1× PhosSTOP (Roche Diagnostics, Tokyo, Japan). For the analysis of EGR2 expression, purified $CD4^+$ T cells (1.5×10^6 cells/well) were stimulated in the above conditions for 24 or 48 h. After incubation, cells were collected and whole-cell lysates were prepared using RIPA buffer. Each sample was resolved by SDS-PAGE in reducing condition. SDS-PAGE and Western blot analysis were conducted as described previously (30). The images were taken using luminescent image analyzer LAS 4000 (GE Healthcare), and data were analyzed with image reader LAS 4000 and Multi Gauge software (GE Healthcare).

T cell proliferation assay

Purified $CD4^+$ T cells (1×10^5) were incubated with plate-bound OKT3 (0.5 μg/ml) and/or 4B10 or 1F7 in 96-well flat-bottom plates for 96 h in the presence or absence of signal inhibitor or culture supernatant. To evaluate T cell proliferation, a cell proliferation ELISA, BrdU Kit (Roche Diagnostics) was used. BrdU was added to each well for the last 2 h of incubation, and proliferation was assessed by measuring BrdU incorporation by ELISA. The absorbance at 450/655 nm was measured in a microplate reader, and data were analyzed with Microplate Manager 6 software. T cell proliferation was also assessed by using Cell Counting

Kit-8 (Dojindo, Kumamoto, Japan). 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt (WST-8) was added to each well at a concentration of 1/10 volume for the last 3 h, and the absorbance at 450/595 nm was measured. For the analysis of the number of cell divisions, a Vybrant CFDA SE cell tracer kit (Molecular Probes, Carlsbad, CA) was used. Purified $CD4^+$ T cells labeled with 2 μmol/l CFSE were incubated for 96 h with plate-bound stimulatory mAbs as described above, and cells were then harvested and analyzed by FACSCalibur.

Small interfering RNA against human EGR2

We selected two target sequences from nucleotides +1802 to 1820 (sense1) and +1890 to 1908 (sense2) downstream of the start codon of human EGR2 mRNA: sense1 small interfering RNA (siRNA), 5′-GAGUUGAUCUAAGACGUUUdTdT-3′, and sense2 siRNA, 5′-CACUUUAUGGCUUGGGACUdTdT-3′. siRNAs against EGR2 were purchased from Sigma-Aldrich, and negative control siRNA (oligonucleotide sequences are not disclosed) was purchased from Qiagen (Valencia, CA). Fifty picomoles siRNA duplexes were transfected into 5×10^5 purified $CD4^+$ T cells by using HVJ-E vector (GenomONE-Si; Ishihara Sangyo Kaisha, Osaka, Japan) according to the manufacturer's instructions (19, 20).

Quantitative real-time RT-PCR assay

Purified $CD4^+$ T cells or siRNA transfected $CD4^+$ T cells (5×10^5) were incubated with plate-bound OKT3 (0.5 μg/ml) and/or 4B10 or 1F7 or Cav-Ig in 24-well flat-bottom plates for 6, 12, 24, 48, or 72 h in the presence or absence of signal inhibitor or culture supernatant. After incubation, cells were collected and total RNA was extracted by the use of an RNeasy Micro kit according to the manufacturer's instructions (Qiagen). cDNA was produced by using a PrimeScript II first strand cDNA synthesis kit (TaKaRa Bio, Shiga, Japan) with oligo(dT) primers. Quantification of mRNA was performed using the 7500 real-time PCR System and SYBR Select Master Mix (Applied Biosystems, Foster City, CA). The obtained data were analyzed with 7500 System SDS software (Applied Biosystems), being normalized to hypoxanthine phosphoribosyltransferase expression. Sequences of primers used in quantitative real-time RT-PCR analysis are shown in Supplemental Table I.

Statistical analysis

Data were analyzed by the paired Student *t* test (two-tailed) for two group comparison, or by the ANOVA test for multiple comparison testing. The assay was performed in triplicate wells, and data are presented as mean ± SD of triplicate samples of the representative experiment, or mean ± SE of triplicate samples of independent experiments. Significance was analyzed using MS Excel (Microsoft) and p values < 0.01 were considered significant and are indicated in the corresponding figures and figure legends.

Results

CD26-mediated costimulation induces the development of $CD4^+$ T cells to a Tr1-like phenotype with high IL-10 production

To explore the negative feedback mechanism associated with CD26-mediated costimulation, we first examined the cytokine production profile of $CD4^+$ T cells, focusing particularly on the production of immunosuppressive cytokines such as TGF-β and IL-10. To characterize the specific phenotype associated with CD26 costimulation, we compared the level of cytokine production following costimulation with either CD26 or CD28, a representative T cell costimulatory pathway. As shown in Fig. 1A, only a small amount of cytokines was produced following stimulation with anti-CD3 mAb alone (gray lines), whereas both anti-CD3 plus anti-CD26 and anti-CD3 plus anti-CD28 costimulation greatly enhanced cytokine production (red or blue lines). Production of IL-10 by $CD4^+$ T cells was preferentially increased following CD26-mediated costimulation compared with CD28-mediated costimulation over the tested time intervals (Fig. 1Aa). IL-21 production was also greatly enhanced in the late phase of CD26 costimulation (Fig. 1Ab). Alternatively, production of IL-2, IL-5, or TNF-α was much lower following CD26 costimulation than CD28 costimulation (Fig. 1Af–h). In contrast, no difference in the production of IL-17A, IFN-γ, or IL-4 was observed following CD26- or CD28-mediated costimulation (Fig. 1Ac–e). Real-time RT-PCR analysis was then conducted to confirm the above results. As shown in Fig. 1B, IL-10 and IL-21 mRNA expression levels were higher after 24 h of CD26 costimulation than CD28 costimulation. Meanwhile, the amount of TGF-β1 was below the detection limit (0.125 ng/ml), and mRNA expression level of IL-35 subunits (EBV-induced 3 and p35) was not elevated following CD26- or CD28-mediated costimulation (data not shown). These data indicate that CD26 and CD28 costimulation of $CD4^+$ T cells results in different profiles of cytokine production, with IL-10 production being preferentially enhanced following CD26 costimulation.

To further characterize the biological process involved in IL-10 production following CD26 costimulation, we evaluated the effect of a wide range of concentrations of anti-CD26 and anti-CD28 mAbs. Additionally, to assess whether caveolin-1, a physiological ligand of CD26, can induce IL-10, we activated $CD4^+$ T cells with anti-CD3 mAb and Cav-Ig as described in *Materials and Methods*. As shown in Fig. 2Aa, CD26-mediated costimulation by both anti-CD3 plus anti-CD26 and anti-CD3 plus Cav-Ig greatly enhanced IL-10 production compared with CD28-mediated costimulation. It is noteworthy that the enhancement in CD26-mediated IL-10 production via either anti-CD26 mAb or Cav-Ig was dose-dependent, whereas IL-10 production decreased with increasing doses of anti-CD28 mAb (Fig. 2Aa). IL-17A production following CD26-mediated costimulation was similar to that observed following CD28-mediated costimulation (Fig. 2Ab). Although the amount of IL-2 produced by $CD4^+$ T cells following CD26 costimulation was 1–10 ng/ml and significantly higher than anti-CD3 mAb alone, it was consistently lower than that induced by CD28 costimulation (Fig. 2Ac, Supplemental Fig. 1A). T cell proliferation in response to CD26 costimulation was significantly decreased in the presence of neutralizing anti–IL-2 mAb, indicating that a relatively small amount of IL-2 induced by CD26 costimulation was sufficiently functional for enhancing T cell proliferation (Supplemental Fig. 1B). For further confirmation, we analyzed the intracellular expression of IL-10 in $CD4^+$ T cells through flow cytometry. Because inhibition of protein transport by monensin or brefeldin A is essential for intracellular cytokine staining, we analyzed IL-10 expression in T cells stimulated by anti-CD3 plus a high dose (50 μg/ml) of anti-CD26 or anti-CD28 with monensin for the last 5 h of culture. As shown in Fig. 2B, IL-10 and IFN-γ were equally induced in $CD4^+$ T cells following CD26 costimulation (Fig. 2Bb), whereas CD28 costimulation induced only IFN-γ but hardly induced IL-10 (Fig. 2Ba). Note that only ~2% of $CD4^+$ T cells expressed IL-10 following CD26 costimulation, but this induction was significant and was consistently seen in all the repeated experiments. In contrast with IFN-γ level, which was clearly detected by restimulation with PMA plus ionomycin, detection of the IL-10 level was relatively difficult because restimulation with PMA plus ionomycin hardly affected the percentage of IL-10–expressing cells following either CD26- or CD28-mediated costimulation (the results with similar stimulatory condition are shown in Fig. 6B). Previous work by Jonuleit et al. (31) investigating IL-10 expression also demonstrated a relatively low level of IL-10 expression when human peripheral blood $CD4^+$ T cells from healthy volunteers were primed and restimulated with allogeneic dendritic cells. We also noted that another anti-human IL-10 mAb from BD Biosciences gave similar results (data not shown). To define whether IL-10–expressing $CD4^+$ T cells following CD26-mediated costimulation expressed CD26, we stained cell surface CD26 and intracellular IL-10. For this purpose, as described in *Materials and Methods*, we used an anti-human CD26 mAb recognizing a different epitope from the one recognized by

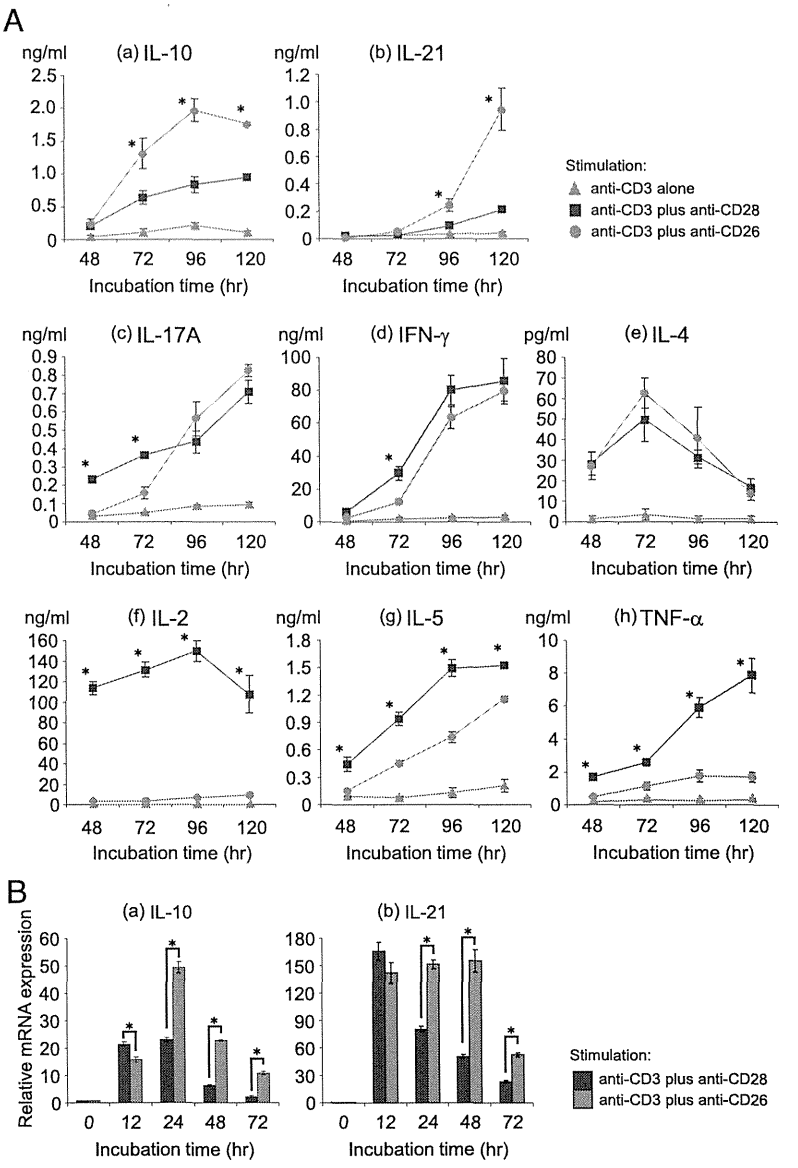

FIGURE 1. CD26-mediated costimulation of $CD4^+$ T cells induces greater levels of IL-10 and IL-21 than does CD28-mediated costimulation. Freshly purified $CD4^+$ T cells were stimulated with anti-CD3 mAb alone, anti-CD3 plus anti-CD28 mAbs (5 μg/ml), or anti-CD3 plus anti-CD26 mAbs (5 μg/ml) for the indicated times. (**A**) Concentrations of IL-10 (**a**), IL-21 (**b**), IL-17A (**c**), IFN-γ (**d**), IL-4 (**e**), IL-2 (**f**), IL-5 (**g**), and TNF-α (**h**) were examined by ELISA. (**B**) mRNA expression of IL-10 (**a**) and IL-21 (**b**) was quantified by real-time RT-PCR. Each expression was normalized to HPRT1, and relative expression levels compared with resting $CD4^+$ T cells (0 h) were shown. Representative data of seven (A) and three (B) independent donors are shown as mean ± SD of triplicate samples, comparing values in anti-CD3 plus anti-CD26 to those in anti-CD3 plus anti-CD28 ($^*p < 0.01$), and similar results were obtained in each experiment.

the immobilized anti-CD26 mAb, 1F7, used for CD26 costimulation. As shown in Fig. 2Bc, all of the IL-10–expressing $CD4^+$ T cells were shown to express CD26. Taken together, these results indicate that CD26-mediated costimulation in $CD4^+CD26^+$ T cells preferentially induces IL-10, and this effect increases with enhancing intensity of CD26-mediated signaling.

We next examined the expression of selected molecules with well-characterized immunosuppressive functions by flow cytometry, including the cell surface expression of CTLA-4 and LAP complexed with TGF-β1, and the intracellular expression of transcription factor FOXP3. Because LAG3 has been reported to be continuously recycled and rapidly translocated to the plasma membrane in response to antigenic stimulation (32), we analyzed both the cell surface and intracellular expression of LAG3. As shown in Fig. 3, both the cell surface and intracellular expression of LAG3 was clearly enhanced with increasing doses of anti-CD26 mAb, and CD26-induced enhancement of LAG3 was more pronounced than the effect of CD28-mediated costimulation (Fig. 3Aa). Alternatively, both CD26- and CD28-mediated costimulation enhanced the expression of CTLA-4 and FOXP3, with no significant difference being detected between these two costimulatory pathways (Fig. 3Ac, d). In contrast with CD28 costimulation, LAP was hardly induced following CD26 costimulation (Fig. 3Ab). Although the mean fluorescence intensity (MFI) results of LAP, CTLA-4, or FOXP3 correlated with those of percentage of cells expressing LAP, CTLA-4, or FOXP3 shown in Fig. 3A, the values of MFI, especially for LAP and FOXP3, were low and the difference between CD26- and CD28-mediated costimulation was minimal (data not shown). Alternatively, as shown in Fig. 3B, all of the $CD4^+$ T cells expressed LAG3 following CD26 or CD28 costimulation, and no difference was observed in the percentage of LAG3-expressing cells, whereas the expression intensity of LAG3 after CD26-mediated costimulation was higher than after CD28-mediated costimulation. LAG3 also serves as a marker of IL-10–producing Tregs (25), suggesting that signaling events via CD26 may induce the development of $CD4^+$ T cells to a Tr1-like phenotype.

NFAT and Raf-MEK-ERK signalings are indispensable for CD26-mediated T cell activation

We next examined the signaling events associated with CD26-mediated enhancement in IL-10 production in $CD4^+$ T cells. Although cytokine receptor signaling through JAK-STAT plays a crucial role in T cell differentiation into specific effector subsets or Tregs, the initial signaling events for inducing cytokine production in T cells are associated with the TCR signaling pathway. Therefore, we first examined the effect of signal inhibitors against

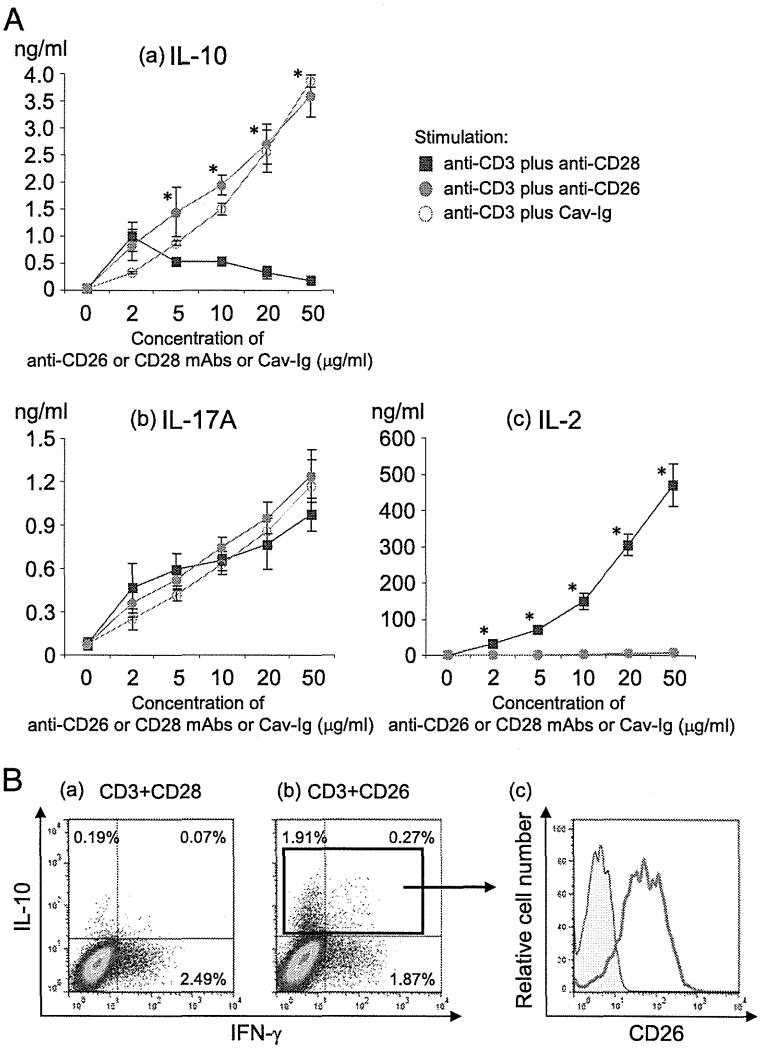

FIGURE 2. CD26-mediated costimulation of $CD4^+$ T cells greatly enhances IL-10 in a stimulation intensity–dependent manner. (**A**) Freshly purified $CD4^+$ T cells were stimulated with anti-CD3 mAb alone, anti-CD3 plus anti-CD28 mAbs, anti-CD3 plus anti-CD26 mAbs, or anti-CD3 mAb plus Cav-Ig at the indicated concentrations for 96 h. Concentrations of IL-10 (A**a**), IL-17A (A**b**), and IL-2 (A**c**) were examined by ELISA. Representative data of five independent donors are shown as mean ± SD of triplicate samples, comparing values in anti-CD3 plus anti-CD26 or anti-CD3 plus Cav-Ig to those in anti-CD3 plus anti-CD28 (*$p < 0.01$), and similar results were obtained in each experiment. (**B**) Freshly purified $CD4^+$ T cells were stimulated with anti-CD3 plus anti-CD28 mAbs (50 μg/ml) (**a**) or anti-CD3 plus anti-CD26 mAbs (50 μg/ml) (**b** and **c**) for 72 h. Cells were treated with monensin in the absence of PMA plus ionomycin restimulation for the last 5 h of culture, and cell surface CD26, intracellular IFN-γ, and IL-10 were detected by flow cytometry. (Ba and b) Two-dimensional dot plot of IFN-γ or IL-10 staining gated for $CD4^+$ T cells is shown. (Bc) Following gating for $CD4^+IL\text{-}10^+$ population as indicated in (Bb), the expression of CD26 was analyzed. The data are shown as histogram of CD26 intensity, and the gray area in the histogram show the data on the isotype control. A representative plot or histogram of four independent donors is shown, and similar results were obtained in each experiment.

NFAT, AP-1, or NF-κB, essential transcription factors for T cell activation located at the terminal end of TCR signaling. We first assessed T cell proliferation by measuring BrdU incorporation by ELISA, which is generally accepted to be equivalent to the [^{3}H]thymidine incorporation assay. As shown in Fig. 4A, the NFAT inhibitor CyA and the MEK1/2 inhibitor U0126 markedly inhibited the proliferative activity of $CD4^+$ T cells following CD26 or CD28 costimulation. Of note, T cells activated with anti-CD3 and anti-CD26 were much more sensitive to these inhibitors as compared with CD28 costimulation, and a high dose of CyA and U0126 suppressed the proliferation of T cells to the same level of proliferative activity as anti-CD3 alone (Fig. 4A). Additionally, we conducted a T cell proliferation assay by using tetrazolium salt, and results similar to those obtained with the BrdU incorporation assay were observed (Fig. 4B). As shown in Fig. 4C, the effects of NFAT and MEK1/2 inhibitors on IL-10 production were very similar to those on T cell proliferation. Whereas the NF-κB inhibitor quinazoline also suppressed T cell proliferation and IL-10 production in a dose-dependent manner, its effect on $CD4^+$ T cells following CD26 costimulation was less apparent as compared with NFAT and MEK1/2 inhibitors (Fig. 4A–C). These three inhibitors had similar effects on IL-17A and IFN-γ production as they did with IL-10 production (data not shown). These results indicate that both NFAT- and Raf-MEK-ERK–related signals are indispensable for CD26-mediated T cell activation, and they suggest that the intensity or persistence of these two signal pathways in $CD4^+$ T cells are different between CD26 and CD28 costimulation.

To further define the difference between CD26- and CD28-mediated costimulatory signaling pathways, we next examined the amount of NFAT and p-ERK1/2 translocated to the nucleus of $CD4^+$ T cells following CD26 or CD28 costimulation. The NFAT family consists of five members (NFAT1–5), and only NFAT5 is regulated by osmotic stress, not by calcium–calcineurin signaling (33). Because NFAT3 is reported to be absent in T cells, we analyzed the expression of NFAT1, NFAT2, and NFAT4. As shown in Fig. 4D, stimulation with both anti-CD3 plus anti-CD28 and anti-CD3 plus anti-CD26 resulted in increased nuclear levels of p-ERK1/2 and NFAT2 compared with anti-CD3 alone. Nuclear levels of p-ERK1/2 peaked at 30 min and then gradually decreased following CD28-mediated costimulation, whereas accumulation of NFAT2 in the nucleus reached a maximum after 2 h of CD28 costimulation. Alternatively, CD26-mediated costimulation resulted in greater enhancement of nuclear level of NFAT2 compared with CD28 costimulation, whereas the maximum level of p-ERK1/2 following CD26-mediated costimulation was lower than that seen with CD28 costimulation, but the effect of CD26 costimulation on p-ERK1/2 persisted until 2 h after stimulation. In contrast, no difference in nuclear level of NFAT1 was observed following CD26 or CD28 costimulation. As shown in Fig. 4D, when each sample was immunoblotted with anti-NFAT2, two bands were detected in molecular mass regions at ~110,000 and

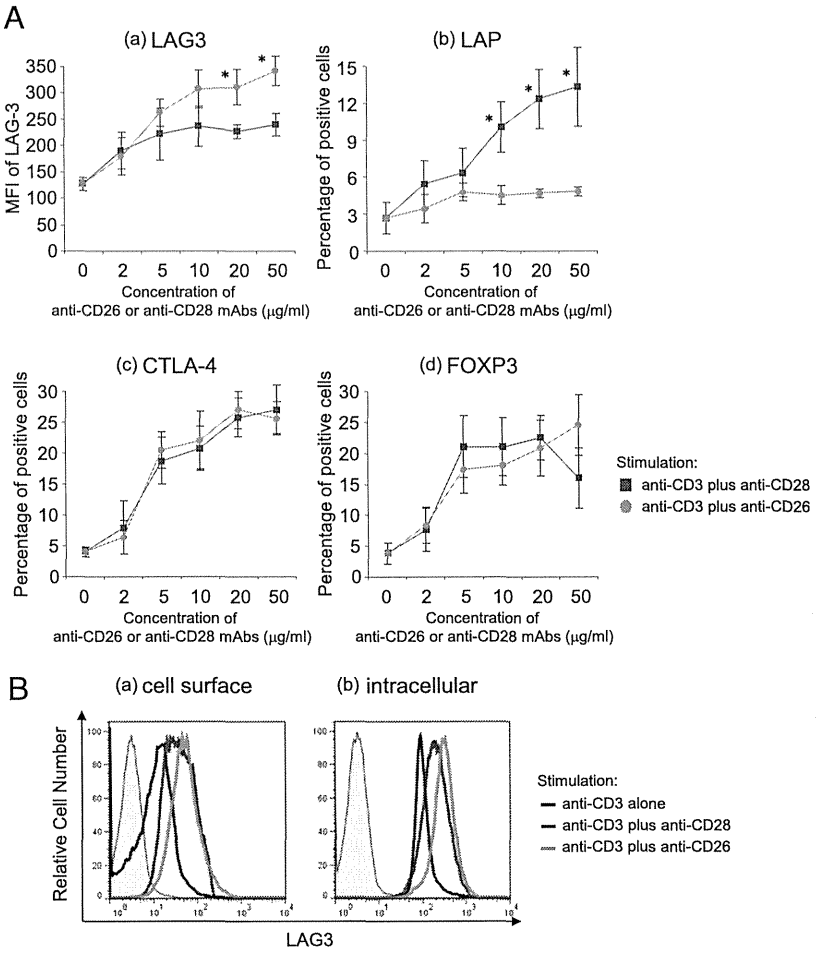

FIGURE 3. CD26-mediated costimulation of $CD4^+$ T cells induces greater LAG3 expression than does CD28-mediated costimulation. (**A**) Freshly purified $CD4^+$ T cells were stimulated with anti-CD3 mAb alone, anti-CD3 plus anti-CD28 mAbs, or anti-CD3 plus anti-CD26 mAbs at the indicated concentrations for 96 h. Cell surface LAP (**Ab**), CTLA-4 (**Ac**), intracellular LAG3 (**Aa**), and FOXP3 (**Ad**) gated for $CD4^+$ T cells were detected by flow cytometry. Data are shown as mean ± SE of MFI (Aa) or percentage positive cells (Ab–d) from five independent donors, comparing values in anti-CD3 plus anti-CD26 to those in anti-CD3 plus anti-CD28 ($^*p < 0.01$), and similar results were obtained in each experiment. (**B**) Freshly purified $CD4^+$ T cells were stimulated with anti-CD3 mAb alone, anti-CD3 plus anti-CD28 mAbs (50 μg/ml), or anti-CD3 plus anti-CD26 mAbs (50 μg/ml) for 96 h. After stimulation, cell surface (a) or intracellular (b) expression of LAG3 gated for $CD4^+$ T cells was analyzed by flow cytometry. Data are shown as histogram of LAG3 and are representative of five independent donors. The gray areas in each histogram show the data of isotype control.

130,000 Da. The band with the higher molecular mass is thought to represent the constitutively expressed NFAT2 isoform, and the one with the lower molecular mass probably represents the inducible NFAT2 isoform (33). In contrast with NFAT4, which was only slightly expressed both in the cytoplasm and the nucleus of peripheral blood $CD4^+$ T cells (data not shown), the protein level of NFAT2 was abundant and clearly increased in response to CD26 or CD28 costimulation compared with NFAT1, suggesting that among the NFAT family, NFAT2 plays a crucial role in regulating human $CD4^+$ T cell activation. NFAT protein is known to synergistically interact with many transcription factors, including AP-1, which is the main transcriptional partner of NFAT during T cell activation. Alternatively, in the absence of AP-1, NFAT directs transcription of a specific program of gene expression that may be responsible for blocking TCR signaling (33). Taken together, these results strongly suggest that persistent NFAT–AP-1 cooperation is responsible for CD26-mediated T cell activation, leading to a specific pattern of gene expression different from CD28-mediated costimulation.

Increased EGR2 expression following CD26-mediated costimulation is associated with preferential IL-10 production

The findings above suggest that persistent NFAT–AP-1 activation following CD26-mediated costimulation is associated with enhanced IL-10 production. The molecular mechanisms involved in the process of IL-10 transcription in T cells have been well characterized, with the involvement of many transcription factors having been reported previously. c-Maf and IFN regulatory factor 4 (IRF4) directly transactivate IL-10 gene expression through binding to the IL-10 promoter (34, 35), whereas Blimp-1 positioned downstream of EGR2, GATA-3, and E4BP4 (also known as NFIL3) enhances IL-10 expression epigenetically by acetylating or methylating histones to change the chromatin structure at the IL-10 locus (36–38). We hypothesized that preferential IL-10 production of $CD4^+$ T cells following CD26-mediated costimulation was associated with the induction of such transcription factors. To validate the above assumption, we conducted a real-time RT-PCR assay to analyze the expression levels of the transcription factors discussed above following CD26 or CD28 costimulation. As shown in Fig. 5Aa, CD26 costimulation resulted in greater enhancement of EGR2 expression in $CD4^+$ T cells during the tested time intervals as compared with CD28 costimulation. Alternatively, both CD26 and CD28 costimulation markedly increased the expression of IRF4 compared with unstimulated T cells, but no difference was observed between these two costimulatory pathways (Fig. 5Ab). In contrast, both CD26 and CD28 costimulation of T cells led to a decrease in c-Maf or Blimp-1 expression (Fig. 5Ac, d), as well as a decline in expression levels of GATA-3 and E4BP4 (data not shown), indicating that EGR2 expression was preferentially induced following CD26-mediated costimulation. Western blot analysis was then conducted to confirm the above results. As shown in Fig. 5B, the protein level of EGR2 was higher after 24 or 48 h of CD26 costimulation than CD28 costimulation. Because it has been reported that EGR2 transcription is regulated by NFAT (39), we next examined the potential involvement of NFAT in the induction of EGR2 expression following CD26 costimulation. As shown in Fig. 5C, stimulation with both anti-CD3 plus anti-CD26 and anti-CD3 plus Cav-Ig clearly enhanced EGR2 expression compared with CD28 costimulation, a process that was partially inhibited by the NFAT

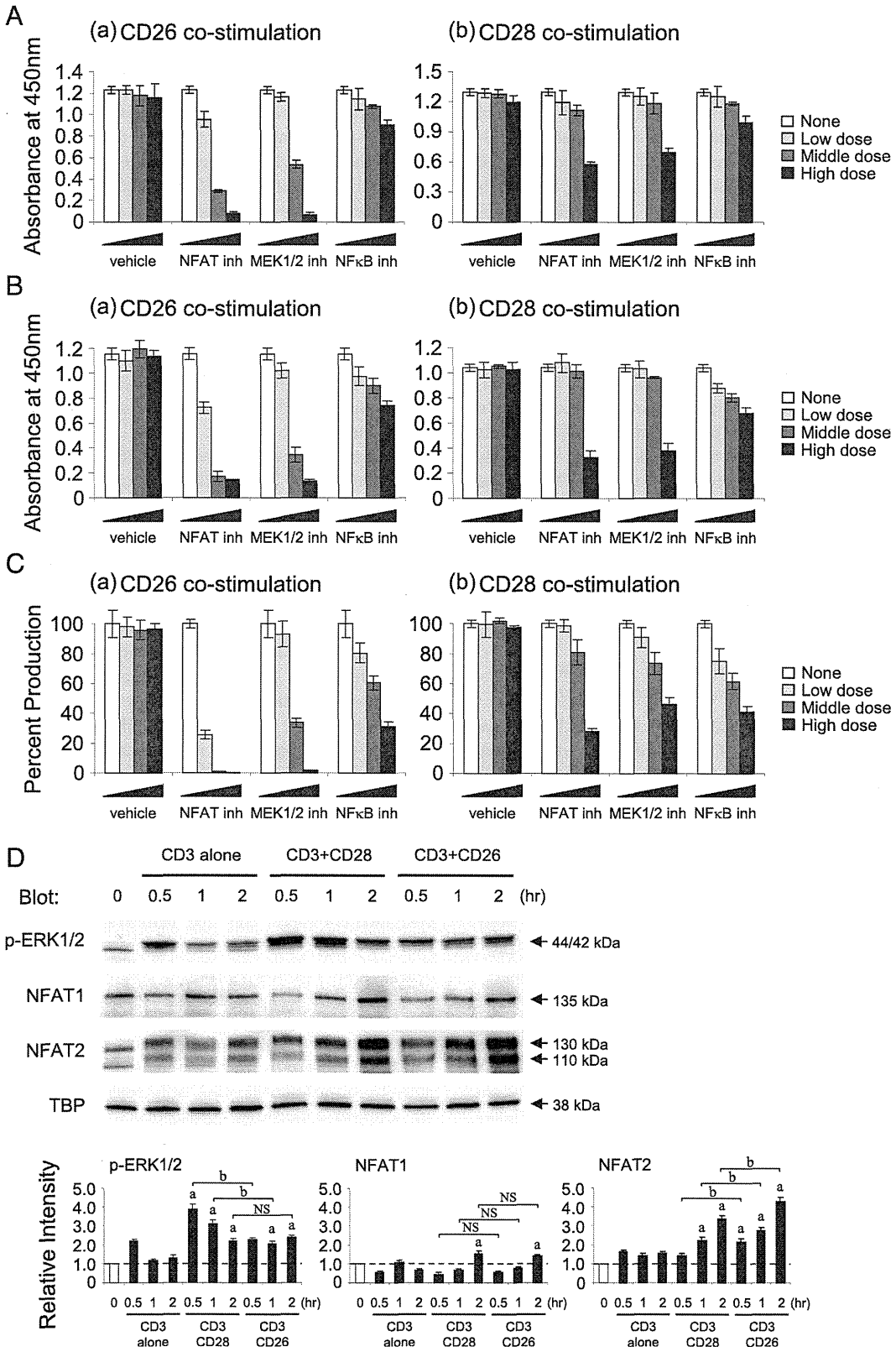

FIGURE 4. NFAT and Raf-MEK-ERK signaling events are indispensable for CD26-mediated CD4$^+$ T cell activation. (**A–C**) Freshly purified CD4$^+$ T cells were stimulated with anti-CD3 plus anti-CD26 mAbs (25 μg/ml) (**a**) or anti-CD3 plus anti-CD28 mAbs (25 μg/ml) (**b**) for 96 h in the presence of increasing doses of vehicle (DMSO, 0.0025, 0.0075, 0.025%), NFAT inhibitor (CyA, 0.01, 0.1, 1 μM), MEK1/2 inhibitor (U0126, 0.5, 1.5, 5 μM), or NF-κB inhibitor (quinazoline, 0.1, 0.5, 2.5 μM). (A) BrdU was added for the last 2 h of culture, and proliferation was assessed by measuring BrdU incorporation by ELISA. (B) Tetrazolium was added for the last 3 h of culture, and the absorbance at 450 nm was measured. (C) Concentration of IL-10 was examined by ELISA. Representative data of two (A) and four (B and C) independent donors are shown as mean ± SD of triplicate samples, and similar results were obtained in each experiment. (**D**) Freshly purified CD4$^+$ T cells were stimulated with anti-CD3 mAb alone, anti-CD3 plus anti-CD28 mAbs (50 μg/ml), or anti-CD3 plus anti-CD26 mAbs (50 μg/ml) for the indicated times. Nuclear extracts were separated by SDS-PAGE (each at 5 μg), and p-ERK1/2, NFAT1, or NFAT2 were detected by immunoblotting. The same blots were stripped and reprobed with Abs specific *(Figure legend continues)*

inhibitor alone and completely abrogated by the combination of the NFAT and MEK1/2 inhibitors. Furthermore, to determine whether EGR2 expression was associated with IL-10 production, we conducted knockdown experiments using siRNA against EGR2 in primary $CD4^+$ T cells. Expression level of EGR2 in T cells following CD26 costimulation was determined by real-time RT-PCR in the presence of control siRNA or two different sequences of EGR2-siRNA. As shown in Fig. 5Da, b, EGR2-siRNA treatment reduced EGR2 expression by ~50% as compared with control siRNA, which was associated with a significant decrease in IL-10 production by $CD4^+$ T cells. Because similar results were also obtained with a different siRNA sense2 designed to target a separate EGR2 site, data obtained with EGR2-siRNA sense1 as described in *Materials and Methods* are shown. Because it has been shown that EGR2-deficient $CD4^+$ T cells in mice produced high levels of IFN-γ and IL-17 following TCR stimulation (40), we therefore examined the effect of EGR2 knockdown on IL-17A and IFN-γ production by human $CD4^+$ T cells. As shown in Fig. 5Dc, d, production of both cytokines was vigorously enhanced by EGR2-siRNA compared with control siRNA. These results rule out the possibility that the decrease in IL-10 production seen with EGR2-siRNA treatment was due to the nonspecific off-target effects of siRNA. Taken together, these observations strongly suggest that CD26-mediated costimulation of $CD4^+$ T cells results in enhanced NFAT/AP-1–dependent EGR2 expression, which is associated with the preferential production of IL-10.

Supernatants of CD3/CD26-stimulated $CD4^+$ T cells suppress activation of bystander T cells in an IL-10–dependent manner

To address the functional significance of enhanced IL-10 production by $CD4^+$ T cells following CD26-mediated costimulation, we prepared culture supernatant of $CD4^+$ T cells stimulated with anti-CD3 plus a high dose (50 μg/ml) of anti-CD26 or anti-CD28 as a control and then examined whether the supernatant of CD3/CD26-stimulated T cells (CD26 sup) suppressed activation of bystander T cells. We confirmed that higher levels of IL-10 and lower levels of IL-2 were contained in CD26 sup as compared with the supernatant of CD3/CD28-stimulated T cells (CD28 sup), as shown in Fig. 2A. As shown in Fig. 6Aa, b, addition of CD26 sup to freshly purified $CD4^+$ T cells incubated under stimulatory condition with anti-CD3 plus anti-CD26 for 24 h resulted in a significant reduction in expression levels of both IL-2 and IFN-γ as compared with AIM-V medium, and this inhibitory effect was abrogated by the addition of anti–IL-10 plus anti–IL-10R. In contrast, IL-10 expression in $CD4^+$ T cells was significantly enhanced by CD26 sup, and this enhancing effect was almost completely reversed by anti–IL-10 plus anti–IL-10R (Fig. 6Ac). These results indicate that IL-10 contained in the CD26 sup suppresses the expression of effector cytokines such as IL-2 and IFN-γ but upregulated the expression of IL-10. Alternatively, treatment with CD28 sup markedly upregulated IL-2 expression, suggesting the presence in CD28 sup of soluble factors such as IL-2 that enhance the process of T cell activation (Fig. 6Aa). In contrast, addition of anti–IL-10 plus anti–IL-10R in the presence of CD28 sup increased the expression of IFN-γ but decreased IL-10 expression, suggesting that the relatively small amount of IL-10 in the CD28 sup was sufficient to regulate $CD4^+$ T cell cytokine production (Fig. 6Ab, c). To analyze the effect of CD26 sup on the proportion of IL-10 or effector cytokine-expressing cells, we next performed intracellular staining of IL-10 and IFN-γ in $CD4^+$ T cells. As shown in Fig. 6Ba, d, stimulation of freshly purified $CD4^+$ T cells with anti-CD3 plus anti-CD26 in the presence of CD26 sup for 3 d resulted in a decrease in the relative percentage of IFN-γ–expressing cells (from 18.5 to 11.1%) as compared with AIM-V medium, whereas the proportion of IL-10–expressing cells was hardly affected by CD26 sup. The effect of CD26 sup on the proportion of IFN-γ–expressing cells was reversed by anti–IL-10 plus anti–IL-10R (Fig. 6Be). Alternatively, addition of CD28 sup to $CD4^+$ T cells did not change the proportion of IFN-γ–expressing cells, but CD28 sup treatment in the presence of anti–IL-10 and anti–IL-10R led to an increase in the level of IFN-γ^+ cells (Fig. 6Bb, c), suggesting that the CD28 sup contained both activating and inhibitory factors such as IL-10. These findings strongly suggest that CD26 sup preferentially suppresses the proliferation of effector cytokine–expressing cells in an IL-10–dependent manner.

We next analyzed the effect of CD26 sup on T cell proliferation. As shown in Fig. 7A, CD26 sup clearly inhibited the proliferative activity of freshly purified $CD4^+$ T cells following CD26 costimulation. Although CD28 sup also slightly suppressed the proliferation of T cells following CD26 costimulation, the suppressive effect of CD26 sup was much more pronounced compared with CD28 sup. Interestingly, the suppressive effect on T cells following CD26 costimulation was more evident compared with T cells following CD28 costimulation (Fig. 7A). To further evaluate this observed difference, we conducted the same T cell proliferation assay in the presence of rIL-10. As shown in Supplemental Fig. 2A and 2B, T cells stimulated with anti-CD3 plus anti-CD26 were much more sensitive to the inhibitory effect of IL-10 as compared with CD28 costimulation, and the difference between CD26 and CD28 costimulation was markedly evident in cytokine production compared with proliferative activity. We further analyzed the expression of IL-10R on $CD4^+$ T cells following CD26- or CD28-mediated costimulation but observed no difference in the expression intensity of IL-10R between these two costimulatory approaches (Supplemental Fig. 2C). To characterize in more detail the effect of CD26 sup on T cell proliferation, we analyzed the cell division process of CFSE-labeled $CD4^+$ T cells by flow cytometry. As shown in Fig. 7Ba, b, d, e, CD26 sup exhibited a very potent suppressive effect on the proliferation of T cells stimulated with anti-CD3 plus anti-CD26 compared with CD28 sup, and this effect was partially reversed by addition of anti–IL-10 plus anti–IL-10R. It is noteworthy that 10 ng/ml rIL-10, a higher concentration than that found in CD26 sup, clearly suppressed T cell proliferation, but this effect was not more potent than CD26 sup (Fig. 7Bd, f). Alternatively, CD26 sup only slightly inhibited the proliferation of T cells stimulated with anti-CD3 plus anti-CD28, and these CD3/CD28-stimulated $CD4^+$ T cells were much less sensitive to the inhibitory effect of IL-10 as compared with CD3/CD26-stimulated T cells (Fig. 7Cc, d). These results correlated strongly with the proliferative activity shown in Fig. 7A. Taken together, these data strongly suggest that soluble factors secreted from $CD4^+$ T cells following CD26-mediated costimulation profoundly suppress bystander T cell proliferation, with IL-10 having a synergistic effect on the suppressive activity of these soluble factors.

for TATA-binding protein (TBP) as a loading control. Band intensity of p-ERK1/2, NFAT1, or NFAT2 was normalized to TBP, and relative intensity compared with resting $CD4^+$ T cells (0 h) is indicated in the *bottom panel*. Data are shown as mean ± SE of relative intensity from three independent donors, comparing values in anti-CD3 plus anti-CD28 or anti-CD3 plus anti-CD26 to those in anti-CD3 alone at the same stimulation period ($^a p < 0.01$), and anti-CD3 plus anti-CD26 to those in anti-CD3 plus anti-CD28 at the same stimulation period ($^b p < 0.01$).

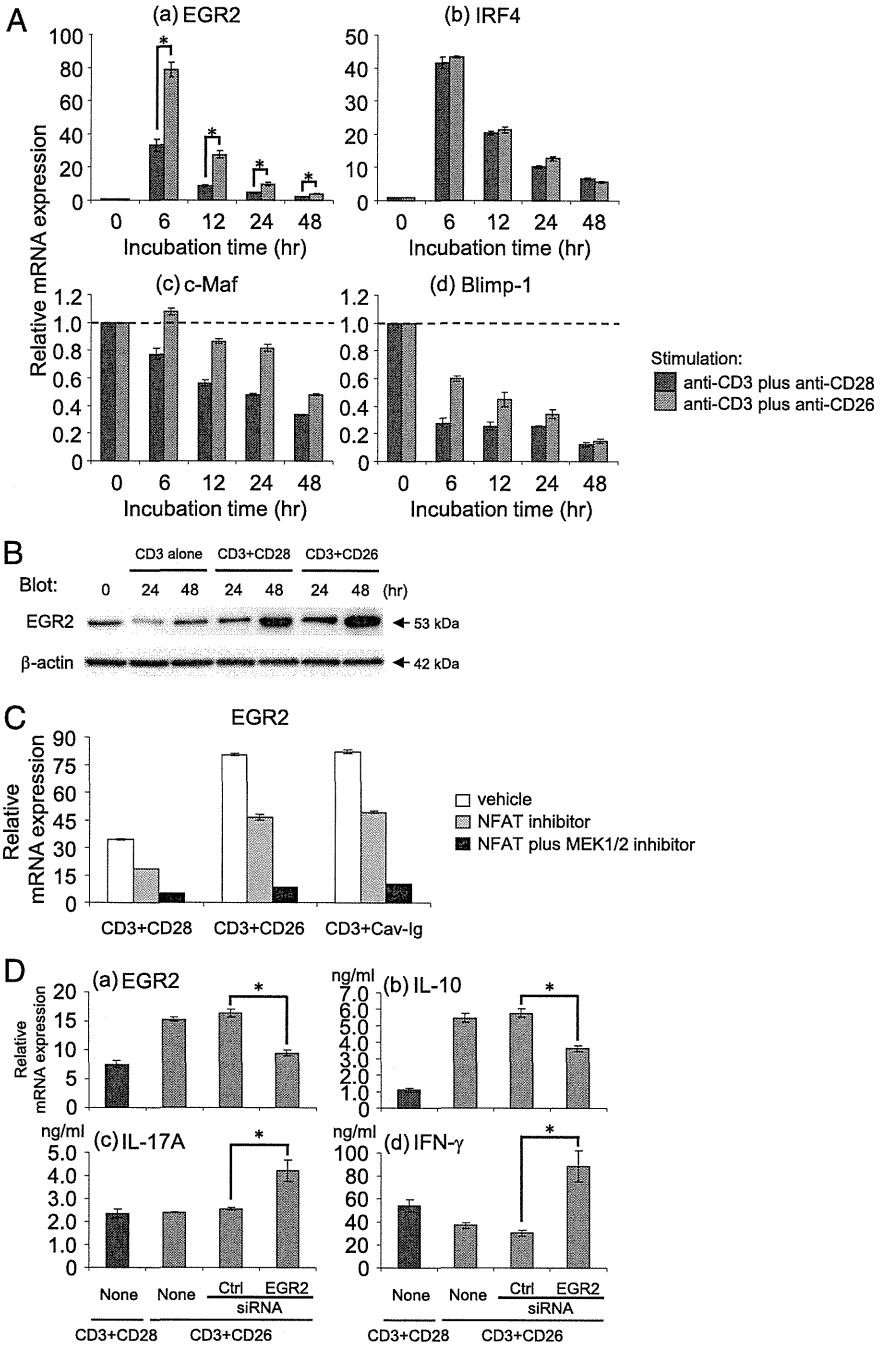

FIGURE 5. EGR2 expression strongly induced via CD26-mediated costimulation is associated with IL-10 production. (**A** and **B**) Freshly purified $CD4^+$ T cells were stimulated with anti-CD3 alone, anti-CD3 plus anti-CD28 mAbs (25 μg/ml), or anti-CD3 plus anti-CD26 mAbs (25 μg/ml) for the indicated times. (A) mRNA expression of EGR2 (**a**), IRF4 (**b**), c-Maf (**c**), and Blimp-1 (**d**) was quantified by real-time RT-PCR. Each expression was normalized to HPRT1, and relative expression levels compared with resting $CD4^+$ T cells (0 h) are shown. Representative data of four independent donors are shown as mean ± SD of triplicate samples, comparing values in anti-CD3 plus anti-CD26 to those in anti-CD3 plus anti-CD28 (*$p < 0.01$). (B) Whole-cell lysates were separated by SDS-PAGE (each 10 μg), and EGR2 was detected by immunoblotting. The same blots were stripped and reprobed with Abs specific for β-actin as a loading control. Data shown are representative of two independent experiments with similar results. (**C**) Freshly purified $CD4^+$ T cells were stimulated with anti-CD3 plus anti-CD28 mAbs (25 μg/ml), anti-CD3 plus anti-CD26 mAbs (25 μg/ml), or anti-CD3 mAb plus Cav-Ig (25 μg/ml) for 6 h in the presence of vehicle (DMSO, 0.025%), NFAT inhibitor (CyA, 1 μM), or NFAT inhibitor plus MEK1/2 inhibitor (U0126, 5 μM). mRNA expression of EGR2 was quantified by real-time RT-PCR, and the relative expression level is shown as in (A). Representative data of three independent donors are shown as mean ± SD of triplicate samples. (**D**) Freshly purified $CD4^+$ T cells were transfected with siRNA against EGR2 or control siRNA (Ctrl) and stimulated with anti-CD3 plus anti-CD28 mAbs (25 μg/ml) or anti-CD3 plus anti-CD26 mAbs (25 μg/ml) for 24 h (**a**) or 96 h (**b–d**). (Da) mRNA level of EGR2 is shown as in (A). (Db–d) Concentrations of IL-10 (b), IL-17A (c), and IFN-γ (d) were examined by ELISA. Representative data of four independent donors are shown as mean ± SD of triplicate samples, comparing values with EGR2 siRNA to those with control siRNA (*$p < 0.01$), and similar results were obtained in each experiment.

Discussion

In the present study, we show that coengagement of CD3 and CD26 induces the development of $CD4^+$ T cells to a Tr1-like phenotype with a high level of IL-10 production and LAG3 expression. CD26 costimulation also induces a high level of EGR2 associated with preferential IL-10 production, possibly via NFAT- and AP-1–mediated signaling. Furthermore, supernatants of these CD3/CD26-stimulated $CD4^+$ T cells clearly suppress the proliferative activity and effector cytokine production of bystander T cells in an IL-10–dependent manner.

Because the maintenance of peripheral tolerance by Tregs is critical to the potential development of autoimmunity, there is considerable interest in elucidating the molecular mechanisms for inducing the differentiation of $CD4^+$ T cells into specific Treg subsets. Although the mechanisms of Treg induction through exogenous cytokine stimulation have been extensively studied, such as TGF-β for $Foxp3^+$ Tregs or IL-27 for Tr1 cells (41), costimulatory signals for the induction of Tregs have not been fully understood. ICOS and CD46 have been reported as costimulatory signals inducing Tr1-type cells (42, 43). ICOS costimulation regulates c-Maf expression possibly through the enhancement of NFAT2 signal (44). CD46 costimulation induces interaction of the cytoplasmic tail of CD46 (CYT-1-BC1) with SPAK, leading to sustained phosphorylation of ERK1/2 (45). The importance of sustained phosphorylation of ERK1/2 has also been reported for the development of IL-10–producing Th1 cells (46). However, sustained phosphorylation of ERK1/2 alone is not sufficient to explain the preferential IL-10 production, and transcription factors such as c-Maf, IRF4, Blimp-1, GATA-3, and E4BP4 (NFIL3) may be associated with the transcription of IL-10 (34–38). Our present study indicates that EGR2 expression is enhanced in $CD4^+$ T cells following CD26-mediated costimulation (Fig. 5A, B), and EGR2

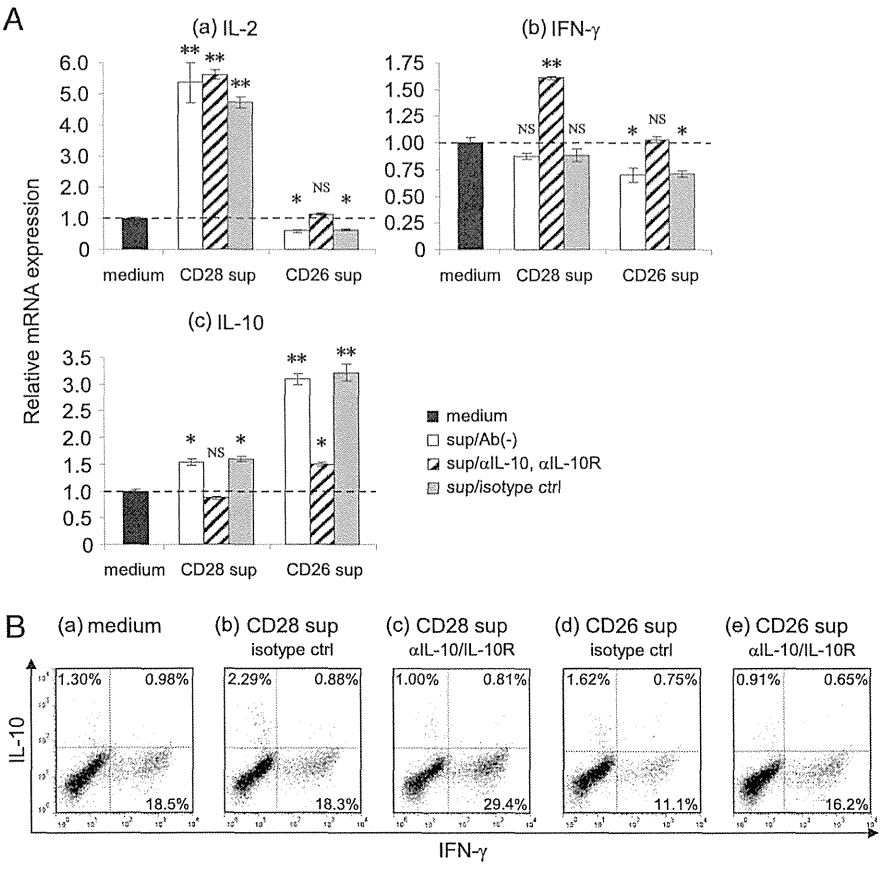

FIGURE 6. Supernatants of CD3/CD26-activated $CD4^+$ T cells suppress effector function of bystander T cells while augmenting IL-10 expression in an IL-10–dependent manner. Culture supernatants of $CD4^+$ T cells stimulated with anti-CD3 plus anti-CD28 mAbs (50 μg/ml) or anti-CD3 plus anti-CD26 mAbs (50 μg/ml) for 72 h were collected, and freshly purified $CD4^+$ T cells were stimulated with anti-CD3 plus anti-CD26 mAbs (5 μg/ml) in the presence of the supernatant (CD28 sup or CD26 sup) or AIM-V medium as a control. Prior to the onset of culture, the combination of anti-human IL-10 mAb and anti-human IL-10 receptor mAb (αIL10/IL10R) or isotype control mAbs (isotype ctrl) were added to the culture wells to give a final concentration of 20 μg/ml each. (**A**) After 24 h of incubation, cells were harvested and mRNA expression of IL-2 (**a**), IFN-γ (**b**), or IL-10 (**c**) was quantified by real-time RT-PCR. Each expression was normalized to HPRT1, and relative expression levels compared with the sample of control medium are shown. Representative data of three independent donors are shown as mean ± SD of triplicate samples, comparing values in each sample to that in control medium. $*p < 0.01$, $**p < 0.0001$. (**B**) On day 3, cells were restimulated with PMA plus ionomycin in the presence of monensin for the last 5 h of culture, and the intracellular expression of IFN-γ and IL-10 was detected by flow cytometry. Two-dimensional dot plot of IFN-γ or IL-10 staining gated for $CD4^+$ T cells is shown as a representative plot of three independent donors, and similar results were obtained in each experiment.

knockdown in $CD4^+$ T cells decreases IL-10 while markedly enhancing IL-17A and IFN-γ production (Fig. 5D). These observations strongly suggest that the induction of EGR2 expression is associated with the preferential production of IL-10 after CD26 costimulation. In mice, it has been recently reported that a high level of EGR2 expression is induced by IL-27/IL-27R–STAT3 signaling, and Blimp-1 induced by EGR2 is important for IL-10 production in $CD4^+$ T cells (47). In contrast with the marked upregulation of EGR2 following CD26-mediated costimulation, the expression level of Blimp-1 is not enhanced as compared with unstimulated T cells, although the decrease is more apparent in T cells following CD28 costimulation (Fig. 5A). To characterize more precisely the molecular mechanisms involved in IL-10 induction following CD26-mediated costimulation, the role of Blimp-1 or c-Maf needs to be better defined. NFAT is considered to be a regulator of EGR2 expression in T cells (39). Our present work shows that EGR2 is highly induced via CD26 costimulatory signal, and its expression is partially decreased by the NFAT inhibitor CyA and completely abrogated by the combination of CyA and the MEK1/2 inhibitor U0126 (Fig. 5C). These results strongly suggest that not only NFAT but also Raf-MEK-ERK signaling is involved in the induction of EGR2 expression. The association of ERK1/2 signaling in the induction of EGR2 expression has been recently reported in osteoprogenitors or breast adipose fibroblasts (48, 49), suggesting that the ERK signaling pathway is also associated with the transcription of EGR2 in T cells.

The importance of the anti-inflammatory effects of IL-10 has been confirmed in IL-10–deficient mice. IL-10–deficient mice develop spontaneous colitis at an early age, indicating that IL-10 is essential for intestinal homeostasis (50). IL-10–deficient mice also exhibit severe neuroinflammation with loss of recovery in experimental autoimmune encephalomyelitis (51). Furthermore, the generation of Tr1 cells from peripheral $CD4^+$ T cells has been shown to be greatly impaired in MS patients in comparison with healthy controls (52). Therefore, Tr1-mediated immunotherapy may be a potentially effective approach for the treatment of many autoimmune disorders, including inflammatory bowel disease or MS. Several groups have attempted to generate large numbers of Tr1 in vitro for clinical applications, and IL-27 is being considered currently to be an essential factor for the generation of Tr1 cells (53). In addition to IL-27, costimulatory signals through ICOS or CD46 play an important role in the generation or expansion of Tr1 cells. Our present study raises the possibility that CD26 is a novel costimulatory molecule inducing preferential IL-10 production in

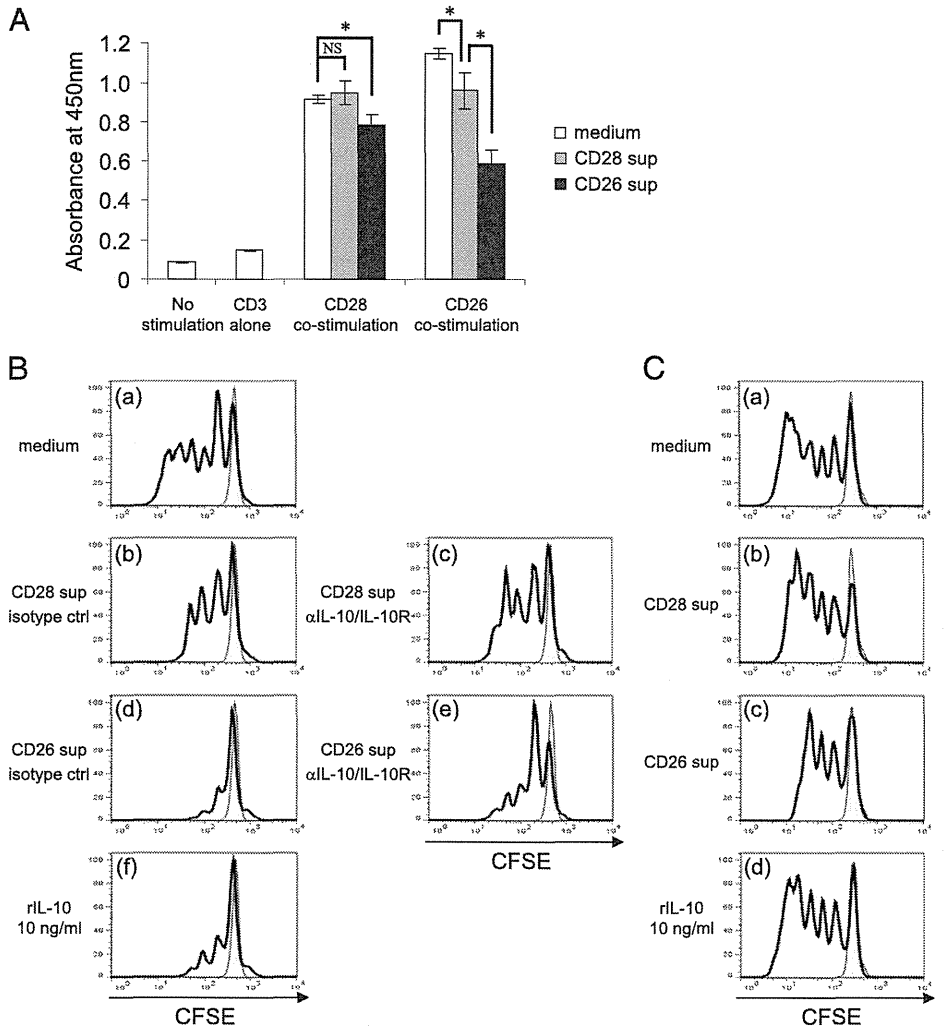

FIGURE 7. Supernatants of CD3/CD26-activated CD4$^+$ T cells significantly suppress the proliferation of bystander T cells through partial dependence on IL-10. Culture supernatants were prepared by the same method as shown in Fig. 6. (**A**) Freshly purified CD4$^+$ T cells were stimulated with anti-CD3 mAb alone, anti-CD3 plus anti-CD28 mAbs (5 μg/ml), or anti-CD3 plus anti-CD26 mAbs (5 μg/ml) for 96 h in the presence of the supernatant (CD28 sup or CD26 sup) or AIM-V medium as a control. Tetrazolium was added for the last 3 h of culture, and the absorbance at 450 nm was measured. Representative data of five independent donors are shown as mean ± SD of triplicate samples, comparing values with culture supernatants to those with control medium (*$p < 0.01$). (**B**) CFSE-labeled freshly purified CD4$^+$ T cells were stimulated with anti-CD3 plus anti-CD26 mAbs (5 μg/ml) in the presence of the supernatant [CD28 sup (**b** and **c**) or CD26 sup (**d** and **e**)] or recombinant human IL-10 (rIL-10, 10 ng/ml) (**f**) or AIM-V medium (**a**) as a control. Prior to the onset of culture, the combination of anti-human IL-10 mAb and anti-human IL-10 receptor mAb (αIL-10/IL-10R) (Bc and e) or isotype control mAbs (isotype ctrl) (Bb and d) were added to the culture wells to give a final concentration of 20 μg/ml each. (**C**) CFSE-labeled freshly purified CD4$^+$ T cells were stimulated with anti-CD3 plus anti-CD28 mAbs (5 μg/ml) in the presence of the supernatant [CD28 sup (**b**) or CD26 sup (**c**)] or recombinant human IL-10 (rIL-10, 10 ng/ml) (**d**) or AIM-V medium (**a**) as a control. After 96 h of incubation, cells were harvested and analyzed by flow cytometry. The data are shown as histograms of CFSE intensity gated for CD4$^+$ T cells and are representative of three independent donors, and similar results were obtained in each experiment. The gray areas in each histogram show the data of T cells cultured for 96 h without any stimulation.

CD4$^+$ T cells. Because CD26, ICOS, and CD46 belong to different families and their downstream signaling events are considered to be different, the combination of these costimulatory pathways may have additive effects on the induction of Tr1 cells. For this purpose, identification of the T cell subsets producing a high level of IL-10 following CD26 costimulation is needed. Because the CD4$^+$ T cells used in this study contained all the T cell subsets such as naive, Th1, Th2, Th17, follicular helper T, or Treg, we conducted costimulation assays following purification of CD4$^+$ naive, memory, or Treg subsets. Although the absolute amount of IL-10 produced by CD45RO$^+$ memory T cells was higher than CD45RA$^+$ naive T cells, both naive and memory CD4$^+$ T cells costimulated through CD26 produced greater levels of IL-10 compared with CD28 costimulation (Supplemental Fig. 3). In contrast, purified CD4$^+$CD25^{high}CD127^{low}FOXP3$^+$ cells apparently produced a low level of IL-10 following CD26 costimulation compared with other populations of CD4$^+$ T cells (Supplemental Fig. 3). These observations indicate that naturally occurring CD4$^+$ CD25^{high}CD127^{low}FOXP3$^+$ cells are not the source of IL-10 production, and they suggest that the subpopulation included in memory CD4$^+$ T cells is the main source of IL-10, whereas naive CD4$^+$ T cells also produce IL-10 in response to CD26 costimulation. Additionally, in vivo studies focusing on the suppressive activity of this population of in vitro–differentiated CD4$^+$ T cells, or the stability or plasticity of this IL-10–producing phenotype, need to be considered in future work.

CD28 is a representative T cell costimulatory pathway, and the negative feedback mechanism through CTLA-4 is associated with this pathway as a means of controlling excessive T cell activation. CD26 also functions as a costimulatory molecule in human T cells,

and CD26$^+$ T cells have been suggested to be involved in the pathophysiology of various immune disorders such as MS, rheumatoid arthritis, and graft-versus-host disease (8, 10, 12). Our present study shows that stimulation through TCR and high-intensity interaction of caveolin-1–CD26-mediated signaling induces the development of CD4$^+$ T cells to a Tr1-like phenotype. Caveolin-1 is a ubiquitously expressed ligand of CD26 in many cell types such as epithelial cells, endothelial cells, fibroblasts, macrophages, and neutrophils. We have previously shown that caveolin-1 was detected on the cell surface of monocytes 12–24 h after Ag uptake, and that CD26 and caveolin-1 colocalized at the T cell/monocyte contact site (19). Moreover, caveolin-1 expression is regulated by NF-κB, and stimulation with LPS or TNF-α increases the expression of caveolin-1 mRNA and protein (54), suggesting that expression of caveolin-1 may be increased on cells accumulated at sites of inflammation, leading to the transduction of intensive CD26-mediated signaling in CD26$^+$ T cells. Taken together, our data strongly suggest that CD4$^+$ T cells receiving robust caveolin-1–CD26-mediated signaling at the inflammatory site produce a high level of IL-10 and potentially other inhibitory factors to curtail the inflammatory process. Because striking defects in the induction of Tr1 cells through CD46 costimulation have been reported in patients with MS (52), it is conceivable that negative feedback mechanisms for regulating excessive CD26-mediated activation may be impaired in patients with autoimmune diseases, and further research is required to evaluate this hypothesis.

The cytoplasmic domain of CD28 has several common motifs that bind to signaling molecules such as PI3K, growth factor receptor–bound protein 2, or IL-2–inducible T cell kinase, whereas the cytoplasmic tail of CD26 consists of only 6 aa without any conserved kinase or protein-binding motifs (18). We have previously shown that CD26 localizes into lipid rafts, and stimulation with anti-CD3 plus anti-CD26 promotes aggregation of lipid rafts, leading to colocalization of CD45 to TCR signaling molecules such as p56^{Lck}, ZAP-70, or TCRζ (30). Our present data strongly suggest that persistent NFAT–AP-1 cooperation is responsible for CD26-mediated T cell activation, and sustained activation of NFAT and ERK1/2 is possibly due to the aggregation of signaling molecules in lipid rafts following CD26-mediated costimulation. Furthermore, we have previously shown that DPPIV enzyme activity is partially involved in the costimulatory activity of CD26 through studies using wild-type CD26 (DPPIV$^+$) or mutant CD26 (DPPIV$^-$)–transfected Jurkat T cell lines (55). Other groups reported that the synthetic competitive DPPIV inhibitor Lys[Z(NO_2)] significantly suppressed the proliferation and production of IL-2, IL-10, and IFN-γ in PWM-stimulated human T cells (56). They showed that these DPPIV inhibitors markedly increased the secretion of latent TGF-β1 by PWM-stimulated T cells, resulting in suppression of T cell activation. To further characterize the role of DPPIV enzyme activity in T cell activation, we are now examining the effect of the clinically used DPPIV specific inhibitor sitagliptin. Our preliminary data indicate that sitagliptin strongly suppresses the proliferative activity and production of cytokines including IL-10 of human CD4$^+$ T cells following CD26-mediated costimulation compared with CD28-mediated costimulation (data not shown), with the precise molecular mechanisms involved in this process being currently investigated.

In conclusion, intensive CD26 costimulatory signaling induces the development of CD4$^+$ T cells to a Tr1-like phenotype with a high level of IL-10 production, possibly for regulating potentially excessive CD26-mediated activation. Whether this possible negative feedback mechanism of CD26 costimulation is normally maintained or impaired in patients with autoimmune diseases is the subject of future investigations.

Disclosures

The authors have no financial conflicts of interest.

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