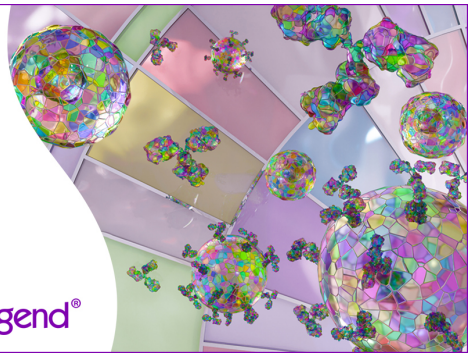

The Journal of Immunology
RESEARCH ARTICLE | APRIL 15 2011

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J Immunol (2011) 186 (8): 4609–4617.
https://doi.org/10.4049/jimmunol.1002361

High TCR Stimuli Prevent Induced Regulatory T Cell Differentiation in a NF-κB–Dependent Manner

Luciana L. Molinero, Michelle L. Miller, César Evaristo, and Maria-Luisa Alegre

The concentration of Ag or mitogenic stimuli is known to play an important role in controlling the differentiation of naive CD4$^+$ T cells into different effector phenotypes. In particular, whereas TCR engagement at low Ag doses in the presence of TGF-β and IL-2 can promote differentiation of Foxp3-expressing induced regulatory T cells (iTregs), high levels of Ag have been shown in vitro and in vivo to prevent Foxp3 upregulation. This tight control of iTreg differentiation dictated by Ag dose most likely determines the quality and duration of an immune response. However, the molecular mechanism by which this high-dose inhibition of Foxp3 induction occurs is not well understood. In this study, we demonstrate that when cells are in the presence of CD28 costimulation, TCR-dependent NF-κB signaling is essential for Foxp3 inhibition at high doses of TCR engagement in mouse T cells. Prevention of Foxp3 induction depends on the production of NF-κB–dependent cytokines by the T cells themselves. Moreover, T cells that fail to upregulate Foxp3 under iTreg-differentiating conditions and high TCR stimulation acquire the capacity to make TNF and IFN-γ, as well as IL-17 and IL-9. Thus, NF-κB helps T cells control their differentiation fate in a cell-intrinsic manner and prevents peripheral iTreg development under conditions of high Ag load that may require more vigorous effector T cell responses. *The Journal of Immunology*, **2011, 186: 4609–4617.**

Upon Ag encounter, T cells undergo proliferation and differentiation into functionally polarized effector cells. Whereas the specific cytokines present during this differentiation are essential to determine the phenotype and function that T cells will acquire, the dose of Ag that T cells encounter also plays an important role (1). Stimulation of CD4$^+$ naive T cells in the presence of TGF-β and IL-2 promotes upregulation of the transcription factor Foxp3 and differentiation into induced regulatory T cells (iTregs) (2, 3). Conversion of naive T cells into iTregs can also occur in vivo, for instance upon oral administration of Ag, systemic injection of soluble Ag in the absence of adjuvant, or solid organ transplantation under cover of anti-CD154 immunosuppressive therapy (4–8). Interestingly, the dose of Ag encountered by T cells is essential in determining whether naive T cells fail or succeed in differentiating to iTregs both in vitro and in vivo. Whereas low doses of peptide or of polyclonal TCR stimuli could induce Foxp3 expression in vitro, high doses of these mitogens prevented Foxp3 upregulation (9). Similarly, the greatest percentage of CD4$^+$CD25$^+$ cells in vivo (before Foxp3 was routinely used to identify regulatory T cells [Tregs]) followed systemic injection of limiting doses of Ag, whereas induction of these cells was abrogated upon administration of high doses of Ag (6). However, the molecular mechanism by which high TCR stimulation prevents iTreg induction is not well understood. A correlation between Ag dose and activation of the Akt–mTOR pathways has been reported (9, 10), and this signaling pathway is known to antagonize thymic natural Treg (nTreg) development and iTreg differentiation (11–13), but whether signaling via this axis is the cause by which high TCR stimulation prevents Foxp3 induction is not completely clear.

NF-κB is a transcription factor activated upon TCR/CD28 engagement that plays a critical role in the thymic development of nTregs (14). Following T cell activation via TCR ligation, the scaffolding molecules CARMA1, Bcl-10, and Malt1 recruit and induce the activity of the IKK complex, resulting in phosphorylation and degradation of the NF-κB inhibitor IκB that normally binds to and retains dimers of NF-κB subunits in the cytoplasm. Release from IκB reveals nuclear localization sequences in the NF-κB subunits that drive their nuclear translocation, allowing their transcriptional activity (15). The NF-κB subunit c-Rel has been shown to bind to enhancer sequences located in the promoter and third intron of the Foxp3 gene, and plays a direct role in Foxp3 expression during thymic nTreg development (16–19). In contrast, the role of c-Rel in driving Foxp3 transcription during iTreg differentiation is more controversial (17, 19, 20). Furthermore, mice lacking CARMA1 or Bcl-10, adaptors that couple the TCR to NF-κB, have been recently shown to lack nTregs, but retain differentiation of naive T cells into iTregs (21–24), suggesting that TCR-driven NF-κB activity is not required for iTreg differentiation, at least if sufficient exogenous IL-2 is present. Surprisingly, our results demonstrate that at high doses of TCR stimulation, NF-κB activity is, at least in part, responsible for the inhibition of TGF-β/IL-2–mediated iTreg differentiation. Therefore, NF-κB is not only dispensable for Foxp3 expression in iTregs, but can in fact antagonize it at greater levels of TCR engagement, via T cell-intrinsic production of effector cytokines that oppose iTreg differentiation. These data shed light on the T cell-intrinsic molecular mechanisms that control conversion of naive T cells into

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Received for publication July 12, 2010. Accepted for publication February 10, 2011.

This work was supported by National Institutes of Health Grant RO1 AI1052352 (to M.-L.A.) and an American Heart Association fellowship (to L.L.M.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AcH3, acetylated histone-3; CA, constitutively active; ChIP, chromatin immunoprecipitation; iTreg, induced regulatory T cell; MOI, multiplicity of infection; mRFP, monomeric red fluorescent protein; nTreg, natural regulatory T cell; qPCR, quantitative PCR; Tg, transgenic; Treg, regulatory T cell.

iTregs and operate in parallel to APC-derived cytokine-mediated signaling to ultimately control the differentiation phenotype of stimulated T cells.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Harlan (Indianapolis, IN). IκBαΔN–transgenic (Tg) mice (25), expressing a superrepressor form of IκBα directed by the Lck promoter and the CD2 enhancer, were bred in house and backcrossed >20 generations to C57BL/6. CARMA1$^{-/-}$ mice (26) were originally generated in the 129 background, but were backcrossed for at least six generations to C57BL/6 animals. DO11.10–Tg (27) mice were provided by A. Sperling (University of Chicago). CAR–Tg mice (28), which express the coxsackie and adenovirus receptor under the Lck promoter, were provided by T. Gajewski (University of Chicago). Bcl-2–Tg mice (29), which overexpress the anti-apoptotic Bcl-2 molecule under the control of the Vav promoter, were provided by F. Gounari (University of Chicago). CD4-Cre × IKKβ-CA × Foxp3-mRFP1 mice were obtained by crossing CD4-Cre (provided by F. Gounari) to Rosa-IKKβ constitutively active (CA)^{FL} and Foxp3-mRFP1 (The Jackson Laboratory). The Rosa26-Stop^{FL}-IKKβ-CA (30) strain possesses a *loxP*-flanked STOP cassette that prevents transcription of the downstream CA form of IKKβ. When bred to mice that express Cre recombinase under the CD4 promoter, the resulting offspring have the STOP cassette deleted in T cells, resulting in expression of the IKKβCA, which leads to constitutive activation of canonical NF-κB signaling. All of the experiments were performed in agreement with the University of Chicago Institutional Animal Care and Use Committee and according to the National Institutes of Health guidelines for animal use.

T cell differentiation in vitro

Naive CD4$^+$ T cells were first enriched from spleen and peripheral lymph nodes with a CD4$^+$ T cell enrichment kit (Stem Cells) and then sorted with a FACSAria (BD Biosciences) as CD4$^+$CD25$^-$(Foxp3-mRFP1$^-$)CD44^{low} cells. Naive CD4$^+$ T cells were stimulated with plate-bound anti-CD3 mAb (0–10 μg/ml; Bio-XCell) and anti-CD28 mAb (1 μg/ml; Bio-XCell) for 3 d in the presence of human rIL-2 and human rTGF-β1 (10 U/ml and 2.5 ng/ml, respectively; R&D Systems). In certain experiments, anti-TNF (10 μg/ml; provided by H. Yagita, Juntendo University, Tokyo, Japan), anti–IL-6 (10 μg/ml, clone MP5-20F3; Bio-XCell), anti–IL-4 (10 μg/ml; clone 11B11; eBioscience), anti–IFN-γ (10 μg/ml, clone XMG1.2; eBioscience), anti–IL-17A (10 μg/ml; R&D Systems), IL-6 (10 ng/ml; PeproTech), IL-17A (10 ng/ml; eBioscience), IFN-γ (10 ng/ml; PeproTech), TNF (10 ng/ml; R&D Systems), or IL-4 (10 ng/ml; PeproTech) was added. BALB/c splenic dendritic cells were purified by digestion of spleens with collagenase IV (400 U/ml; Sigma-Aldrich) for 30 min and then purified with CD11c$^+$ microbeads (Miltenyi Biotec) and AutoMacs, following the manufacturer's instructions. Dendritic cells were cocultured in vitro with naive T cells in the presence of $OVA_{323–339}$ peptide (0–5 μg/ml), human rTGF-β1 (2.5 ng/ml; R&D Systems), and human rIL-2 (10 U/ml) for 3 d. In some experiments, purified CD4$^+$CD25$^-$CD44^{low} cells were stained with CFSE (2.5 μM; Molecular Probes). Alternatively, cells were treated with PD98059 (50 μM, ERK inhibitor; Calbiochem), cyclosporine A (100 ng/ml, calcineurin inhibitor; Sigma-Aldrich), Akt1/2 inhibitor (1 μM; Calbiochem), SB203580 (10 μM, p38 MAPK inhibitor; Calbiochem), or SP600125 (10 μM, JNK inhibitor; Calbiochem).

Adenoviral vectors

An adenoviral vector containing IKKβ-CA (IKKβEE) and an adenoviral vector without a coding cDNA (EV) were generated in HEK 293 cells, as described by Marks et al. (31).

Adenoviral transduction of CAR–Tg T cells

CAR–Tg CD4$^+$CD25$^-$CD44^{low} naive T cells were FACS cell sorted in the FACSAria (BD Biosciences) and resuspended at 2 × 10^7 cells/ml in DMEM supplemented with 2% FCS. The cells were then mixed with an equal volume of DMEM containing 2% FCS and the indicated multiplicity of infection (MOI) of adenoviral particles and incubated at 37°C for 1 h. The cell/virus mixture was then transferred to a 10-cm tissue cell culture dish, incubated overnight at 37°C in an 8% CO_2 atmosphere, and then washed and used for experiments.

Flow cytometry

To assess intracellular cytokine expression, the cells were restimulated with PMA (150 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10 μg/ml; Biolegend) for 5 h. Cells were fixed and permeabilized with 1% paraformaldehyde in FACS buffer (0.1% BSA, 0.1 NaN_3 in PBS) with 0.1% saponin (IC buffer), and then stained in IC buffer with fluorochrome-conjugated Abs to Foxp3, IL-17A, IL-17F, IFN-γ, and IL-9, or their respective isotype controls. For the assessment of phosphoproteins, cells were stimulated for 18 h, fixed with 1% formaldehyde at 37°C for 10 min, permeabilized with ice-cold methanol for 30 min on ice, washed in FACS buffer, and stained with fluorochrome-conjugated Abs to CD4, phospho-Smad2 ($Ser^{465/467}$)/Smad3 ($Ser^{423/425}$), or phopsho-RelA (Ser^{536}) in FACS buffer. All experiments were analyzed in BD LSR II flow cytometers (BD Biosciences). Abs were purchased from eBioscience, except for anti–IL-9 (Biolegend) and all phospho-Abs (Cell Signaling Technology).

Western blot

Whole-cell extracts (10 μg proteins) were analyzed by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with rabbit polyclonal Ab against phospho-Smad2/3, phospho-Akt, phospho-S6, and mouse cyclophilin B (Cell Signaling Technology), or mouse anti–β-actin (Millipore/Upstate Biotechnology). Bound Abs were detected using peroxidase-labeled anti-rabbit or anti-mouse IgG (Bio-Rad) and ECL Plus chemiluminescent reagent (GE Healthcare) on Kodak BioMax films.

Reverse transcription and quantitative PCR

Total RNA was prepared from T cells with the use of RNEasy Plus Mini Kit (Qiagen). cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad), and the samples were diluted in water (1:10). A total volume of 25 μl containing 5 μl cDNA template, 0.3 μM of each primer, and SYBR Green PCRMaster Mix (Applied Biosystems) was analyzed in triplicate. Gene expression was analyzed with an ABI PRISM 7300 Sequence Detector and ABI Prism Sequence Detection Software version 1.9.1 (Applied Biosystems). Results were normalized by division of the value for the tested gene by that obtained for β-actin. The primers used were as follows: Foxp3-F, 5′- TCTTCGAGGAGCCAGAAGAG-3′; Foxp3-R, 5′-TACTGGTGGCTACGATGCAG-3′; β-actin–F, 5′-TGGAATCCTGTGGCATCCATGAAAG-3′; β-actin–R, 5′-TAAAACGCAGCCTCAGTAACAGTCCG-3′.

Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay kit was used according to the manufacturer's instructions (Upstate Biotechnology). Acetylated histone-3 (AcH3) and NF-κB/RelA were immunoprecipitated with anti–acetyl-H3 (Millipore/Upstate Biotechnology) and anti-RelA Abs (Santa Cruz Biotechnology), respectively. Purified DNA was then analyzed by quantitative PCR (qPCR), as described above, and normalized to input DNA. The primers spanned Foxp3 sequence +6144 to +6280 from transcription start site, as follows: Foxp3-F, 5′-CAACTTCTCCTGACTCTGCCTTCA-3′; Foxp3-R, 5′-GGAACTGTGCTAGTGGGAAGTGTACT-3′, as previously described (11). The primers for the amplification of the NF-κB–containing site in the IL-2 promoter (−266 to −100 bp to transcription start site) were as follows: IL-2NF-κB–F, 5′-ATATGGGGGTGTCACGATGT-3′ and IL-2NF-κB–R, 5′-GCCACCTAAGTGTGGGCTAA-3′.

Cytokine ELISAs

IFN-γ and TNF ELISAs (kits from eBioscience) were performed, according to the instructions of the manufacturer, using purified mAbs as capture Abs and biotinylated mAbs as developing Abs, followed by incubation with streptavidin-alkaline phosphatase and substrate. Plates were read in a 96-well spectrophotometer (Spectra Max 250; Molecular Devices), and data were analyzed using Softmax software (Molecular Devices) by comparison against a standard curve generated using recombinant cytokines at known concentrations.

EMSAs

Nuclear proteins were extracted from T cells that had been stimulated with immobilized anti-CD3 (0, 0.05, 0.5, or 5 μg/ml) mAb and anti-CD28 mAb (1 μg/ml each) for 24 h. Nuclear extracts were quantified by Bradford, and 0.5 μg was mixed with a 5′-biotin IL-2–NF-κB consensus oligonucleotide, ACC AAG AGG GAT TTC ACC TAA ATC. The EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce/Thermo Scientific), as recommended by the manufacturer.

Immunofluorescence detection and analysis of RelA nuclear translocation

CD4-Cre and CD4-Cre × IKKβ-CA freshly isolated lymphocytes were fixed with 1% formaldehyde for 10 min, permeabilized with 90% methanol for 30 min on ice, washed in FACS buffer, and stained with goat anti-RelA

(sc372G; Santa Cruz Biotechnology), Alexa Fluor 647-labeled anti-goat IgG (Invitrogen), allophycocyanin-Alexa750–conjugated anti-CD4 (eBioscience), and DAPI (Invitrogen). Intracellular expression of RelA and its nuclear translocation were assessed by image-based fluorescence using the ImageStream 100 multispectral imaging flow cytometer (Amnis), as described previously (32). Briefly, a minimum of 740 $CD4^+$ T cells was collected and analyzed. RelA translocation was assessed by the similarity of pixel intensities between the RelA image in channel 11 (Alexa Fluor 647) and the nuclear image in channel 7 based on the image mask of DAPI on a pixel-by-pixel basis for each cell. The similarity score for each cell was calculated using a log transformation of Pearson's correlation coefficient. Positive translocation events were assessed by comparison with the similarity score for the wild-type negative control (correlation of dark field scatter image with the nuclear image). Data were analyzed using the IDEAS software package (Amnis).

Statistical analysis

Statistical significance was evaluated using the two-tailed unpaired *t* test. The *p* values <0.05 were considered significant.

Results

High-dose TCR stimulation prevents Foxp3 transcription and locus accessibility

It has been shown previously that high dose of TCR stimulation prevents iTreg differentiation in peripheral naive $CD4^+$ T cells (9), although the molecular mechanism of this inhibition remains to be characterized. To study this phenomenon, OVA-specific DO11.10–Tg $CD4^+CD25^-CD44^{low}$ sorted naive T cells were stimulated for 72 h with increasing concentrations of $OVA_{323–339}$ peptide in the presence of dendritic cells, TGF-β, and IL-2. The highest proportion and numbers of cells upregulating Foxp3, as assessed by intracellular flow cytometry, were observed when T cells were stimulated with 0.01–0.5 μg/ml OVA peptide (Fig. 1*A*, 1*B*). In contrast, higher concentrations of Ag inhibited iTreg differentiation. To determine whether this inhibition was T cell intrinsic or depended on the presence of APCs, polyclonal purified $CD4^+$ naive T cells were stimulated on plate-bound anti-CD28 mAb with increasing doses of plate-bound anti-CD3 mAb in the presence of TGF-β and IL-2. As observed with OVA peptide, stimulation with anti-CD3 mAb concentrations higher than 0.5–1 μg/ml resulted in a reduced percentage and total number of $Foxp3^+$ cells (Fig. 1*C*), suggesting that high doses of TCR stimulation decrease the number of Foxp3-expressing cells in a T cell–intrinsic manner. Unless otherwise stated, 0.5 μg/ml anti-CD3 mAb was chosen as the prototypic low dose and 5 μg/ml as the prototypic high dose of TCR stimulation. The inhibitory effect of high-dose TCR stimulation on the percentage of cells upregulating Foxp3 expression was observed at all time points following TCR stimulation (Fig. 1*D*).

CD28 costimulation augments TCR engagement-mediated T cell activation (33). To determine the role of CD28 costimulation in the prevention of iTreg differentiation by high dose of TCR stimulation, sorted CD4 naive cells were activated for 3 d in the presence or absence of agonistic anti-CD28 mAb. Inhibition of iTreg differentiation upon high anti-CD3 stimulation was dependent on the presence of costimulation as the percentage $Foxp3^+$ cells remained high in the absence of CD28 mAb (Fig. 1*E*). For the remainder of the experiments, the dose of anti-CD28 mAb was kept

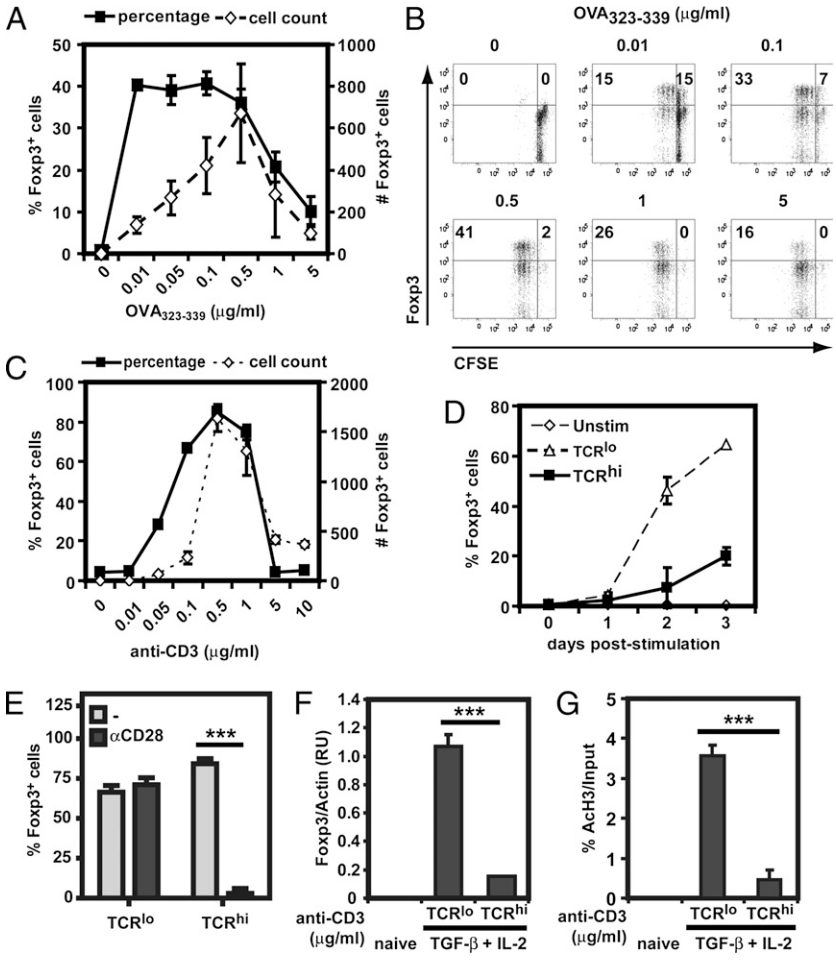

FIGURE 1. High dose of TCR stimulation reduces iTreg differentiation. *A*, DO11.10–Tg $CD4^+$ $CD25^-CD44^{low}$ cells were stimulated with BALB/c splenic dendritic cells, TGF-β, IL-2, and increasing concentrations of $OVA_{323–339}$ peptide. Percentage and numbers of Foxp3-expressing cells were assessed 72 h later by intracellular staining. Results show mean ± SD of triplicates. *B*, CFSE-labeled $CD4^+CD25^-CD44^{low}$ cells were stimulated as in *A*, and proliferation was assessed by flow cytometry. *C*, Wild-type C57BL/6 $CD4^+CD25^-CD44^{low}$ cells were stimulated with plate-bound anti-CD28, TGF-β, IL-2, and increasing doses of anti-CD3 mAb. Percentage and numbers of Foxp3-expressing cells were assessed at 72 h, as in *A*. *D*, Wild-type C57BL/6 $CD4^+CD25^-CD44^{low}$ cells were stimulated with plate-bound anti-CD28, TGF-β, IL-2, and anti-CD3 mAb at 0 (unstim), 0.5 (TCR^{low}), or 5 μg/ml (TCR^{high}). Percentage of cells expressing Foxp3 was assessed at the indicated times, as in *C*. *E*, Cells were stimulated, as in *D*, in the absence (−) or presence (αCD28) of anti-CD28. Percentage of Foxp3-expressing cells was assessed, as in *C*. *E*, C57BL/6 $CD4^+CD25^-CD44^{low}$ cells were untreated (naive) or stimulated with anti-CD28, TGF-β, and IL-2 in the presence of either 0.5 (TCR^{low}) or 5 μg/ml (TCR^{high}) anti-CD3 mAb. Three days later, cells were processed and Foxp3 mRNA was assessed by RT-qPCR and normalized to levels of β-actin gene expresion. *F*, T cells were stimulated, as in *E*, and processed for ChIP using anti-AcH3 Ab, with subsequent amplification of the Foxp3 locus by qPCR. Results shown are representative of two independent experiments. All experimental points represent average values and SD from three replicates of representative experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

constant to examine the mechanism by which a high dose of TCR stimulus prevents iTreg differentiation in the presence of costimulation. To investigate whether high TCR stimulus-mediated inhibition of iTreg differentiation occurred at the transcriptional level, Foxp3 mRNA was analyzed by RT-quantitative PCR (qPCR). mRNA expression of FoxP3 was much lower in T cells cultured under high than low dose of anti-CD3 mAb (Fig. 1*F*). Foxp3 locus accessibility was probed by determining the presence of AcH3 by ChIP assay. AcH3 levels in the Foxp3 locus were significantly higher when cells were stimulated with low, but not high dose of anti-CD3 mAb (Fig. 1*G*). These results suggest that high doses of TCR stimuli prevent accessibility of the Foxp3 locus and subsequent gene transcription.

The reduced percentage of Foxp3-expressing cells at high dose of TCR stimulation is not due to cell death

TCR stimulation results in T cell activation, but also cell death (34). To determine whether the reduced conversion of naive T cells into iTregs at high doses of TCR stimuli was due to cell death, iTreg differentiation experiments were performed using T cells transgenic for the anti-apoptotic molecule Bcl-2. Although apoptosis of T cells induced by high concentration of anti-CD3 mAb was largely prevented by overexpression of Bcl-2, increased cell survival did not restore iTreg differentiation at high doses of anti-CD3 mAb (Fig. 2), indicating that the reduced percentage of Foxp3-expressing cells observed upon high TCR stimulation is not due to cell death.

Reduced iTreg differentiation at high dose of TCR stimulation is not due to impaired TGF-β or IL-2 signaling

De novo expression of Foxp3 in peripheral $CD4^+$ naive T cells requires signaling by the cytokines TGF-β and IL-2, through phosphorylation of Smad2 and Smad3 and STAT5, respectively (35, 36). To investigate whether high doses of TCR stimulation impaired TGF-β signaling, the expression of phospho-Smad2/3 was assessed by Western blot (Fig. 3*A*) and flow cytometry (Fig. 3*B*). Increasing amounts of TGF-β resulted in a dose-dependent augmentation phospho-Smad2/3 at both low and high concentrations of anti-CD3 mAb (Fig. 3*B*), but even high concentrations of TGF-β could not restore iTreg differentiation in cells stimulated with high dose of anti-CD3 mAb (Fig. 3*C*). Levels of phospho-STAT5 were also equivalent in cells stimulated with low and high doses of anti-CD3 mAb (Fig. 3*D*). Hence, TGF-β and IL-2 signaling do not appear affected by the concentration of anti-CD3 mAb. To exclude that TGF-β and IL-2 were consumed in cells stimulated with high doses of anti-CD3 mAb, therefore preventing iTreg differentiation, daily addition of TGF-β and IL-2 was provided. These culture conditions did not increase the percentage of Foxp3-expressing cells upon stimulation with high doses of anti-CD3 mAb (Fig. 3*E*). Taken together, our results suggest that inhibition of iTreg differentiation under conditions of high-dose TCR stimulation is not due to impaired IL-2 or TGF-β signaling.

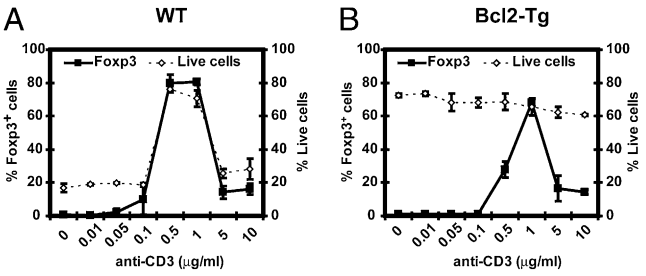

FIGURE 2. Inhibition of iTreg differentiation at high dose of TCR stimulation is not due to cell death. C57BL/6 (*A*) or Bcl-2-Tg (*B*) $CD4^+$ $CD25^-CD44^{low}$ cells were stimulated and analyzed, as in Fig. 1*C*, in the presence of increasing doses of anti-CD3. Results shown are representative of two independent experiments. All experimental points represent average values and SD from three replicates.

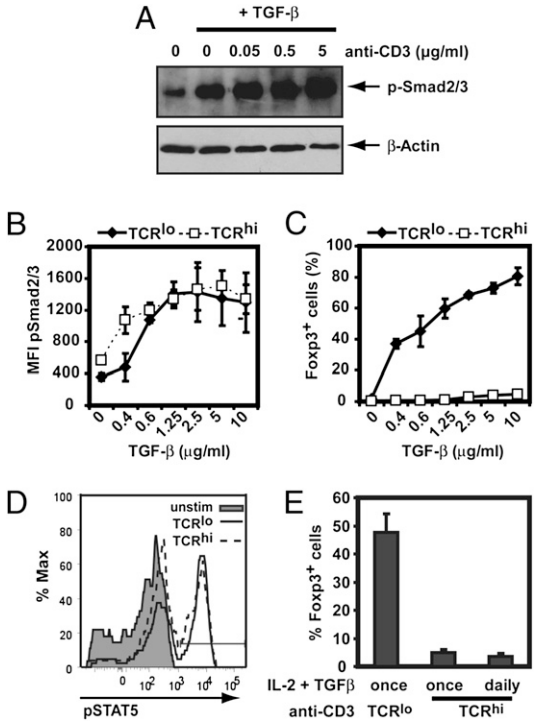

FIGURE 3. TGF-β and IL-2 signaling is not impaired at high dose of anti-CD3 stimulation. *A*, C57BL/6 $CD4^+CD25^-CD44^{low}$ cells were stimulated for 30 min with plate-bound anti-CD28 in the presence or absence of TGF-β and increasing doses of plate-bound anti-CD3 mAb. Smad2/3 phosphorylation was assessed by immunoblot and normalized to β-actin. *B* and *C*, C57BL/6 $CD4^+CD25^-CD44^{low}$ cells were stimulated with IL-2 and plate-bound anti-CD28 in either 0.5 (TCR^{low}, solid line) or 5 μg/ml (TCR^{high}, dashed line) plate-bound anti-CD3 mAb, in the presence of increasing doses of TGF-β. Smad2/3 phosphorylation at 18 h (*B*) or Foxp3 expression at 72 h (*C*) was assessed by flow cytometry. *D*, C57BL/6 $CD4^+CD25^-CD44^{low}$ cells were stimulated with IL-2, TGF-β, plate-bound anti-CD28, and either 0 (unstim, filled histogram), 0.5 (TCR^{lo}, solid line), or 5 μg/ml (TCR^{hi}, dashed line) anti-CD3 mAb. STAT5 phosphorylation was assessed by flow cytometry at 18 h. *E*, C57BL/6 $CD4^+CD25^-CD44^{low}$ cells were stimulated with TGF-β and IL-2, and 0.5 (TCR^{lo}) or 5 (TCR^{hi}) μg/ml anti-CD3 mAb. TGF-β and IL-2 were added either at the beginning of the culture (once) or every 24 h (daily). Results shown are representative of two independent experiments. All experimental points represent average values and SD from three replicates.

Akt/mTOR activity may not explain high TCR stimuli-mediated inhibition of iTreg differentiation

It has been previously shown that Akt and mTOR negatively regulate Foxp3 expression (11, 12). Therefore, it was conceivable that higher concentrations of TCR stimuli resulted in increased Akt/mTOR activity, in turn preventing Foxp3 induction. However, the percentage of cells expressing phospho-S6, a downstream target of mTOR, was very similar at low and high doses of anti-CD3 mAb, as assessed by intracellular flow cytometry (Fig. 4*A*, 4*C*) and immunoblot (Fig. 4*C*). In addition, Akt phosphorylation was also comparable at both concentrations of anti-CD3 mAb (Fig. 4*B*, 4*C*). Furthermore, inhibition of Akt resulted in an increased percentage of Foxp3-expressing cells at both low and high doses of anti-CD3 stimulation (Fig. 4*D*), suggesting that Akt/mTOR

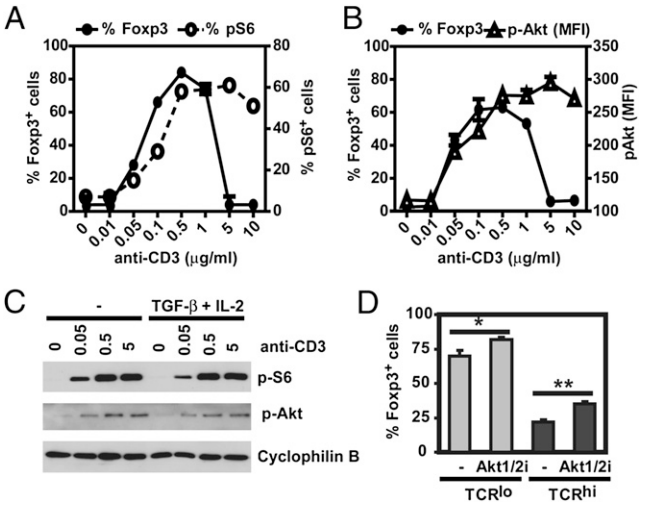

FIGURE 4. Activation of Akt may not explain inhibition of Foxp3 expression at high doses of TCR stimulation. *A* and *B*, C57BL/6 $CD4^+CD25^-CD44^{low}$ cells were stimulated and analyzed, as in Fig. 1*C*. Phospho-S6 (*A*) and phospho-Akt (*B*) were assessed by flow cytometry 18 h poststimulation and correlated with levels of Foxp3 in parallel cultures at 72 h. Results shown are representative of three independent experiments. All experimental points represent average values and SD from three replicates. *C*, C57BL/6 $CD4^+CD25^-CD44^{low}$ cells were stimulated for 30 min with plate-bound anti-CD28 and increasing doses of anti-CD3 in the presence or absence of TGF-β and IL-2. Phosphorylation of S6 and Akt was assessed by immunoblot, and levels were normalized to cyclophilin B. *D*, C57BL/6 $CD4^+CD25^-CD44^{low}$ cells were stimulated with TGF-β, IL-2, plate-bound anti-CD28, and 0.5 (TCR^{low}) or 5 μg/ml (TCR^{high}) anti-CD3 in the presence or absence of an Akt1/2 inhibitor. Percentage of Foxp3-expressing cells was evaluated 72 h poststimulation by flow cytometry. All experimental points represent average values and SD from three replicates. $*p < 0.05$, $**p < 0.01$.

signaling has a negative effect on iTreg differentiation regardless of TCR stimulation dose. Thus, Akt/mTOR signaling does not seem to explain the selective inhibitory effects of high TCR stimulation.

High-dose TCR-mediated inhibition of iTreg differentiation is NF-κB dependent

TCR/CD28 stimuli are known to be potent inducers of NF-κB activity (33). High-dose TCR triggering resulted in significantly increased recruitment of RelA/NF-κB to the NF-κB binding site of the IL-2 promoter compared with low-dose anti-CD3 mAb (Fig. 5*A*), demonstrating a correlation between the level of NF-κB activity and the inhibition of iTreg differentiation. To determine whether NF-κB activity was necessary for high-dose TCR-mediated inhibition of iTreg differentiation, IκBαΔN–Tg and CARMA1-deficient $CD4^+$ T cells were used as genetic models of NF-κB–impaired T cells. Increasing concentrations of anti-CD3 mAb resulted in a dose-dependent induction of NF-κB activity in wild-type, but not IκBαΔN–Tg or CARMA1-deficient cells, as assessed by EMSA (Fig. 5*B*). Both IκBαΔN–Tg and CARMA1-deficient $CD4^+$ T cells were partially resistant to the high-dose TCR-mediated inhibition of iTreg differentiation (Fig. 5*C*, 5*D*). This was not due to reduced proliferation by NF-κB–impaired T cells, as the exogenous IL-2 present in iTreg culture conditions compensated for the reduced production of IL-2 by these T cells (23, 25), allowing NF-κB–impaired T cells to proliferate as well as wild-type T cells (Supplemental Fig. 1). These results suggest that T cell NF-κB is required for high TCR stimulation-mediated prevention of iTreg differentiation.

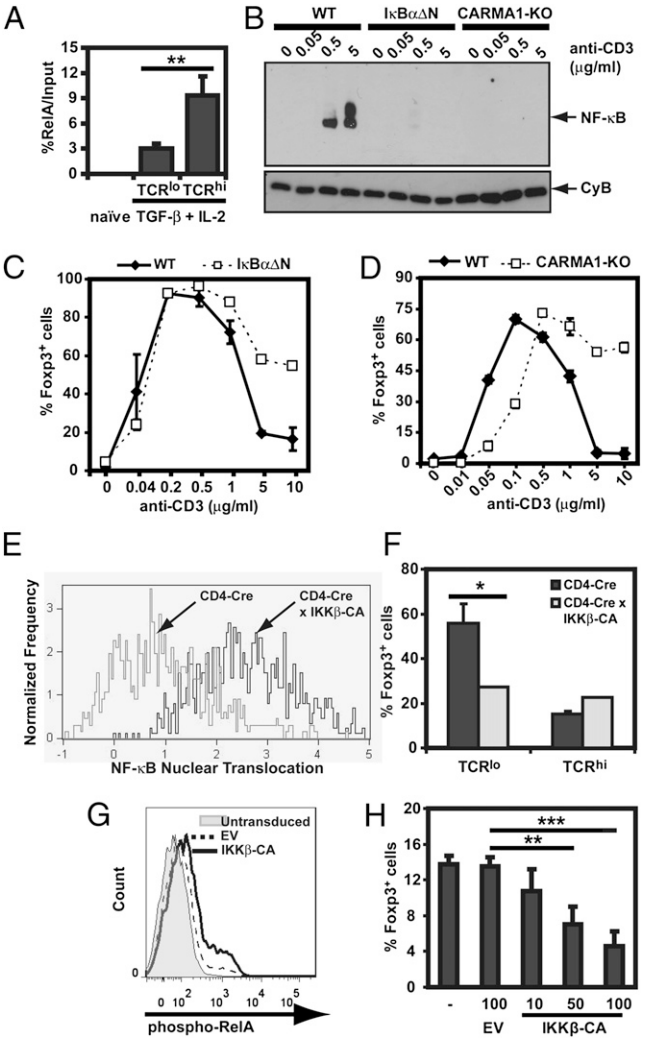

FIGURE 5. Inhibition of iTreg differentiation at high doses of TCR stimulation is NF-κB dependent. *A*, Wild-type $CD4^+CD44^{low}CD25^-$ cells were left alone or stimulated for 20 h with anti-CD28 and anti-CD3 (0.5 or 5 μg/ml) in the presence of TGF-β and IL-2. Chromatin was immunoprecipitated with anti-RelA Ab, and qPCR was performed spanning the NF-κB site in the IL-2 promoter. *B*, Wild-type (WT), IκBαΔN, and CARMA1-deficient $CD4^+CD25^-CD44^{low}$ cells were stimulated for 24 h with TGF-β, IL-2, plate-bound anti-CD28, and increasing doses of anti-CD3 mAb. Nuclear extracts were prepared and NF-κB activity was assessed by EMSA. Immunoblot for cyclophilin B (CyB) was used for protein normalization. *C*, Expression of Foxp3 was assessed in wild-type and IκBαΔN–Tg $CD4^+CD25^-CD44^{low}$ cells, stimulated, and analyzed, as in Fig. 1*C*. *D*, $CD4^+CD25^-CD44^{low}$ wild-type and CARMA1-deficient cells were stimulated and analyzed, as in Fig. 1*C*. *E*, Freshly isolated $CD4^+$ cells from CD4-Cre × $Foxp3^{RFP}$ and CD4-Cre × IKKβ-CA × $Foxp3^{RFP}$ were analyzed for RelA nuclear translocation using the ImageStream 100 multispectral imaging flow cytometer, as described in *Materials and Methods*. *F*, $CD4^+CD25^-CD44^{low}Foxp3^{RFP-}$ from CD4-Cre × $Foxp3^{RFP}$ and CD4-Cre × IKKβ-CA × $Foxp3^{RFP}$ were stimulated with TGF-β, IL-2, plate-bound anti-CD28, and 0.5 (TCR^{low}) or 5 μg/ml (TCR^{high}) plate-bound anti-CD3. Percentage of Foxp3-expressing cells was evaluated 72 h poststimulation by intracellular flow cytometry. *G* and *H*, CAR–Tg $CD4^+CD25^-CD44^{low}$ cells were left untreated, or infected with either control adenovirus (empty vector, EV) or increasing MOIs of adenovirus encoding for IKKβ-CA. Twenty hours later, RelA phosphorylation was evaluated by intracellular flow cytometry (*G*) or Foxp3 expression was assessed in cells further stimulated for 72 h with TGF-β and IL-2 in the presence of anti-CD3 and anti-CD28 mAbs. Results shown are representative of three independent experiments. All experimental points represent average values and SD from three replicates. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

To test the sufficiency of NF-κB in inhibiting Foxp3 induction, we used two complementary approaches. First, we analyzed T cells from CD4-Cre × Rosa26-Stop^{FL}IKKβCA (CD4-Cre × IKKβ-CA) mice that express constitutive NF-κB activity, as demonstrated by the constitutive nuclear translocation of RelA assessed by flow-based immunofluorescence (Fig. 5*E*). To enable purification of FoxP3-negative T cells, these mice were crossed to Foxp3-mRFP Tg mice (37), in which cells expressing Foxp3 are comarked with monomeric red fluorescent protein (mRFP). CD4$^+$ CD25$^-$CD44^{low}Foxp3-mRFP$^-$ cells sorted from CD4-Cre × Foxp3-mRFP and CD4-Cre × IKKβ-CA × Foxp3-mRFP mice were stimulated in the presence of IL-2, TGF-β, anti-CD28, and either low or high doses of anti-CD3 mAb. Constitutive activity of NF-κB reduced the percentage of iTregs induced by low-dose anti-CD3 stimulation (Fig. 5*F*). As an alternative approach, CAR–Tg CD4$^+$ naive T cells were transduced with adenovirus encoding IKKβ-CA, resulting in a subset of cells expressing phospho-RelA (Fig. 5*G*). T cells transduced with IKKβ-CA at increasing MOIs, but not empty vector-transduced T cells, displayed a dose-dependent reduction in the percentage of FoxP3$^+$ cells (Fig. 5*H*), further indicating that increased activation of NF-κB is sufficient to reduce iTreg differentiation. Taken together, these results show correlation, necessity, and sufficiency, and therefore strongly implicate the TCR–CARMA1–NF-κB axis in the inhibition of iTreg differentiation by high doses of TCR stimulation.

NF-κB–dependent cytokines induced by high doses of anti-CD3 stimulation prevent iTreg differentiation

At high doses of TCR stimulation, NF-κB may either directly inhibit gene expression of Foxp3 or may induce the transcription of NF-κB–target genes capable of suppressing Foxp3 expression. Target genes of NF-κB include a number of cytokines. To determine whether cytokines produced by T cells stimulated with high doses of anti-CD3 mAb could reduce iTreg differentiation, congenic wild-type and CARMA1-deficient naive CD4$^+$ T cells were cultured in the presence of TGF-β, IL-2, and high doses of anti-CD3 mAb. Whereas CARMA1-deficient T cells were resistant to the inhibitory effect of high doses of TCR stimulation on iTreg differentiation when cultured alone, coculture with wild-type T cells resulted in a significantly reduced percentage of CARMA1-deficient T cells expressing Foxp3 (Fig. 6*A*). Similar results were obtained whether the coculture was carried out in the same well or whether wild-type and CARMA1-deficient T cells were separated in transwells (Fig. 6*B*), suggesting that soluble factors produced by wild-type T cells were suppressing iTreg differentiation by CARMA1-deficient and wild-type T cells. Whereas individual neutralization of IFN-γ, IL-6, TNF, IL-17, and IL-4 did not restore iTreg differentiation of wild-type T cells upon high TCR stimulation, combined blockade of these cytokines almost abrogated the ability of high doses of anti-CD3 mAb to suppress Foxp3 induction (Fig. 6*C*). To address which cytokines were sufficient to inhibit Foxp3 expression, wild-type naive T cells were subjected to iTreg differentiation under low concentration of anti-CD3 mAb. The only combination of cytokines that reduced the percentage of FoxP3$^+$ cells was TNF and IFN-γ (Fig. 6*D*). Consistent with this result, secretion of both cytokines was maximal when wild-type cells were stimulated with high doses of anti-CD3 mAb that simultaneously prevented iTreg differentiation (Fig. 6*E*). Thus, NF-κB–dependent inhibition of Foxp3 expression upon high TCR stimulation is secondary to the production of TNF and IFN-γ by the activated T cells.

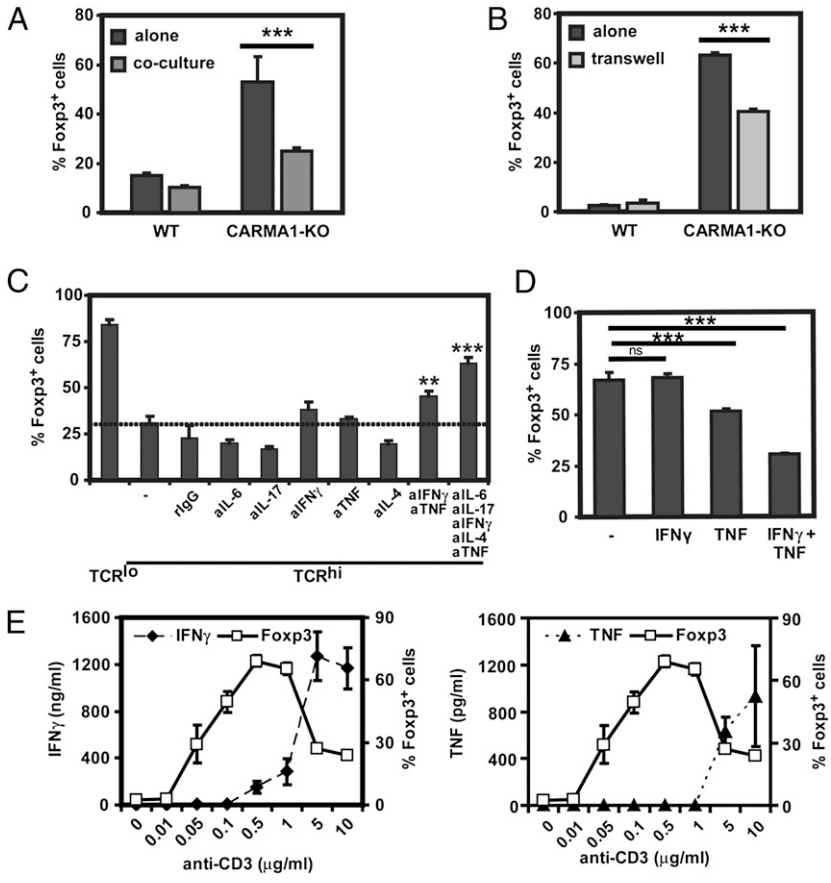

FIGURE 6. High doses of anti-CD3 stimulation induce NF-κB–dependent cytokines that prevent Foxp3 expression. *A*, Wild-type (CD45.1$^+$) and CARMA1-KO (CD45.2$^+$) CD4$^+$CD25$^-$CD44^{low} congenic cells were stimulated with TGF-β, IL-2, anti-CD28, and 5 μg/ml (TCR^{high}) anti-CD3 mAb. Cells were cultured alone or together. Three days later, expression of Foxp3 was assessed by intracellular staining in wild-type (CD45.1$^+$) or CARMA1-KO (CD45.2$^+$) cells. *B*, Wild-type and CARMA1-KO CD4$^+$CD25$^-$CD44^{low} cells were stimulated with TGF-β, IL-2, anti-CD28, and 5 μg/ml anti-CD3 mAb. Cells were cultured apart or separated by transwell. Three days later, expression of intracellular Foxp3 was assessed by flow cytometry. *C*, Expression of Foxp3 was assessed in wild-type cells, stimulated as in *A*, in the presence of neutralizing Abs to IL-6, IL-17A, IFN-γ, TNF, IL-4, alone, or in combination. *D*, Wild-type cells were stimulated with 0.5 μg/ml anti-CD3 with TGF-β and IL-2 in the presence or absence of IFN-γ and TNF. Foxp3 expression was assessed 72 h later by intracellular staining. *E*, Wild-type CD4$^+$CD25$^-$ CD44^{low} cells were stimulated for 3 d, as in Fig. 1*C*. Foxp3 expression was assessed by intracellular flow, and IFN-γ and TNF secretion was evaluated by ELISA. Results shown are representative of three independent experiments. All experimental points represent average values and SD from three replicates. $^{**}p < 0.01$, $^{***}p < 0.001$. ns, not significant.

Upon high doses of TCR stimulation, differentiating iTregs acquire effector phenotypes

The high levels of TNF and IFN-γ produced by cells stimulated with high doses of TCR triggering suggested that cell differentiation had switched from iTreg to Th1. In addition, TGF-β can promote not only the generation of suppressive iTregs, but also differentiation of naive T cells into cells producing IL-17A/F or IL-9 (38). To assess whether high doses of TCR stimulation also diverted iTreg differentiation into Th17 or Th9 development, expression of IL-17A, IL-17F, and IL-9 was analyzed. High concentrations of anti-CD3 mAb induced the expression of IL-17A, IL-17F, and IL-9, both by intracellular flow cytometry (Fig. 7*A*) and ELISA (data not shown). However, the percentage of cells expressing these proinflammatory cytokines was low unless IFN-γ was blocked (Fig. 7*B*), as it has been shown that IFN-γ inhibits differentiation of non-Th1 subsets (38). These results suggest that despite a cytokine milieu that promotes the differentiation of iTregs (TGF-β and IL-2), the strength of TCR stimulation can determine in a T cell–intrinsic manner whether differentiation proceeds toward iTreg or toward proinflammatory T cell subsets.

Discussion

High doses of TCR stimuli have been shown to prevent conversion of naive T cells into iTregs and overrule the ability of TGF-β/IL-2 to drive Foxp3 expression, although the molecular mechanism by which expression of Foxp3 is inhibited was not well understood. Our results indicate that TCR-driven NF-κB activity is, at least in part, responsible for this high-dose inhibition of Foxp3 expression, and operates via the induction of NF-κB–dependent, T cell–intrinsic effector cytokines that antagonize iTreg differentiation. These data highlight the potential of T cells to undergo a fate differentiation distinct to that specified by the APC-produced cytokine milieu surrounding them, and identify a signaling pathway that enables such fate changes in a T cell–intrinsic manner.

Our data, as well as those previously published using CARMA1- or Bcl10-deficient T cells, suggest that TCR-mediated NF-κB activity is not required for expression of Foxp3 in peripheral T cells stimulated in the presence of exogenous TGF-β and IL-2 (21, 23). This is in contrast to the high dependency on NF-κB activity for the thymic development of nTreg and of their precursors (22–24, 39), and the demonstration of direct transcriptional control of Foxp3 at least by c-Rel in nTregs (16, 17). These results suggest independence from TCR-driven NF-κB for Foxp3 transcription in iTregs, but not nTregs. However, an alternative explanation is that TCR-mediated NF-κB activity is only required in the context of iTreg differentiation for the induction of IL-2 production. Because exogenous IL-2 is provided for iTreg differentiation in vitro, developing iTregs may be able to upregulate Foxp3 in a NF-κB–independent manner. This hypothesis is supported by our results showing that CARMA1-KO, but not wild-type T cells, fail to upregulate Foxp3 if IL-2 is omitted from the tissue culture, as only wild-type T cells can produce their own IL-2 upon TCR stimulation (L. Molinero, unpublished observations). This hypothesis is also consistent with results on iTreg differentiation using c-Rel–deficient T cells. Indeed, it has been suggested that c-Rel is essential for the differentiation of iTregs (17) either via its regulation of IL-2 production (20) or of peripheral homeostatic proliferation of Tregs (19). Regardless of the requirement for NF-κB–mediated Foxp3 expression at low dose of TCR stimulation, our results demonstrate that NF-κB upon high TCR stimulation abrogates Foxp3 induction.

Our data support the notion that inhibition of iTreg differentiation at high dose of TCR stimulation depends on CD28 costimulation. The role of CD28 in iTreg differentiation has been controversial, whereby some authors claim that CD28 requirement is due to its ability to promote IL-2 production (40), whereas others show that costimulation can be detrimental (41). However, the dose of TCR stimulation was not taken into account in those studies and may explain the opposite results. Our results indicate that CD28 costimulation inhibits iTreg differentiation at high doses of TCR stimulation, whereas it helps differentiation at very low doses of TCR triggering (≤0.1 μg/ml; data not shown). Consistent with an impact of costimulation on iTreg differentiation, Francisco et al. (42) have recently shown that triggering of the inhibitory receptor PD-1 resulted in increased percentage of Foxp3-expressing cells.

Whereas NF-κB is activated by combined CD3 and CD28 engagement, other signaling pathways such as NFAT, ERK, and JNK are also induced upon CD3/CD28 ligation (43, 44). Thus, one

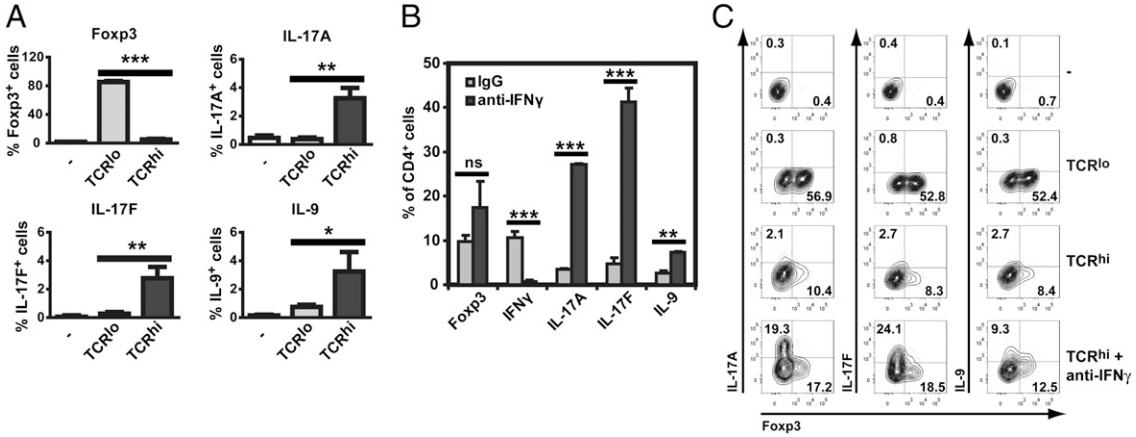

FIGURE 7. High doses of TCR stimulation switch differentiating iTregs to IL-17 or IL-9 production. *A*, Wild-type $CD4^+CD25^-CD44^{low}$ cells were stimulated with TGF-β, IL-2, and anti-CD28 in the absence (−) or presence of 0.5 (TCR^{low}) or 5 μg/ml (TCR^{high}) anti-CD3 mAb. Three days later, cells were restimulated with PMA and ionomycin, and expression of Foxp3, IL-17A, IL-17F, and IL-9 was assessed by intracellular flow cytometry. *B*, Wild-type $CD4^+CD25^-CD44^{hi}$ cells were stimulated with TGF-β and IL-2 with 5 μg/ml of anti-CD3 mAb, in the presence of an irrelevant IgG or an IFN-γ–neutralizing Ab. Three days later, cells were restimulated with PMA and ionomycin, and expression of Foxp3, IFN-γ, IL-17A, IL-17F, and IL-9 was assessed by intracellular flow cytometry. Results shown are representative of three independent experiments. *C*, Representative contour plots for expression of IL-17A, IL-17F, IL-9, and Foxp3 in $CD4^+$ cells treated as in *A* and *B*. All experimental points for *A* and *B* represent average values and SD from three replicates. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns, not significant.

hypothesis is that any TCR-driven signal may be detrimental for Foxp3 induction at high level of TCR ligation. However, pharmacological inhibition of the calcineurin–NFAT pathway reduced iTreg differentiation at both low and high doses of anti-CD3 (Supplemental Fig. 2), confirming the absolute requirement for this pathway during de novo expression of Foxp3 (45) rather than a selective detrimental effect at high Ag dose. In contrast, pharmacological inhibition of JNK and ERK signaling promoted iTreg differentiation upon high TCR triggering, but had minimal or no effect upon stimulation with low doses of anti-CD3 mAb (Supplemental Fig. 2). However, because TGF-β signaling also induces activation of JNK and ERK (46), we cannot distinguish whether the increase in iTreg differentiation upon inhibition of ERK and JNK is due to reducing those signaling pathways downstream of TGF-β or TCR engagement. Taken together, our data suggest that NF-κB, along with other signaling pathways, is required for high-dose TCR-mediated inhibition of iTreg differentiation.

Our results indicate that effector cytokines expressed by the differentiating naive T cells following activation with high doses of TCR stimuli can antagonize their differentiation into iTregs despite the presence of TGF-β and IL-2. Furthermore, our data reveal a mechanism for this effect showing its dependence on NF-κB activity. In fact, the two cytokines that were sufficient to prevent Foxp3 induction, TNF and IFN-γ, are direct target genes of NF-κB (47–49). These data complement those by Hill et al. (50), who recently described the ability of memory T cells capable of producing the effector cytokines IFN-γ, IL-4, and IL-21 to prevent Foxp3 induction in naive T cells. Interestingly, retinoic acid was able to curtail production of these cytokines and restore iTreg differentiation. Remarkably, retinoic acid has been shown to prevent CD28-mediated inhibition of de novo Foxp3 expression (41). It remains to be established whether retinoic acid or other proregulatory factors can also antagonize the inhibitory effects of high TCR stimuli on iTreg differentiation.

Although we show that NF-κB–dependent, T cell–derived, effector cytokines antagonize Foxp3 induction in differentiating iTregs, NF-κB may also prevent Foxp3 upregulation by other means. Our data demonstrate that high TCR stimulation is associated with reduced AcH3 at specific sites in the Foxp3 locus when compared with low TCR stimulation. NF-κB activity induced at high TCR stimulation may directly or indirectly maintain a closed conformation of the Foxp3 locus, although NF-κB family members have generally been associated with opening rather than closing of chromatin structures in T cells (51, 52).

The Akt–mTOR signaling pathway is known to prevent iTreg differentiation (11, 12, 53). For instance, premature termination of TCR signaling, inhibition of PI3K, Akt, or mTOR have all been shown to facilitate Foxp3 induction, whereas maintenance of TCR signaling or constitutive PI3K/Akt/mTOR activity antagonized its expression (11). The level of Akt activity has recently been correlated with Ag dose in T cells stimulated with pulsed dendritic cells, and has therefore been hypothesized to drive the inhibition of Foxp3 expression at high doses of TCR stimuli (9). However, differences in phospho-S6 mean fluorescence intensity were more modest when T cells were stimulated with anti-CD3 mAb alone in the absence of APCs (9). Our data confirm a slight increase in the proportion of T cells expressing phospho-Akt or phospho-S6 at high versus low TCR stimulation by flow cytometry, although this difference is unlikely to account for the dramatic decrease in cells expressing Foxp3 at high versus low TCR activation. Our data indicate that inhibition of Akt (Fig. 4*B*) and PI3K (data not shown) can promote iTreg differentiation at any dose of TCR stimulation (data not shown), suggesting that this pathway may act in parallel to, rather than downstream of, the TCR signaling cascade.

Our data also demonstrate that a proportion of naive T cells activated with high TCR stimuli under iTreg conditions that fail to upregulate Foxp3 instead expresses IL-17A, IL-17F, or IL-9, cytokines whose acquisition depends on TGF-β–mediated signaling. It has been recently shown that the concentration of anti-CD3 mAb positively correlates with the percentage of cells induced to express IL-17A upon Th17 differentiation, which itself depends on NFAT activity (54). Conversely, it is known that Foxp3 associates with NFAT upon iTreg differentiation preventing it from binding AP-1 (55), and can also bind the Th17 lineage-determining transcription factor RORγt (56), inhibiting the latter from driving Th17 differentiation. When Foxp3 induction is inhibited at high TCR stimulation during iTreg differentiation, these transcription factors may therefore be available for promoting effector rather than iTreg differentiation, which is reflected by the emergence of IL-17– and IL-9–expressing cells in our cultures. This tendency was exacerbated when IFN-γ production was blocked, as IFN-γ is known to prevent differentiation of Th17 cells (57). These results suggest that specific subsets of effector Th subsets may be preferentially generated upon high Ag exposure even in proregulatory environments.

Overall, our data prompt the speculation that inhibition of NF-κB in T cells, or of NF-κB–dependent T cell effector cytokines, may restore regulation under circumstances of high Ag exposure, such as in autoimmunity settings.

Acknowledgments

We are grateful to Hideo Yagita for providing anti-TNF Ab as well as to Fotini Gounari, Anne Sperling, Daniel Littman, and Thomas Gajewski for providing Bcl-2–Tg, DO11.10–Tg, CARMA1-deficient, and CAR–Tg mice, respectively. We thank the University of Chicago Flow Cytometry Core Facility for expert technical help.

Disclosures

The authors have no financial conflicts of interest.

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