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Activin A Functions as a Th2 Cytokine in the Promotion of the Alternative Activation of Macrophages¹

Kenji Ogawa,^{2*} Masayuki Funaba,[†] Yan Chen,[‡] and Masafumi Tsujimoto*

Activin A, a member of the TGF- β superfamily, is a pluripotent growth and differentiation factor. In this study, we report that murine Th cells produce activin A upon activation. Activin activity in the cultured CD4⁺ T cells was induced by anti-CD3 cross-linking. Activin β A mRNA level was increased in response to activation, indicating that activin production in CD4⁺ T cells is regulated at the mRNA level. Activin production was detected exclusively in CD4⁺CD25⁻ T cells, but not in CD4⁺CD25⁺ regulatory T cells. When CD4⁺ T cells were differentiated into Th cell subsets, higher activin secretion was detected when cultured under Th2-skewing conditions. The mRNA level of activin β A was abundant in Th2, but not in Th1 cells. Furthermore, secretion of activin was significantly higher in activated Th2 clones than in Th1 clones. The activin β A-proximal promoter contains a binding site for c-Maf, a Th2-specific transcriptional factor, at close proximity with an NF-AT binding site. c-Maf was able to synergize with NF-AT to transactivate activin β A gene, and both factors are implicated in activin β A transcription in Th2 cells. Activin A induced macrophages to express arginase-1 (M-2 phenotype), whereas it inhibited inducible NO synthase expression (M-1 phenotype) induced by IFN- γ . Taken together, these observations suggest that activin A is a novel Th2 cytokine that promotes differentiation of macrophages toward the M-2 phenotype. *The Journal of Immunology*, 2006, 177: 6787–6794.

The Th cells play an important role in specific immunity by producing cytokines. Upon Ag stimulation, naive CD4⁺ T cells differentiate into distinct populations of effector cells that exhibit characteristic patterns of cytokine production and immune regulation. Th1 cells secrete higher levels of IFN- γ and TNF- α , and are involved in cell-mediated immunity (1). In contrast, Th2 cells produce IL-4, IL-5, and IL-13, and are involved in humoral immune responses implicated in the promotion of IgE production and eosinophil proliferation.

Activins, members of the TGF- β superfamily, are pluripotent growth and differentiation factors. The diverse physiological activities of activins are found in neural and endocrine tissues (2). Activins are also expressed in bone marrow cells (2, 3), monocytes (2, 3), macrophages (4), and mast cells (5, 6). However, the function of activins in immune cells is largely unknown.

Activins have overlapping biological activities with TGF- β (7), a prototype of the TGF- β superfamily proteins, partly due to the fact that activins and TGF- β s use the same proteins (Smad2 and/or Smad3) in signal transduction (8). Targeted disruption of TGF- β 1 in mice resulted in a severe multifocal inflammation, indicating a role of TGF- β in immune suppression (9). TGF- β inhibits T cell functions through a wide array of processes. It inhibits the proliferation and differentiation of T cells into effector cells, and induces apoptosis of T cells (10). However, targeted disruption of Smad3, a signal mediator of TGF- β s and activins, reduced immune re-

sponses in mice, whereas an inactivating Smad3 mutation impaired neutrophil chemotaxis (11) and local inflammation (12). These observations indicate that Smad3 signaling is required for the stimulation of immune responses by TGF- β family members. Furthermore, the difference in the phenotype of Smad3-null mice compared with that of TGF- β 1-null mice suggests that activins may act as a stimulatory modulator of T cell-mediated immune response contrasting to the inhibitory function of TGF- β . Therefore, we investigated this hypothesis and demonstrated that bioactive activin A is produced in Th2 cells in response to TCR-mediated activation. In addition, our study indicates that activin A produced in activated Th2 cells is involved in alternative activation of macrophages.

Materials and Methods

Reagents

Recombinant human activin A and follistatin-288 were provided by the National Hormone and Pituitary Program. Recombinant mouse IL-2, IL-4, IL-12, and TGF- β 1 were obtained from R&D Systems. mAbs against mouse CD3 (145-2C11), CD28 (37.51), CD25 (7D4), B7-1 (1G10), IL-4 (11B11), and IL-12 (C17.8) were purchased from BD Pharmingen. An anti-activin A mAb was obtained from R&D Systems. mAbs against mouse CD4, CD8, and Thy1.2 were purified from culture supernatants of the hybridomas GK-1.5, HO-2.2, and HO-13-4 (American Type Culture Collection), respectively.

Animals

Female specific pathogen-free BALB/c and BALB/c *nu/nu* mice were obtained from SLC and used at 8–12 wk of age. All animal experiments were conducted in accordance with the guidelines for animal experiments in RIKEN.

Isolation and culture of spleen cells, CD4⁺ T cells, and macrophages

Single-cell suspensions were prepared from the spleens of BALB/c mice or *nu/nu* mice with BALB/c background. T cell-depleted spleen cells were prepared by treatment of whole spleen cells from normal BALB/c mice with anti-CD4, anti-CD8, and anti-Thy1.2 mAbs, followed by guinea pig complements (Cedarlane Laboratories). CD4⁺ T cells were isolated from mouse spleens by negative selection using mouse CD4⁺ T cell isolation kit (Miltenyi Biotec) in a MACS preparation column. CD4⁺ T cells were

*Laboratory of Cellular Biochemistry, RIKEN, Saitama, Japan; [†]Laboratory of Nutrition, Azabu University School of Veterinary Medicine, Kanagawa, Japan; and [‡]Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202

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² Address correspondence and reprint requests to Dr. Kenji Ogawa, Laboratory of Cellular Biochemistry, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. E-mail address: kkgawa@riken.jp

further fractionated into CD25⁺ and CD25⁻ cells on a MACS column after reacting with anti-CD25 mAb and streptavidin microbeads (Miltenyi Biotec). The cells were treated with various reagents (Con A, 5 μ g/ml; plate-bound anti-CD3 mAb, 2 μ g/ml; soluble anti-CD28 mAb, 2 μ g/ml), and activin activity in the supernatants was measured. Peritoneal macrophages were collected and cultured, as described previously (4).

In vitro differentiation of Th cells

CD4⁺ T cells were differentiated in vitro into Th1 and Th2 cells according to a previously described method (13). The cells were stimulated in vitro with plate-bound anti-CD3 mAb alone (nonskewing conditions), or with anti-IL-4 mAb (5 μ g/ml; Th1-skewing conditions) and anti-IL-12 mAb (20 μ g/ml; Th2-skewing conditions). After a 24-h incubation, IL-2 (50 U/ml) was added to all cultures. In addition, IL-12 (50 U/ml) or IL-4 (500 U/ml) was added into Th1 or Th2 cultures, respectively. After an additional 6 days of culture, cells were harvested, washed thoroughly, and restimulated for 48 h with plate-bound anti-CD3. The supernatants were harvested and measured for activin, IFN- γ , and IL-4.

Generation of OVA-specific Th1 and Th2 clones

OVA-specific Th1 and Th2 clones were generated according to a previously described method (14), with some modifications. Briefly, mice were injected i.p. with 100 μ g of OVA in CFA. To generate Th2 clones, mice were immunized with OVA, followed by daily i.p. administration of 100 μ g of anti-B7-1 mAb on days 0–2. Spleen cells were prepared 10 days after immunization, activated in the presence of OVA and mitomycin C-treated spleen cells as APCs, and propagated in the presence of IL-2. The cytokine profile of each Th clone was established by cytokine ELISAs.

Bioassay for activin activity

Activin activity in culture supernatant was assayed by erythroid differentiation assay using mouse erythroleukemia F5-5.f1 cells (RIKEN Cell Bank), as described previously (15). Briefly, serially diluted samples were added to F5-5.f1 cells at a cell density of 1000 cells/well in 0.2 ml. After incubation for 6 days, 20 μ l of 1% *o*-dianisidine was added to stain the differentiated (hemoglobin-positive) cells. After a 15-min incubation, the supernatant was removed, and cells were incubated in lysis buffer (20% SDS/50% dimethylformamide (pH 4.7)) for an additional 6 h at room temperature. Absorbance was determined at a wavelength of 405 nm. Activin activity of each sample was determined from a standard curve derived from the absorbance at 405 nm induced by serial dilutions of recombinant human activin A. In this assay, F5-5.f1 cells were differentiated into hemoglobin-positive cells in the presence of activin A, activin AB, or activin B, but not TGF- β 1, BMP-4, IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, IFN- γ , and TNF- α (15, 16). To examine whether erythroid differentiation activity of the supernatant was due to activin A, samples were incubated with 400 ng/ml recombinant human follistatin-288 or 1 μ g/ml anti-human activin A neutralizing mAb.

RT-PCR and quantitative RT-PCR

Total RNA isolation, cDNA synthesis, and competitive PCR were conducted, as described previously (4). Oligonucleotides in primer sets for activin β A, β B, and G3PDH were used, as previously described (4). Primer sets and expected size of PCR products for inducible NO synthase (iNOS)³ and arginase-1 are as follows: iNOS, 5'-TGAAGAGTTCCTTCCTTGC-3' (forward) and 5'-GGATGCTCTGAACGTAGACC-3' (reverse), 530 bp; arginase-1, 5'-CAGTTGGAAGCATCTCTGGC-3' (forward) and 5'-TCCCAAGAGTTGGGTTCACT-3' (reverse), 540 bp.

Western blotting

For Western blotting, CD4⁺ T cells cultured for 6 days with plate-bound anti-CD3 were washed and resuspended with serum-free S-Clone SF-O2 medium (Sanko Junyaku), incubated with or without plate-bound anti-CD3 mAb for 48 h. The supernatant was subjected to SDS-PAGE under non-reducing condition, blotted to Immobilon-P (Millipore), and immunostained with anti-activin A mAb (R&D Systems). Bands were visualized using peroxidase-conjugated goat anti-mouse IgG (Jackson Immuno-Research Laboratories) and ECL plus reagents (Amersham Biosciences).

Plasmid and GST-fusion proteins

The mouse activin β A promoter region (17) that spans nt -278 to +57 relative to the transcription initiation site was amplified by PCR and sub-

cloned into pGL3-basic luciferase vector (Promega). Mutation and deletion constructs were generated by overlap extension PCR, followed by subcloning into pGL3-basic vector. Expression plasmid encoding full-length NF-ATp (pREP4-NF-ATp) was a gift from T. Hoey (Tularik, San Francisco, CA) (18). The c-Maf expression plasmid was generated by inserting the full-length cDNA-encoding mouse c-Maf (19) into the pCI-neo mammalian expression vector (Promega). The GST-fusion protein of c-Maf was generated by in-frame fusion of the DNA binding domain (aa 172–370) of c-Maf cDNA with pGEX-6P-1 (Amersham Biosciences). The plasmid constructs were transformed into *Escherichia coli* BL21 strain (Amersham Biosciences), and the GST fusion proteins were purified according to the manufacturer's protocol.

Promoter assay

Mouse T cell lymphoma EL4 cells (RIKEN Cell Bank) were transiently transfected with different combinations of plasmid DNA by SuperFect transfection reagent (Qiagen). A *Renilla*-luciferase vector, phRL-SV40 (Promega), was cotransfected to serve as an internal control to monitor transfection efficiency. The transfected cells were lysed 36 h later and used in dual luciferase assay. In some groups, cells were treated for 8 h with PMA (100 nM) and/or ionomycin (1 μ M) in the presence or absence of cyclosporin A (CsA; 100 nM) before harvest. The samples were counted for 10 s in a MicroLumat LB96P luminometer (EG & G Berthold), and the data were represented as the relative light unit/second.

EMSA

Double-stranded oligonucleotides were labeled with [³²P]ATP by T4 polynucleotide kinase. The labeled probes (~5 \times 10⁴ cpm) were incubated with ~2 μ g of GST or GST-c-Maf fusion proteins in a buffer containing a final concentration of 10 mM HEPES (pH 7.4), 100 mM KCl, 1 mM EDTA, 5 mM MgCl₂, 2 mM DTT, 10% glycerol, 0.5 mg/ml BSA, and 50 μ g/ml poly(dI-dC) for 1 h on ice, followed by electrophoresis in 5% non-denaturing polyacrylamide gel in 0.5 \times TBE.

Statistical analysis

Data are presented as mean \pm SD. Comparisons between groups were conducted by Student's *t* test.

Results

Production of activin A in CD4⁺ T cells in response to activation

To elucidate the potential role of activins on T cells, we first examined the expression of activins by erythroid differentiation assay that is able to detect the specific bioactivity of activin (15). Treatments with Con A and plate-bound anti-CD3 mAb increased the activity of activin in the culture supernatant of whole spleen cells, but not in T cell-depleted spleen cells from normal mice as well as the spleen cells from athymic *nul/nul* mice (Fig. 1A). These results suggest that activin is produced in T cells upon activation. The activin induction by anti-CD3 cross-linking was also detected in purified CD4⁺ T cells (Fig. 1B). Activin production was further augmented when CD4⁺ T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 mAbs (Fig. 1C). Treatment of CD4⁺ T cells with CsA abolished the activin production (Fig. 1C), suggesting that the expression of activin in CD4⁺ T cells is activation dependent. The activin activity induced by anti-CD3 cross-linking was neutralized by addition of follistatin, an activin-binding protein (20), and an anti-activin A neutralizing mAb (Fig. 1D), further confirming the specificity of activin produced by the T cells. Activins consist of homo- and heterodimers of activin β A or β B subunits that form activin A (β A β A), activin AB (β A β B), and activin B (β B β B). Western blotting showed that activin A, homodimer of activin β A, was detected in the culture supernatant by treatment with anti-CD3 mAb in CD4⁺ T cells (Fig. 1E). RT-PCR also revealed the expression of activin β A, but not β B, in CD4⁺ T cells (Fig. 1F), indicating that CD4⁺ T cells are able to express activin A, but not activin AB and activin B. Quantitative competitive RT-PCR demonstrated that

³ Abbreviations used in this paper: iNOS, inducible NO synthase; CsA, cyclosporin A; MARE, Maf recognition element; TGC, thioglycolate.

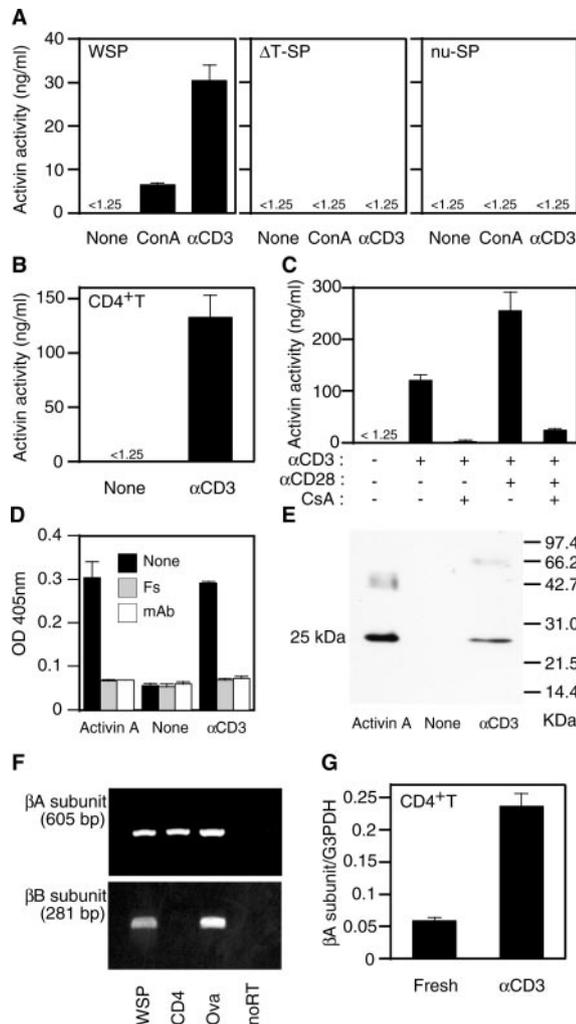


FIGURE 1. Production of activin A in CD4⁺ T cells upon activation. *A*, Activin production from whole spleen cells (WSP) and T cell-depleted spleen cells (Δ T-SP) from normal mice and the spleen cells from *nu/nu* mice (nu-SP) after incubation with Con A or anti-CD3 mAb. *B*, Activin production in CD4⁺ T cells in response to CD3-cross-linking. *C*, Inhibitory effect of CsA on activin production in CD4⁺ T cells stimulated with anti-CD3 and anti-CD28 mAbs. CD4⁺ T cells cultured for 6 days with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb in the presence or absence of CsA (100 nM). The supernatants were collected and assayed for activin. *D*, Neutralization effect of addition of excess follistatin or anti-activin A neutralizing mAb to recombinant human activin A or to the culture medium. Erythroid differentiation activity in the supernatant of anti-CD3-stimulated CD4⁺ T cells was abolished by excess follistatin (Fs; 400 ng/ml) or anti-activin A mAb (1 μ g/ml) as well as that of recombinant human activin A. *E*, Western blotting of activin A in the supernatants of activated CD4⁺ T cells restimulated with (α CD3) or without (None) anti-CD3 mAb. Immunoreactive activin A was detected in the culture supernatant as a 25-kDa band only when the cells were restimulated with anti-CD3 mAb. *F*, RT-PCR products of activin β A and β B subunits from the mouse spleen (WSP), CD4⁺ T cells, and ovary (Ova). Negative control: no reverse transcriptase control of CD4⁺ T cells (noRT). *G*, Activin β A mRNA levels in CD4⁺ T cells primed with anti-CD3 mAb (α CD3) were compared with those in freshly prepared CD4⁺ T cells (Fresh) by competitive RT-PCR relative to G3PDH mRNA level.

activin β A mRNA level was augmented by anti-CD3 cross-linking (Fig. 1*G*), suggesting that the increased production of activin A protein during CD4⁺ T cell activation is regulated at the mRNA level of the β A gene.

Activin A is produced exclusively in CD4⁺CD25⁻ T cells and is not involved in suppressor function in T cell

Recently, CD4⁺CD25⁺ regulatory T cells have emerged as a unique population of suppressor T cells that produce high levels of TGF- β that is involved in maintaining peripheral immune tolerance (21–23). A previous study has reported that CD4⁺CD25⁺ T cells exert immunosuppressive activity via cell-cell interaction involving cell surface TGF- β 1 (24). However, another study failed to demonstrate a role of TGF- β in CD4⁺CD25⁺ T cells, suggesting that the immune suppressive function of these cells is independently of TGF- β 1 (25). Thus, the potential role of TGF- β in CD4⁺CD25⁺ T cell-mediated suppression remains controversial. We determined whether or not activin A is produced in CD4⁺CD25⁺ regulatory T cells and acts as a suppressor of T cell functions. Consistent with the previous studies (18–20), CD4⁺CD25⁺ T cells did not proliferate well in response to plate-bound anti-CD3 mAb, and the addition of soluble anti-CD28 mAb resulted in proliferation (data not shown). As shown in Fig. 2*A*, we clearly detected activin production in CD4⁺CD25⁻ T cells upon activation. On the contrary, activin was not detected in CD4⁺CD25⁺ T cells even when they are stimulated with anti-CD3 and anti-CD28 mAbs (Fig. 2*A, top*). As expected, high level of TGF- β 1 production was found in CD4⁺CD25⁺ T cells after they were treated with anti-CD3 and anti-CD28 mAbs (Fig. 2*A, bottom*). In addition, neither exogenous activin A nor neutralization of activin with either follistatin or anti-activin mAb affected in vitro suppression of T cell proliferation by CD4⁺CD25⁺ regulatory T cells (Fig. 2*B*). Although TGF- β 1 clearly inhibited CD4⁺CD25⁻ T cell proliferation in a dose-dependent manner, activin A did not show significant effect on the proliferation of T cells (Fig. 2*C*). These results indicate that the expression and function of activin in CD4⁺ T cells were distinct from those of TGF- β , suggesting different roles of these two structurally related peptides on immune regulation.

Activin A is preferentially produced in Th2 cells

As we demonstrated, activin A is produced exclusively in CD4⁺CD25⁻ T cells upon their activation, contrasting to TGF- β 1 that is produced in CD4⁺CD25⁺ regulatory T cells. Furthermore, we also found that activin A is not involved in suppressor function in T cell, suggesting that activin A may play a role in immune responses differently from TGF- β 1. Activated CD4⁺CD25⁻ T cells are able to differentiate into two distinct subsets of effector cells, Th1 and Th2, that are defined by their distinct cytokine profiles and their immune regulatory functions (1). We next determined whether activin A is expressed exclusively within Th cell subsets. Under Th1-skewing conditions, CD4⁺ T cells are differentiated into Th1 cells that secrete high level of IFN- γ and low level of IL-4, whereas under Th2-skewing conditions Th2 cells are characterized by high IL-4 and low IFN- γ secretion (Fig. 3*A*). High level of activin secretion was detected in T cells cultured under Th2-skewing conditions on day 7 after the primary stimulation (Fig. 3*B, left*) and at 48 h after the secondary stimulation (Fig. 3*B, right*). Consistent with the results, the mRNA level of activin β A was abundant in Th2, but not in Th1 cells (Fig. 3*C*). We further established a total of 12 OVA-specific Th clones that were categorized into either Th1 or Th2 clones on the basis of the secretion profile of IFN- γ and IL-4 (Fig. 3*D, table*). When each Th clone was treated with Ag or anti-CD3 mAb, significant production of activin was detected in the Th2 clones, but not in the Th1 clones (Fig. 3*D*). However, TGF- β 1 secretion was unchanged by these treatments (data not shown).

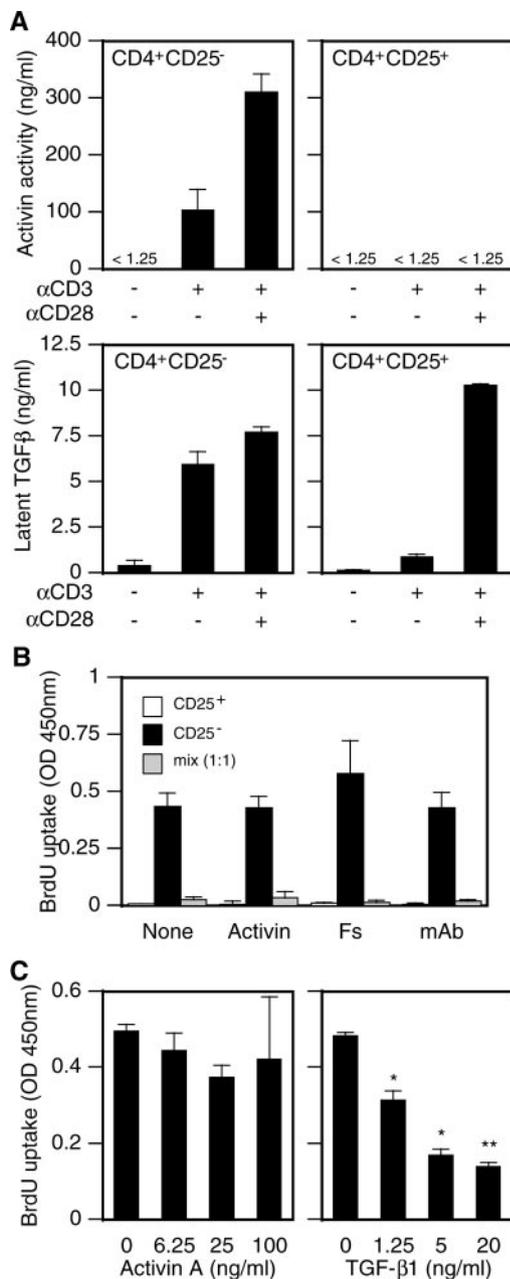


FIGURE 2. Expression and function of activin A and CD25⁺ and CD25⁻ subsets of CD4⁺ T cells. **A**, Expression of activin A in CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells. CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells isolated from mouse spleen were cultured with plate-bound anti-CD3 mAb (2 μ g/ml) with or without soluble anti-CD28 mAb (2 μ g/ml) for 6 days. Activin level in the supernatants was assayed and compared with that of latent TGF- β 1. **B**, Effects of activin A, follistatin, and anti-activin A mAb on CD4⁺CD25⁺ T cell-mediated suppression of T cell proliferation. CD4⁺CD25⁻ T cells (2.5×10^4) were stimulated with soluble anti-CD3 mAb (2 μ g/ml) and mitomycin C-treated T cell-depleted spleen cells (5×10^4) either alone or with CD4⁺CD25⁺ T cells (2.5×10^4) in the presence or absence of activin A (100 ng/ml), follistatin (400 ng/ml), or anti-activin A mAb (1 μ g/ml). Cell proliferation was measured by BrdU uptake after 72 h. **C**, Effects of activin A and TGF- β 1 on T cell proliferation. CD4⁺CD25⁻ T cells (2.5×10^4) were stimulated with soluble anti-CD3 mAb (2 μ g/ml) and mitomycin C-treated T cell-depleted spleen cells (5×10^4) in the presence of different dose of activin A (0–100 ng/ml) and TGF- β 1 (0–20 ng/ml) for 72 h. Cell proliferation was measured by incorporation of BrdU. The results are shown as mean \pm SD. * and **, $p < 0.01$ and $p < 0.001$, respectively.

NF-AT and c-Maf synergistically transactivate the activin β promoter

Our finding revealed that activin A production is markedly induced in the cells that differentiate into Th2 pathway, suggesting that activin β A gene expression is regulated similarly to other Th2-specific cytokines. Then, we studied about Th2-specific transcriptional regulation of activin β A gene. Sequence analysis of the activin β A promoter revealed the presence of a putative NF-AT site (nt -153 to -145 bp) and a Maf recognition element (MARE)-like sequence (nt -136 to -113 bp) (Fig. 4A). Reporter assays using the 5'-flanking region of the mouse activin β A fused to the luciferase gene revealed that the transcription of activin β A was stimulated by treatment with PMA and ionomycin in EL4 T cells, and that the stimulation was blocked by pretreatment with CsA (Fig. 4B), suggesting the involvement of NF-AT in transactivation of the activin β A gene. Expression of NF-ATp was also able to increase the luciferase activity in a dose-dependent manner (Fig. 4C). Mutations or deletion of the NF-AT site abolished responsiveness of the promoter to PMA and ionomycin (Fig. 4D), indicating the necessity of the NF-AT site for activation-induced activin β A gene expression.

c-Maf, a basic region/leucine zipper transcription factor, is expressed in Th2, but not in Th1 cells, and is induced during the differentiation along a Th2 lineage (26). There are two nucleotide sequences known to be able to bind Maf homodimers, a 13-bp TPA-responsive element-type MARE and a 14-bp cAMP response element-type MARE (27). Analysis of the nucleotide sequence of activin β A promoter revealed two putative MAREs (nt -136 to -113 bp) in close proximity to the NF-AT site. MARE-1 (nt -136 to -123 bp) has 10 of 14 nt matching the cAMP response element-type MARE, and MARE-2 (nt -125 to -113 bp) is similar to the TPA-responsive element-type MARE with 8 of 13 nt matching (Fig. 4A). Activin β A transcription was stimulated by the expression of c-Maf (Fig. 4E), and coexpression of c-Maf and NF-ATp synergistically increased the transcription of the activin β A gene (Fig. 4F). Gel-shift assays revealed that an oligonucleotide probe containing the two MAREs was able to form a complex with the GST-c-Maf DNA binding domain (Fig. 4G, left). Mutations of the MARE-1, but not the MARE-2, led to reduction of the c-Maf complex formation (Fig. 4G, left). Correspondingly, the MARE-1-mutated oligonucleotide was not able to compete off the complex formation of c-Maf with the wild-type oligonucleotide (Fig. 4G, right). Reporter assays also showed that mutations of the MARE-1 led to unresponsiveness to c-Maf expression (Fig. 4H). These results indicate that the nucleotide sequence spanning -136 to -123 bp of activin β A gene is crucial for transactivation activity of c-Maf. Furthermore, both NF-AT and c-Maf appear to be important for efficient transcription of activin β A. A reporter mutated at the NF-AT site was unresponsive to c-Maf expression, and the mutation of the MARE-1 rendered the reporter unresponsive to NF-ATp expression (Fig. 4I).

Activin A promotes alternative activation of macrophages

Our finding that activin A is produced in Th2 cells suggests that activin A has a role in Th2-mediated immune responses. However, activin A hardly affected T cell proliferation, suggesting that activin A produced by Th2 cells may have an effect on other immune cells. Our previous studies have shown that activin A alters the functions of macrophages (4) and mast cells (5). Macrophages play important roles in both Th1- and Th2-mediated immune responses, whereas mast cells are critical effector cells for Th2-type immune responses (28). Exposure of macrophages to Th1 or Th2 cytokines results in two distinct phenotypes, classically (M-1 phenotype) or

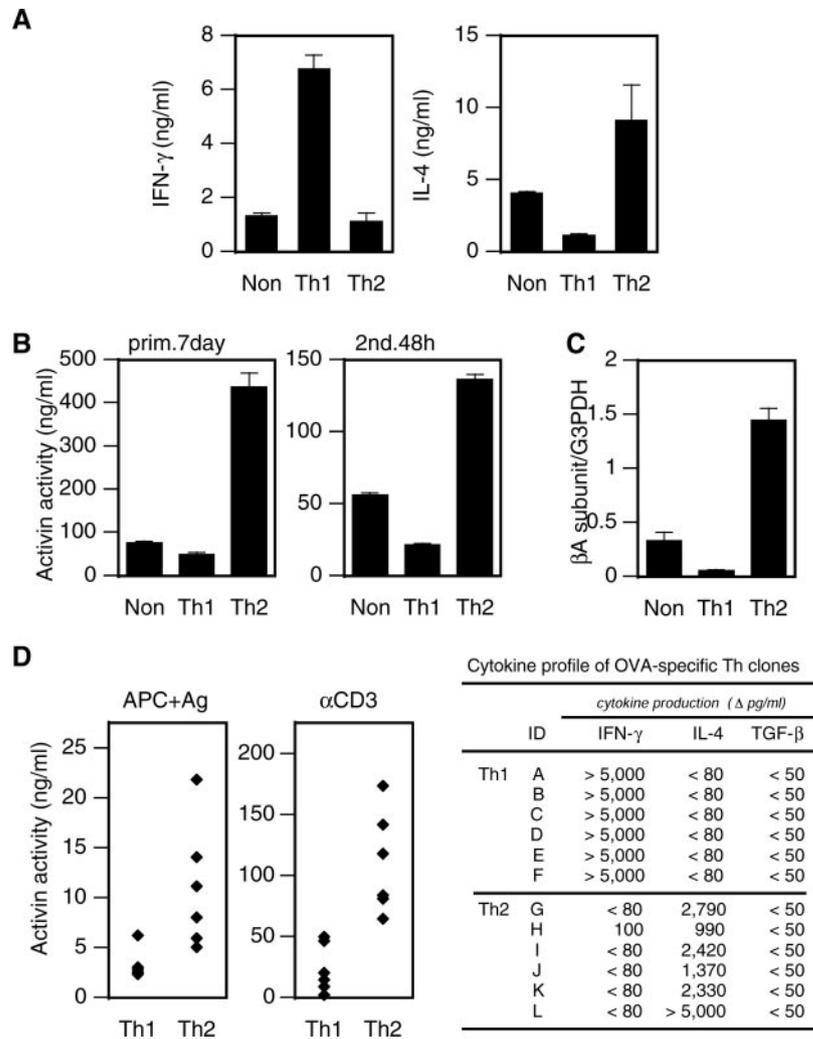


FIGURE 3. Preferential expression of activin A in Th2 cells. *A*, Cytokine profiles of in vitro differentiated Th1 and Th2 cells. CD4⁺ T cells were differentiated in vitro under non-skewing (Non), Th1-skewing (Th1), or Th2-skewing (Th2) conditions, as described in *Materials and Methods*. Supernatants collected from cells 48 h after the secondary stimulation were assessed for IFN-γ and IL-4 by ELISA. *B*, Activin production in in vitro differentiated Th1 and Th2 cells. Supernatants from the cells at 7 days after the primary stimulation (prim.7day) and 48 h after the secondary stimulation (2nd.48h) were assessed for activin activity. *C*, Activin βA mRNA levels in Th cells generated in in vitro differentiation were analyzed by competitive RT-PCR. *D*, Activin production by OVA-specific Th clones in response to Ag (APC+Ag) or anti-CD3 cross-linking (αCD3). Cytokine profiles of Th clones cultured with syngenic spleen cells in the presence of OVA (table).

alternatively (M-2 phenotype) activated macrophages, respectively (29–34). We determined whether activin A has an effect on the alternative activation of macrophages similar to other Th2 cytokines such as IL-4 and IL-13. The effect of exogenous activin A (120 ng/ml) to thioglycolate (TGC)-elicited peritoneal macrophages and macrophage-like cell line RAW264.7 cells on NO²⁻ production was examined at 48 h of the IFN-γ treatment (100 U/ml). Like other Th2 cytokines, activin A inhibits IFN-γ-induced NO²⁻ production in both peritoneal macrophages and RAW264.7 cells (Fig. 5A). The mRNA expression of arginase-1, a marker of alternatively activated macrophages (M-2 phenotype), was clearly augmented in the macrophages after treatment with activin A (Fig. 5B). In contrast, IFN-γ-induced expression of iNOS, a marker of classically activated macrophages (M-1 phenotype), was decreased in macrophages treated with activin A (Fig. 5B). These findings suggest that activin A is functionally implicated in the differentiation of macrophages into M-2 phenotype.

Discussion

Activin-deficient mice are not viable and die within the first 24 h after birth, primarily due to craniofacial defects and the lack of a lower incisor (35, 36). Thus, the in vivo expression and function of activin in immune system remain to be determined. In contrast, the immune suppressive function of TGF-β is elegantly illustrated by the in vivo studies. Targeted disruption of TGF-β1 in mice resulted in a severe multifocal inflammation, indicating a role of TGF-β in immune suppression (9). TGF-β inhibits the proliferation and dif-

ferentiation of T cells into effector cells, and induces apoptosis in these cells (10). In most studies, activins have overlapping biological activities with TGF-β (7). Thus, it was expected that activin A also exhibits immune suppressive function. However, our previous studies suggest that activin A positively regulates immune responses. Activin A induces the expression of matrix metalloproteinase-2 in peritoneal macrophages (4), and increases the migration and gene expression of mast cell-specific protease-1 in mast cell progenitors (5). In addition, the expression of activin A, but not TGF-β1, was up-regulated in peritoneal macrophages (4) and mast cells (37) in response to their activation. The present study demonstrated that CD4⁺CD25⁻ T cells exclusively express activin A, whereas CD4⁺CD25⁺ regulatory T cells produce TGF-β1 upon activation. Furthermore, activin A did not have significant effect on the proliferation of T cells, which is inhibited by TGF-β1 in a dose-dependent manner. These findings indicate that the expression and function of activin A in immune cells are different from those of TGF-β. Thus, the activity of activin A produced in CD4⁺ T cells, different from the function of TGF-β, is a unique example of the roles of activin A as implicated in the regulation of immune response as a local regulator.

Previous studies revealed the enhanced expression of activin A in peritoneal macrophages (4), monocytes (2, 3), and mast cells (5, 6) in response to cell activation. T cell-derived cytokines enhanced activin A secretion in monocytes and bone marrow stromal fibroblasts (38). Therefore, it appears that the activation-induced expression of activin A is most likely a common feature in immune

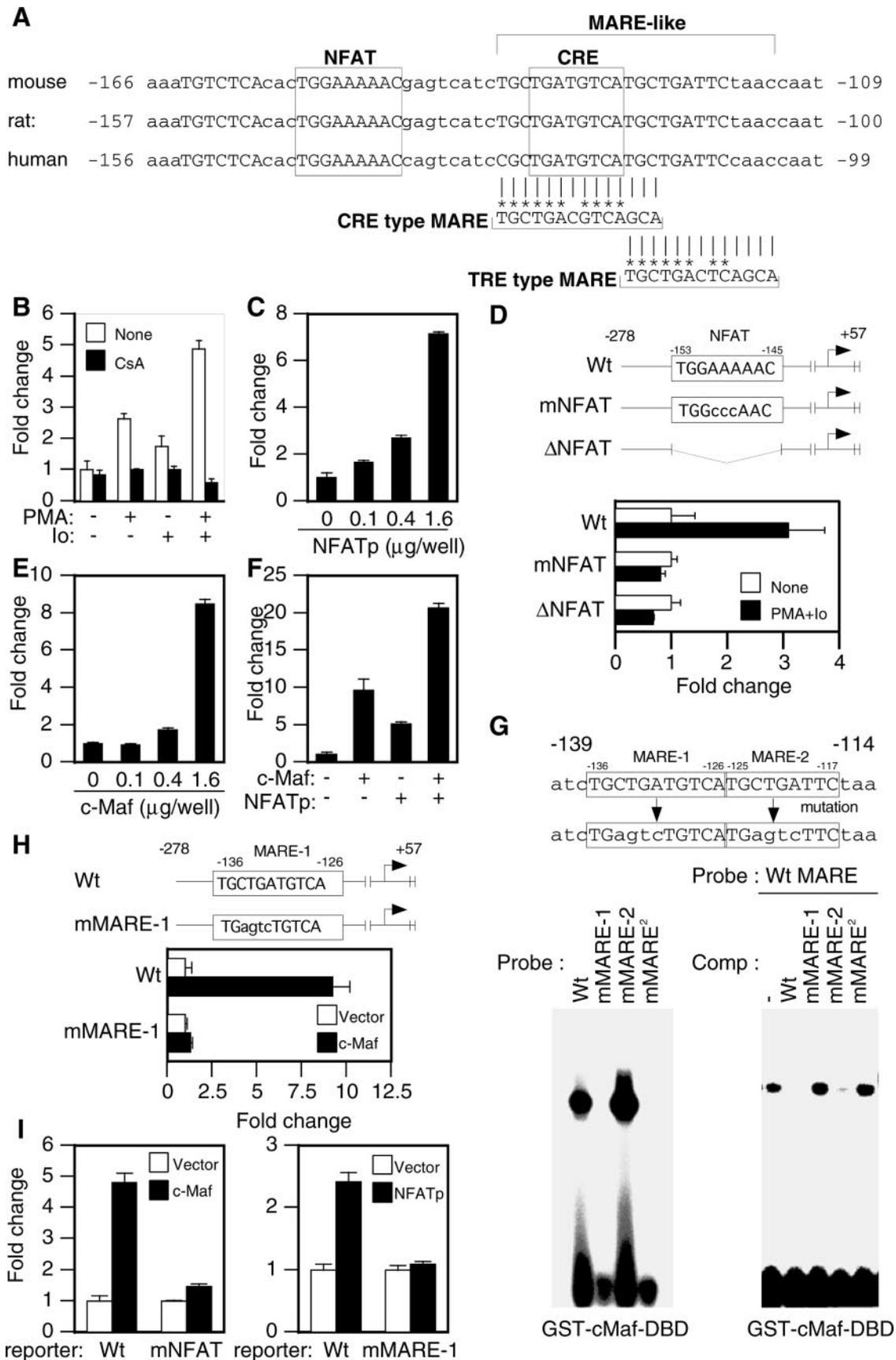


FIGURE 4. Transcriptional regulation of Th2-specific expression of activin β A gene. *A*, The nucleotide sequence of 5'-flanking region of activin β A gene. *B*, Effect of PMA, ionomycin (Io), and CsA on transactivation of activin β A promoter. EL4 T cells transfected with activin β A promoter construct were treated with PMA and/or Io in the presence or absence of CsA. *C*, Effect of the expression of NFATp (0–1.6 μ g/well) on transactivation of the activin β A promoter. *D*, Effects of mutation and deletion of the putative NFAT site (mNFAT and Δ NFAT, respectively) on promoter activity induced by PMA and ionomycin. *E*, Effects of the expression of c-Maf (0–1.6 μ g/well) on transactivation of activin β A promoter. *F*, Effect of NFATp and c-Maf (0.8

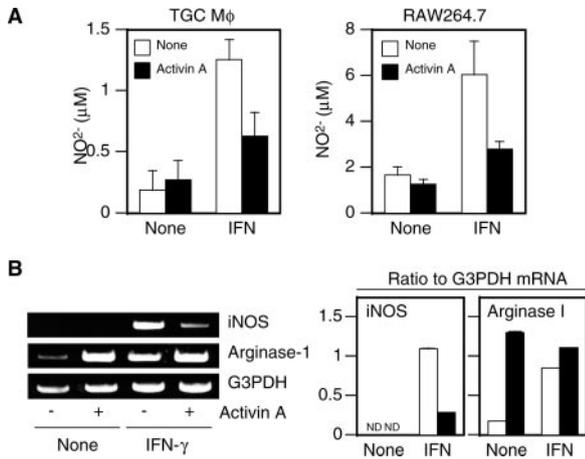


FIGURE 5. Involvement of activin A in the alternative activation of macrophages. *A*, Effect of activin A on NO production in peritoneal macrophages and macrophage-like cell line. TGC-elicited peritoneal macrophages and macrophage-like cell line RAW264.7 cells were cultured for 48 h with IFN- γ (100 U/ml) in the presence or absence of activin A (120 ng/ml). NO²⁻ in the culture supernatant was used as an indicator of NO generation and measured with the Griess reagent. The results are shown as mean \pm SD. *B*, Effect of activin A on mRNA expression of iNOS and arginase-1 in peritoneal macrophages. Total RNAs from TGC-elicited peritoneal macrophages cultured for 24 h with IFN- γ and/or activin A were isolated and used for RT-PCR with specific primers for iNOS, arginase-1, and G3PDH. The PCR products were run in a 2% agarose gel and stained with ethidium bromide (*left*). The intensities of the PCR products of iNOS and arginase-1 were normalized to G3PDH levels (*right*).

cells. In fact, systemic administration of bacterial LPS leads to an increased level of activin A in the circulation of sheep (39, 40). In Th cells, increased expression of activin A was found only in Th2 cells, but not in Th1 cells, indicating that activin A production is associated with Th2-type immune responses. This was supported by a previous study showing that secretion of activin A was increased in the airway of mice after OVA sensitization, followed by Ag challenge (6). Activin A in bronchoalveolar lavage fluid from OVA-sensitized mice was also elevated after Ag challenge (41). Furthermore, activin β A mRNA was highly induced in murine bone marrow mast cells after stimulation by IgE receptor cross-linking (6). In a human study, the serum level of activin A was increased in patients with asthma, and T cells from these patients had an increased level of activin A mRNA (42). Taking these results with the up-regulation of activin A in activated Th2 cells shown in this study, activation-induced expression of activin A may be a common feature in Th2-type immune responses.

Our study demonstrating that activin A production is markedly induced in the cells differentiated into Th2 subset prompted us to hypothesize that activin β A gene expression is regulated in a manner similar to other Th2-specific cytokines. To address this hypothesis and explore the molecular basis of activin induction in CD4⁺ Th cells, we studied the transcriptional regulation of the mouse activin β A gene. Analysis of the activin β A promoter sequence revealed the presence of a putative NF-AT site, indicating the necessity of the NF-AT site for activation-induced activin β A gene expression in T cells. NF-AT, however, binds to and trans-

activates the promoter of multiple genes, including cytokines expressed in Th1 cells as well as in Th2 cells (43). Thus, they are not likely to direct Th2-specific cytokine transcription. Further analysis of the promoter sequence revealed the presence of possible MAREs immediately downstream of the NF-AT site. c-Maf, a basic region/leucine zipper transcription factor, is expressed in Th2, but not in Th1 cells and is induced during the differentiation along a Th2 lineage (26). Our analyses suggest that NF-AT and c-Maf synergistically transactivate the activin β A promoter in murine thymoma EL4 cells. These factors may play an important key role for transcription of activin A, especially in Th2-specific expression. The cooperative regulation of activin β A gene by NF-ATp and c-Maf is consistent with the transcriptional regulation of a representative Th2 cytokine IL-4, in which c-Maf binds to a MARE immediately downstream of an NF-AT site in the proximal IL-4 promoter and transactivates IL-4 gene in synergy with the NF-ATp (26).

Our finding demonstrates that activin A is produced in Th2 cells, and suggests that activin A has a role in Th2-mediated immune responses. However, activin A hardly affected T cell proliferation, suggesting that activin A produced by Th2 cells has effects on other immune cells. Our previous studies have shown that activin A has effects on other immune cells, including macrophages (4) and mast cells (5). Mast cells are known to be critical effector cells of Th2-induced immune responses such as allergic inflammation and immediate hypersensitivity (28). In contrast, macrophages play important roles in both Th1- and Th2-mediated immune responses. Classical activation of macrophages with Th1 cytokines results in free-radical release and increased cytokine secretion, implicated as essential signaling components of a successful response to infection by intracellular bacteria and viruses (29, 30). In contrast, the alternative activation of macrophages with Th2 cytokines is required for defense against extracellular pathogens and parasites (31). In parallel with the separation of Th cells into Th1 or Th2 cells, several groups have proposed that the exposure of macrophages to a specific set of cytokines also biases them toward either an M-1 (activated macrophages) or an M-2 (alternatively activated macrophages) phenotype (32–34). Activated macrophages metabolize L-arginine by two alternative pathways involving the enzymes iNOS or arginase (44, 45). Th1 cytokines induce macrophages to produce iNOS, whereas Th2 cytokines induce arginase-1 (45–47). Both iNOS and arginase compete for the same substrate L-arginine. iNOS converts L-arginine into NO and L-citrulline, whereas arginase catalyzes its conversion to urea and L-ornithine. Thus, iNOS and arginase-1 effectively compete for L-arginine, and thereby negatively regulate the function of each other. Like the other Th2 cytokines, treatment of macrophages with activin A markedly induced expression of arginase-1 and decreased IFN- γ -induced expression of iNOS, indicating that activin A is involved in the alternative activation of macrophages.

Activin production in activated Th2 cells is much higher than that in peritoneal macrophages (4) and B cells (K. Ogawa, M. Funaba, and M. Tsujimoto, unpublished observation). In view of ~200 pg/ml blood activin levels (48), activation of Th2 cells leads to significantly higher concentration of activin A at the affected site. One of the important implications is that activin A is not

μ g/well each) on transactivation of the activin β A promoter. *G*, Binding of c-Maf to the MARE in the activin β A promoter. Th nucleotide sequences of the putative MARE-containing sequence and mutants are shown in the *top panel*. The GST-c-Maf was subjected to EMSA with probe and competitors, as indicated. *H*, Effects of the mutations of the MARE-1 (mMARE-1) on the activin β A promoter activity induced by c-Maf expression (1.6 μ g/well). *I*, Effects of the mutants (mNFAT and mMARE-1) of activin β A promoters on c-Maf- and NF-ATp (1.6 μ g/well each)-induced transcription. The fold change of normalized luciferase activity is shown as mean \pm SD for *B*, *C*, *D*, *E*, *F*, *H*, and *I*.

involved in T cell-suppressor function, in contrast to the structurally and functionally related TGF- β 1. Thus, activin A produced in the inflamed site may act on immune responses without suppressing T cell functions.

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Disclosures

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