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Smad and NFAT Pathways Cooperate To Induce CD103 Expression in Human CD8 T Lymphocytes

M’Barka Mokrani,*†‡ Jiheène Klibi,*†‡ Dominique Bluteau,§‡ Georges Bismuth,¶‖# and Fathia Mami-Chouaib*†‡

The interaction of integrin αEβ7, often expressed on tumor-infiltrating T lymphocytes, with its cognate ligand, the epithelial cell marker E-cadherin on tumor cells, plays a major role in antitumor CTL responses. CD103 is induced on CD8 T cells upon TCR engagement and exposure to TGF-β1, abundant within the tumor microenvironment. However, the transcriptional mechanisms underlying the cooperative role of these two signaling pathways in inducing CD103 expression in CD8 T lymphocytes remain unknown. Using a human CTL system model based on a CD8+/CD103+ T cell clone specific of a lung tumor–associated Ag, we demonstrated that the transcription factors Smad2/3 and NFAT-1 are two critical regulators of this process. We also identified promoter and enhancer elements of the human ITGA2 gene, encoding CD103, involved in its induction by these transcriptional regulators. Overall, our results explain how TGF-β1 can participate in CD103 expression on locally TCR-engaged Ag-specific CD8 T cells, thus contributing to antitumor CTL responses and cancer cell destruction. The Journal of Immunology, 2014, 192: 2471–2479.

Cytotoxic T lymphocytes are major effector cells of the immune system, predominantly responsible for Ag-specific clearance of tumors and infected cells. These effector cells exert their lytic activity following interaction of TCR with the specific peptide–MHC class I complex on target cells, mainly through exocytosis of cytotoxic granules. Upon initial TCR-dependent target cell recognition, adhesion/costimulatory molecules, including integrins, are repositioned at the T cell–target cell contact zone referred to as the immune synapse. This results in the formation of a signaling complex with intracellular proteins and the initiation of a transduction cascade, leading to execution of CTL effector functions, mainly killing of target cell and cytokine secretion.

Among integrin family members, the αEβ7 integrin plays an essential role in TCR-mediated cancer cell lysis by interacting with its ligand, the epithelial cell marker E-cadherin, on tumor cells, triggering the release of cytotoxic granules by specific CTL (1–4). CD8+αEβ7+ T lymphocytes have been reported to play a critical role in mediating tubular injury following allogeneic renal transplantation (5) and in promoting renal allograft rejection (6, 7). They are also involved in selective destruction of pancreatic islet allografts (8) and host intestinal epithelium during graft-versus-host disease (9–12), which can be prevented by CD103 deficiency (13). The αEβ7 integrin is expressed at high levels by mucosal CD8+ T lymphocytes, in particular intestinal epithelium lymphocytes (14), psoriatic skin epidermal CD8+ T cells (15), and cervicovaginal Ag-specific CTL (16). It is also found on mucosal mast cells and dendritic cells (17), CD4+ and CD8+ T regulatory (Treg) cells (18, 19), and on a large proportion of CD8+ effector T cells infiltrating epithelial tumors, including bladder (20), colorectal (21), pancreatic (22), ovarian (23), and lung cancers (1, 3). The restricted distribution of the αEβ7 integrin is attributed to expression of the αE subunit (CD103), because the β7 subunit is widely distributed on T lymphocytes (24).

It is now widely admitted that CD103 can be induced on CD8 T cells residing in tissue microenvironments in which TGF-β1 is abundant, including epithelia, chronic inflammatory lesions (25), and tumors (26, 27), or during autoimmune processes (28) and renal allografts (6, 29, 30). Indeed, accumulating evidence indicates that TGF-β1 is directly involved in CD103 induction upon T cell activation (31, 32) and that its regulation occurs at the transcription level (33, 34). However, little is known about the molecular mechanisms that regulate expression of the ITGA2 gene, which encodes CD103. A proximal promoter region has been previously described, but it did not confer TGF-β1 responsiveness, suggesting the existence of distal control elements (34). In this report, we demonstrate that Smad and NFAT pathways cooperate to induce ITGA2 gene expression in CD8 T cells. We also identify promoter and enhancer regulatory elements of the human ITGA2 gene with effective Smad3 and NFAT-1 binding sites whose partnership activates CD103 expression and antitumor cytotoxicity of CD8 T cells after TCR engagement in the presence of TGF-β1.

Materials and Methods

T cell clone and tumor cell lines

The CD103+ T cell clone H32-22 was isolated from PBL of a patient suffering from a nonsmall cell lung carcinoma (35). This clone recognizes,
Constitution of ITGAE promoter and enhancer reporter plasmids and luciferase assay

ITGAE promoter and enhancer sites were predicted by Genomatix software (http://www.genomatix.de). A 899-bp fragment of the ITGAE promoter and a 843-bp fragment of the ITGAE enhancer were amplified by PCR from genomic DNA of the autologous CD103 T cell clone Heu171 (1) and cloned into the pGL4.12 vector (Promega) between SacI and XhoI sites. Primer pairs used for amplification were as follows: promoter forward, 5'-TATAGAGCTCCATCCGGCACTCTCGACTCTCAGACGAC-3' and promoter reverse, 5'-ATATCGGACGATCCTGCTGGACGAGAAGGGCTTGTTG-3'; enhancer forward, 5'-TAGATGATCCATTACATGGGGCTTTTTCGTCACCCAGG-3' and enhancer reverse, 5'-ATAGTGACCATGACGAAAGGATAGATAGATAGAAC-3'. Then we cloned the ITGAE enhancer fragment into a BamHI site located downstream of the luciferase gene in the ITGAE promoter reporter plasmid. Small site deletion mutants were created from this reporter plasmid by site-directed mutagenesis using a mutagensis kit (Quickchange II XL Site-Directed Mutagenesis Kit; Agilent Technologies) and the following primer pairs: promoter forward, 5'-CGAGAGGACCTGGCCTGACAGCTGGGGCTAGG-3' and enhancer forward, 5'-GGGCACTCCTGCGTGCAGTTGGGCTGTG-3'.

Luciferase assays were performed in 293-T cells or Jurkat-T cells. A total of 2 x 10^5 cells was transfected with 1 µg luciferase reporter plasmid and 1 µg Reulla luciferase control plasmid using jetPEX transfection and then cultured for 17 h at 37°C in six-well flat-bottom cell culture plates (Corning Life Sciences). Transfected cells, untreated or treated with the TGF-β1 kinase inhibitor SB, were either kept in medium or stimulated with TGF-β1. Cells were then collected and analyzed for luciferase activity by the dual-luciferase reporter assay system (Promega). All assays were repeated at least three times, and the activity of firefly luciferase (pGL4) was normalized to that of the internal control, Reulla luciferase (pRL-CMV).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed with the H32-22 T cell clone, unstimulated or stimulated for 30 min with a combination of anti-CD3 mAb and rTGF-β1 using the SimpleChIP enzymatic ChIP kit (Cell Signaling). Cells were fixed (1% formaldehyde) for 10 min at room temperature. chromatin was sheared by sonication, and lysates were clarified by centrifugation. Immunoprecipitating mAb (anti-Smad2, anti-phospho-Smad3, anti–NFAT-1, or anti-IgG negative control), validated for the ChIP assay, were then added to the cross-linked chromatin preparation and incubated at 4°C overnight. Immune complexes were collected by addition of protein G magnetic beads, washed, and eluted by spin columns. To determine the percentage of each analyzed region against the input DNA, quantitative RT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and primers specific to the region of interest. The PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. The band intensities were quantified using ImageJ software (National Institutes of Health). The enrichment of the input DNA was calculated as the ratio of the PCR product band intensities to the input DNA band intensities. The fold enrichment was calculated by dividing the enrichment of the input DNA by the enrichment of the input DNA.

DNA pull-down assay

A DNA fragment containing the ITGAE promoter or ITGAE enhancer was amplified by PCR using a 5’ biotin-labeled forward primer. Primer sequences were as follows: ITGAE promoter, forward, 5’-ATAGAGCTCCATCCGGCACTCTCGACTCTCAGACGAC-3’ and reverse, 5’-TATACGAGCATCCTGCTGGACGAGAAGGGCTTGTTG-3’; and for ITGAE enhancer, forward, 5’-TATAGAGCTCCATCCGGCACTCTCGACTCTCAGACGAC-3’ and reverse, 5’-TATACGAGCATCCTGCTGGACGAGAAGGGCTTGTTG-3’. The biotinylated probes (1 µg) were hybridized with cell lysates in 400 µl of a binding buffer (25 mM HEPES [pH 7.9], 0.5 M NaCl, 1 mM MgCl2, 1 mM DTT, 2 µg poly(dI-dC) and incubated for 2 h at 4°C. Then 25 µl streptavidin–agarose beads (Invitrogen) were added and incubated for an additional 1 h. The streptavidin–agarose beads were washed five times with the binding buffer, and then SDS-SDS coprecipitated buffer was added. The complexes were subjected to SDS-PAGE, followed by immunoblotting with anti-phospho-Smad3 or anti–NFAT-1 mAb.

Statistical analysis

Data were compared using the two-tailed Student t test. Two groups were considered as significantly different if p < 0.05.

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**Results**

**Smad2 and Smad3 transcription factors are activated after TGF-β1 stimulation of CD8 T cells**

Using a human cytotoxic T cell clone model, H32-22, generated from PBL of a lung cancer patient, we had previously demonstrated the key role played by integrin CD103, induced upon stimulation with TGF-β1 and anti-CD3 mAb, in triggering specific TCR-mediated tumor cell lysis (1). As Smad2 and Smad3 are critical transcription factors in TGF-β1 signaling (37), we investigated their contribution to this biological effect using our T cell model system. Smad2 and Smad3 transcriptional activity is governed by their rapid phosphorylation and translocation into the nucleus after cell stimulation. Therefore, we analyzed these two processes in the H32-22 T cell clone. Fig. 1A and Supplemental Fig. 1 show that both Smad2 and Smad3 were rapidly phosphorylated after T cell clone activation with TGF-β1 or a combination of TGF-β1 and anti-CD3 mAb. Moreover, phosphorylation of the two proteins was inhibited in the presence of the TGF-βR1 kinase inhibitor SB or, for Smad3, the Smad3-specific inhibitor SIS3 (Fig. 1A).

Phosphorylated Smad2 and Smad3 form complexes with Smad4 and translocate into the nucleus, where they regulate target gene transcription (38, 39). Experiments were therefore also performed to analyze Smad2 and Smad3 locations in untreated T cells or in T cells treated with a combination of TGF-β1 and anti-CD3 mAb. Confocal microscopy analyses indicated that H32-22 T cells cultured in the presence of TGF-β1 plus anti-CD3 displayed nuclear accumulation of Smad2 and Smad3 proteins (Fig. 1B, **upper panels**). Indeed, 92% ± 6 and 76% ± 6 of stimulated cells, compared with 5% ± 1 and 3% ± 4 of unstimulated cells, exhibited a nuclear localization of Smad2 and Smad3, respectively (Fig. 1B, **lower panels**). We then assessed the effect of SB and SIS3 inhibitors on Smad protein localization after T cell treatment with TGF-β1 and anti-CD3 mAb. Results indicated that addition of the TGF-βR1 kinase inhibitor SB resulted in total inhibition of nuclear translocation of both transcription factors (Fig. 1B, **upper panels**). As expected, SIS3 only abrogated Smad3 translocation into the nucleus, whereas it had no effect on Smad2 localization. Indeed, only 2% ± 3 and 5% ± 2 of SB-treated cells displayed nuclear Smad2 and Smad3 expression, whereas 86% ± 20 and 1% ± 1 of SIS3-treated cells showed nuclear accumulation of Smad2 and Smad3, respectively (Fig. 1B, **lower panels**).

**Smad3 cooperates with Smad2 in TGF-β1-induced CD103 expression on TCR-activated CD8 T cells and TCR-mediated cytotoxicity**

Next, we used SB and SIS3 inhibitors to analyze the involvement of Smad2 and Smad3 in CD103 induction at the T cell clone surface. Unstimulated H32-22 T cells do not spontaneously express CD103. However, as we had previously reported (1), whereas TGF-β1 plus anti-CD3 mAb, in triggering specific TCR-mediated tumor cell lysis (1). As Smad2 and Smad3 are critical transcription factors in TGF-β1 signaling (37), we investigated their contribution to this biological effect using our T cell model system. Smad2 and Smad3 transcriptional activity is governed by their rapid phosphorylation and translocation into the nucleus after cell stimulation. Therefore, we analyzed these two processes in the H32-22 T cell clone. Fig. 1A and Supplemental Fig. 1 show that both Smad2 and Smad3 were rapidly phosphorylated after T cell clone activation with TGF-β1 or a combination of TGF-β1 and anti-CD3 mAb. Moreover, phosphorylation of the two proteins was inhibited in the presence of the TGF-βR1 kinase inhibitor SB or, for Smad3, the Smad3-specific inhibitor SIS3 (Fig. 1A).

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Next, we used SB and SIS3 inhibitors to analyze the involvement of Smad2 and Smad3 in CD103 induction at the T cell clone surface. Unstimulated H32-22 T cells do not spontaneously express CD103. However, as we had previously reported (1), whereas TGF-β1 or anti-CD3 mAb used alone were unable to induce integrin expression on H32-22 CTL, their combination induced strong expression of CD103 (Fig. 2A). This increase was completely inhibited by SB and partially by SIS3. Similar results were obtained with the CD8+ leukemia T cell line TALL-104, in which CD103 expression induced by TGF-β1 and anti-CD3 was again strongly inhibited by SB and to a lesser extent by SIS3 (Supplemental Fig. 2). Because Smad2-specific chemical inhibitors are not available, and to address the role of this transcription factor in CD103 expression, we used a specific siRNA that completely blocks Smad2 expression in H32-22 cells (Fig. 2B). Results shown in Fig. 2C indicate that Smad2 knockdown poorly affects CD103 induction by TGF-β1 plus anti-CD3 mAb.

We had previously reported, at the functional level, the essential role exerted by CD103 in triggering TCR-mediated cytotoxicity of CTL toward specific tumor cells (1, 2). Indeed, as shown in Fig. 2D, untreated H32-22 T cells, which do not express CD103 (Fig. 2A), are unable to lyse the autologous lung cancer cell line IGR-Heu. In contrast, T lymphocytes expressing high levels of CD103, after TGF-β1 plus anti-CD3 mAb treatment, efficiently kill the specific target. In concordance with CD103 expression analyses (Fig. 2A), we found that the TGF-βR1 inhibitor SB totally inhibited tumor cell lysis, whereas SIS3 had only a partial effect (Fig. 2D). Together these results demonstrate the essential role of the Smad pathway in CD103 induction and CTL effector function triggered by TGF-β1 plus TCR signaling.

**NFAT-1 is involved in CD103 expression**

Induction of CD103 on CD8 T lymphocytes requires, along with exposure to TGF-β1, the engagement of the TCR through recognition of the peptide–MHC class I complex on target cells (2) or via an anti-CD3 mAb. As data from Fig. 2A showed that TGF-β1 alone is not sufficient to induce CD103 expression and that the signal given by the anti-CD3 mAb was also required for integrin induction, we assumed that another pathway in addition to the Smad pathway could be involved in regulating CD103 expression. Among the various transcription factors triggered through the TCR–CD3 complex in T cells is NFAT-1, a key protein in the network of molecules that initiate T cell activation. Moreover, molecular analyses of the human ITGA8 gene (see below) indicated the presence of consensus NFAT binding sites in its regulatory regions. We therefore investigated the activation of NFAT-1 and its involvement in CD103 expression in H32-22 T lymphocytes. We first monitored NFAT-1 nuclear localization induced by a combination of TGF-β1 and anti-CD3 mAb. Confocal microscopy analyses indicated that, compared with unstimulated cells, H32-22 T cells stimulated with TGF-β1 plus anti-CD3 displayed nuclear translocation of NFAT-1 (Fig. 3A, **left panel**). Indeed, although only 14% ± 7 of untreated cells displayed spontaneous nuclear localization of NFAT-1, 93% ± 4 of T lymphocytes stimulated with TGF-β1 plus anti-CD3 showed accumulation of the transcription factor in the nucleus (Fig. 3A, **right panel**).

We then assessed the effect of the calcineurin inhibitor CsA, which inhibits NFAT-1 activation (40). Results indicate that CsA totally blocked NFAT-1 relocalization in the nucleus (Fig. 3A). CsA also led to a dramatic decrease in CD103 expression on H32-22 and TALL-104 T cells induced by TGF-β1 plus anti-CD3 treatment (Fig. 3B, Supplemental Fig. 2) and suppressed H32-22-mediated cytotoxicity toward autologous IGR-Heu tumor cells (see Fig. 2D). These data support a role for NFAT-1 in induction of CD103 on CD8 T lymphocytes upon TCR engagement. They also indicate that TGF-β1 and TCR signaling pathways cooperate to induce CD103 and to trigger the cytotoxic function of CD8+ CTL.

**ITGA8 gene regulatory regions include promoter and enhancer elements with Smad and NFAT binding sites**

ITGA8 gene regulatory elements are still poorly defined. To identify human ITGA8 gene regulatory regions, we used Genomatix software that can predict gene promoter sequences and transcription factor binding sites. Analyses of the whole sequence of the ITGA8 gene (86.62 Kb) identified two potential regulatory regions with consensus binding sites for both Smad and NFAT proteins, which may correspond to the ITGA8 promoter and enhancer regions (Supplemental Fig. 3A). The potential promoter (regulatory sequence 1, see Supplemental Fig. 3B) starts upstream of exon 1 and ends 32 bp before its 3′-terminal end (~801 to +101). It contains both a NFAT consensus binding site (5′-CCCTTCCA-3′) and a sequence (5′-CTGAGATGTCTGCGG-3′) encompassing a Smad binding site (5′-GTCT-3′) separated by 145 nucleic acids (Fig. 4A,
The potential enhancer (regulatory sequence 2, see Supplemental Fig. 3C) is located within intron 1 and extends from position +16,561 to +17,462. It also includes a Smad binding site (5′-AGAC-3′) and a NFAT consensus binding site (5′-TTTTTCCA-3′) separated by 27 nucleic acids (Fig. 4A, lower panel). Next, using the ChIP assay, we analyzed the binding capacity of Smad2, Smad3, and NFAT-1 to these DNA regulatory sequences using ChIP assay-validated anti-Smad2, anti–phospho-Smad3, and anti–NFAT-1 Ab. An anti-IgG mAb was included as a control. Substantial binding of phospho-Smad3 to the Smad binding site of the potential ITGAE promoter was detected in immune complexes with DNA prepared from H32-22 T cells stimulated with TGF-β1 and/or anti-CD3 mAb (green fluorescence). Nuclei were stained with TO-PRO-3 iodide (red fluorescence). Original magnification x63. Lower panels show percentages of T cells displaying Smad2 (left) or Smad3 (right) nuclear relocalization. Data shown represent mean ± SD of two independent experiments. Numbers of cells analyzed (n = 50).

Binding of the two transcription factors to potential ITGAE gene regulatory elements was further confirmed by the DNA pull-down assay using nuclear extracts from H32-22 T cells stimulated with TGF-β1 and/or anti-CD3 mAb (Supplemental Fig. 4B).
binding sites in the promoter and enhancer core (Fig. 5B) or use of an antisense enhancer (Supplemental Fig. 4C) inhibited luciferase activity. It should be noted that stronger activity was not observed when cells were stimulated with TGF-$\beta$ (Supplemental Fig. 4B, Fig. 5B). These results are most likely due to the presence of constitutive active Smad molecules in 293-T cells, as suggested by Western blot analysis revealing spontaneous phosphorylation of both proteins in this cell line (Supplemental Fig. 4D). Therefore, and to confirm their involvement in the observed activity, cells were pretreated with the TGF-$\beta$R1 kinase inhibitor SB. Results show that cell treatment with SB totally abrogated luciferase activity (Fig. 5C). A spontaneous luciferase activity was also obtained with the ITGAE reporter plasmid transfected in Jurkat-Tag T cells (Supplemental Fig. 4E). The constitutive phosphorylation of Smad2 and Smad3 in this cell line (see Supplemental Fig. 4F) is most likely responsible, a hypothesis also supported by its inhibition in the presence of SB. Importantly, stimulation of Jurkat-Tag T cells with anti-CD3 mAb induced an increase in luciferase activity, further supporting a role of TCR signal in ITGAE gene expression regulation (Supplemental Fig. 4E).

We then assessed the contribution of NFAT-1 to ITGAE enhancer activity. With this aim, we analyzed the activity of a reporter vector containing the two regulatory sequences in 293-T cells, displaying constitutively phosphorylated Smad2 and Smad3 (see Supplemental Fig. 4D), cotransfected with a NFAT-1–encoding plasmid. No luciferase activity was observed in untreated cells. In contrast,
FIGURE 3. Role of NFAT-1 in CD103 induction on the T cell clone surface. (A) Confocal microscopy analysis of nuclear translocation of NFAT-1 transcription factor. H32-22 T cells, untreated or pretreated with CsA, were stimulated for 1 h with anti-CD3 plus TGFβ-1 and then stained with anti–NFAT-1 mAb (green fluorescence). Nuclei were stained with TO-PRO-3 iodide (red fluorescence). Original magnification ×63. Right panel, Percentages of T cells displaying NFAT-1 nuclear relocalization. Data shown represent mean ± SD of two independent experiments. Numbers of cells analyzed (n = 50). (B) Expression of the αββ1 integrin on the CD8+ T cell clone surface. H32-22 T cells, untreated or pretreated with CsA, were stimulated with a combination of plastic-coated anti-CD3 mAb and rTGFβ-1 for 4 d and then analyzed by immunofluorescence staining using anti-CD103 (black fill) mAb. T cells stimulated with anti-CD3 alone or rTGFβ-1 alone and an isotypic control (without fill) mAb were included. Percentages of positive cells are indicated. Numbers in parentheses correspond to mean fluorescence intensity of positive cells.

Discussion

TGF-β1 is involved in CD103 induction upon TCR engagement (31, 32). Indeed, we show in this work that both signaling pathways are required for CD103 induction on CD8+ T cell surface. TGF-β1 is frequently described as an immunosuppressive cytokine used by cancer cells to escape from the immune system and target CTL functions (41). Therefore, the paradoxical role of TGF-β1 in optimization of antitumor CTL reactivity via regulation of the CD103 integrin is an important observation that warrants intensive investigation. In this report, using a human tumor Ag-specific CTL system model, we demonstrated that Smad3/2/3 transcription factors, in cooperation with NFAT-1, are directly involved in CD103 induction on CD8+ T cells upon TCR engagement in the presence of TGF-β1. We also identified both promoter and enhancer regulatory sequences of the human CD103-encoding ITGAE gene involved in this process.

TGF-β1 initiates signaling by binding to TGF-βR at the cell membrane to form a heterotetrameric complex composed of TGF-βR type I and type II dimers. These receptors are serine/threonine kinase receptors, and, in this complex, TGF-βR2 catalyzes phosphorylation of the cytoplasmic domain of TGF-βR1. This leads to recruitment and phosphorylation of receptor-associated Smad2 and Smad3 molecules (37). Upon their phosphorylation, Smad2 and Smad3 undergo homotrimerization and formation of heterooligomeric complexes with the co-Smad molecule, Smad4. These activated Smad complexes enter the nucleus and, in cooperation with other transcription factors, regulate transcription of target genes (37). Our data indicate that T cell treatment with TGF-β1 induces Smad2 and Smad3 phosphorylation and their nuclear translocation. They also indicate that CD103 induction at the surface of CD8+ T cells requires activation of these transcription factors. Indeed, inhibition of TGF-βR1 abrogated CD103 induction by TGF-β1 and, concomitantly, specific TCR-mediated tumor cell lysis. In contrast, inhibition of either Smad2 or Smad3 separately had only a weak or marginal effect, suggesting that a Smad-independent pathway is involved in CD103 expression regulation, or, alternatively, that Smad2 and Smad3 are redundant for TGF-β-mediated induction of CD103. This result is intriguing because, in principle, Smad3 binds to DNA, whereas Smad2 does not (38), and we did not find any binding of Smad2 to ITGAE promoter or enhancer elements. Thus, it remains unclear as to how Smad2 upregulates expression of ITGAE when Smad3 is inhibited. One possibility is that Smad2 activates transcription of the gene by interacting with other yet unknown transcription factors, whose binding regions are distinct from the Smad3 binding sites identified in the present report. Further studies will be necessary to clarify this issue, but a recent report showing that Smad2 and Smad3 are redundantly essential for TGF-β-mediated regulation of Treg cell plasticity and Th1 development reached the same conclusion (42). CD103 induction on CD8+ T cells also requires an...
additional signal given by the TCR. Our data point to the important role played by NFAT-1 in this function, as illustrated by the dramatic blocking effect of the calcineurin inhibitor CsA upon CD103 expression on T lymphocytes treated with TGF-β1 plus anti-CD3 and also the complete inhibition of CTL-mediated lysis of autologous tumor cells. Collectively, these results show that Smad and NFAT transcription factors cooperate to induce CD103 expression and thus trigger the CTL effector function.

In the current study, we also identified an ITGAE promoter located upstream of exon 1, which roughly corresponds to that previously suggested by Robinson et al. (34). We also identified an ITGAE enhancer located within intron 1. Each regulatory region includes Smad and NFAT consensus binding sites, but whereas both ITGAE regulatory sequences effectively bind Smad3, only the enhancer region is able to bind NFAT-1. In agreement with a previous report (34), we found that the ITGAE promoter sequence by itself, without any enhancer, is inactive in a reporter gene activation assay in 293-T cells. However, enhancer activity was detected with a reporter plasmid containing both ITGAE promoter and enhancer elements. This activity was constitutive in 293-T cells, most likely because these cells constitutively express phosphorylated Smad2 and Smad3. Indeed, it was abrogated with the TGF-β1R1 inhibitor or after deletion of the Smad binding site in the regulatory sequence. Taken together, these data show that transcription regulation of the ITGAE gene is more complex than that of other integrin genes, such as ITGAL, ITGA2, ITGA6, and ITGAX (43-47), for which promoter regions directed tissue-specific functional activity, and that ITGAE expression is regulated through promoter and enhancer elements with the participation of both Smad and NFAT-1 transcription factors. It is to note that, as opposed to CD103 expression, we observed that TGF-β1 had an inhibitory effect on LFA-1 (αLβ2) expression levels on the surface of the T cell clone used in the current study (data not shown).

We previously reported, in a xenograph model, that stable expression of CD103 can be induced in vivo following H32-22 T cell transfer into the autologous tumor and interaction of TCR with the specific tumor epitope–MHC-I complex presented on the surface of malignant cells. We also reported that neutralization of TGF-β1, secreted within the tumor microenvironment with a soluble AdTGF-β1RII-Fc molecule, inhibited ITGAE expression in tumor-reactive
CD8+ T cells (2). These results are fully consistent with the conclusion that Smad and NFAT pathways are key regulators in initiating transcription of the \textit{ITGAE} gene. However, additional transcription factors, such as Runx, may also be involved in \textit{ITGAE} gene regulation under in vivo conditions. Indeed, evidence for involvement of Runx factors in the TGF-\(\beta\) pathway and interactions between Smad and Runx proteins have been previously reported (48–51). Runx binding regions were also found in the \textit{ITGAE} regulatory sequences identified in this work (data not shown), and Runx3 has been shown to induce CD103 expression during development of CD4+/CD8+ T cells having Treg cell characteristics (52).

Regulation of CD103 expression appears similar to that of Foxp3 in several aspects, as follows: 1) both proteins are expressed following T lymphocyte activation by a combination of TGF-\(\beta\) and anti-CD3; 2) \textit{ITGAE} and Foxp3 genes are regulated by enhancer elements, including both Smad3 and NFAT binding sites; and 3) they are induced by enhancers through the actions of Smad3 and NFAT (53). Interestingly, a subset of Treg cells coexpresses both Foxp3 and CD103 molecules (54). These Treg cells might be generated through interaction of TCR with tumor Ag, and, in this case, CD103 may control their retention at the tumor site (55), as suggested by studies in epithelial tissues (56), including epithelial tumors (2). However, the effective role of CD103 in Treg cell functions is to date unknown, especially in the immunosuppressive Treg cell subpopulation that accumulates within tumors and favors cancer cell escape from immune responses.

In summary, we have identified an \textit{ITGAE} gene enhancer element and defined the role of Smad and NFAT-1 transcription factors in CD103 induction. These results support the conclusion that TGF-\(\beta\)1 plays a major role in activation of CD103 in CD8 T cells and that this cytokine contributes to regulating antitumor immune responses. Our studies also suggest a rational immunotherapeutic approach for inducing CD103+ T cells by specific activation of signals that increase Smad-dependent \textit{ITGAE} gene expression in tumor-specific TCR-activated T cells. These will not only increase the capacity of TGF-\(\beta\)1 to enhance CD103 expression on tumor-infiltrating T lymphocytes and their killing activity but will also improve their retention at the tumor site.

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**Disclosures**

The authors have no financial conflicts of interest.

**FIGURE 5.** Role of Smad3 and NFAT-1 in \textit{ITGAE} promoter and enhancer activities. (A) Luciferase assay of the \textit{ITGAE} reporter plasmid. Promoter activity was analyzed in 293-T cells, either kept unstimulated (medium) or stimulated with TGF-\(\beta\)-1. An empty vector negative control was included. Bars correspond to the mean of relative light units (RLU) ± SD of two independent experiments from four. (B) Enhancer activity of the luciferase reporter plasmid containing \textit{ITGAE} promoter and enhancer. The 293-T cells were transfected with the reporter plasmid containing both \textit{ITGAE} promoter and enhancer sequences, either unmutated or mutated for Smad binding sites, and then cultured in medium or in the presence of TGF-\(\beta\)-1. (C) Role of Smad in \textit{ITGAE} enhancer activity. The 293-T cells, pretreated with the TGF-\(\beta\)-1 kinase inhibitor SB, were transfected with the reporter plasmid containing both the \textit{ITGAE} promoter and enhancer and then cultured in medium or in the presence of TGF-\(\beta\)-1. (D) Role of NFAT-1 in \textit{ITGAE} enhancer activity. Luciferase assay was performed in 293-T cells transfected with the reporter plasmid containing both the \textit{ITGAE} promoter and enhancer and then transduced with a NFAT-1 plasmid in the absence or presence of PMA plus ionomycin to induce translocation of the transcription factor into the nucleus. *\(p < 0.05\), **\(p < 0.01\).
References


Supplementary figure 3: A. Schematic localization of the ITGAE gene promoter and enhancer. Partial ITGAE gene organization in introns/exons is shown. The ITGAE gene, of approximately 86.62 kb, consists of 31 exons and 30 introns. The potential promoter and enhancer sequences of the ITGAE gene were predicted by Genomatix software (http://www.genomatix.de). The potential promoter sequence is located just before exon 1 and ends in this exon. The potential enhancer sequence is located in intron 1 of the gene. B. The promoter sequence includes 901 bp and is a proximal region of the human ITGAE gene. Numbers shown refer to nucleotide positions in the ENSEMBL database entry accession number (ENST00000263087). The transcription start site is indicated in bold and underlined. Binding sites of Smad and NFAT proteins are in italic and underlined lower case letters. C. The enhancer sequence includes 901 pb and is located in intron 1 of the ITGAE gene. Binding sites of Smad and NFAT proteins are in italic and underlined. Numbers refer to nucleotide positions in the ENSEMBL database.