Table I. *Induction of IL-10 in T Cells and Macrophages by TGF-β1–expressing Constructs*

Cell line[a]	Plasmid/retrovirus	TGF-β 1 (pg/ml)[b]		IL-10 (pg/ml)	
		mock	TGF-β	mock	TGF-β
T cell					
OVA-3 T cell clone	regional LN	<30	327	<25	1,620
EL-4 (anti-CD3/anti-CD28)	thymus	184	353	<25	<25
Macrophage					
RAW264 (LPS 0.5 μg)	Mθ	214	924	181	930
MH-S (LPS 0.5 μg)	alveolar Mθ	119	636	<25	161
Epithelial					
CMT93	colon epithelial	440	1,270	<25	<25
C10	lung epithelial	<30	468	44	<25
E10	lung epithelial	<30	1,028	<25	<25
LA-4	lung epithelial Ca	<30	128	<25	<25
KLN205	lung squamous	88	552	48	<25
Fibroblast					
MLg	lung fibroblast	<30	268	<25	<25
3T3	embryo fibroblast	128	1,430	<25	<25

The value obtained for each cell line is the mean value from three independent experiments.
[a]Mouse cell lines were transfected with pCI-TGF-β1 plasmid using TransfectAMINE 2000 or by electroporation (EL-4). The OVA-3 T cell clone was infected with a retrovirus of pBMN-TGF-β1-GFP.
[b]TGF-β1 ELISA was performed after acid treatment.

produce IL-10, similar cells infected with retro-TGF-β1 (retrovirus encoding both TGF-β1 and GFP) produced IL-10 whether or not they were GFP$^+$. On the other hand, as shown in Fig. 3 b, developing Th2 cells exhibited IL-10 production when infected with either retrovirus regardless of GFP positivity. These studies show quite clearly that Th1 cells produce IL-10 either when they produce TGF-β1 themselves or are exposed to TGF-β1 produced by other cells.

In complementary studies, we measured the secretion of IL-10 by naive cell populations infected with retro-mock or retro-TGF-β1 and then subjected them to two rounds of stimulation under either Th1 and Th2 conditions. As shown in Fig. 3 c, in this case, cells stimulated under Th1 conditions again required infection with retro-TGF-β1 to produce IL-10 and cells stimulated under Th2 conditions produced (even higher levels of) IL-10 when infected with either retro-TGF-β1 or retro-mock. In addition, IL-10 production was increased in both Th1 and Th2 cells after the second round of stimulation. Finally, we noted that Th1 cells infected with retro-mock could produce IL-10 with repeated addition of rTGF-β1 to the culture, but only after the second round of stimulation. These studies show that repeated stimulation of Th1 cells in the presence of TGF-β1 (from retro-TGF-β1 or soluble rTGF-β1) leads to Th1 cells capable of IL-10 production. In addition, they suggest that exogenous TGF-β1 is a less efficient means of inducing IL-10 than endogenous TGF-β1. Parenthetically, as shown in Fig. 3 d, repeated stimulation of Th1 cells exposed to TGF-β1 become dual producers of IFN-γ and IL-10.

The Molecular Basis of TGF-β1 Induction of IL-10. On the assumption that TGF-β1 induction of IL-10 observed above could be related to TGF-β1 signaling via Smad activation of the IL-10 promoter, we searched the murine IL-10 promoter region for an SBE (17, 18, 23, 24). Indeed, we found an "SBE variant" site GTCCAGAC at −901 that closely resembled the consensus SBE, GTCTAGAC, and contained overlapping CAGA sequences (termed a CAGA box; reference 25). In additional studies of whether this sequence actually binds to Smad proteins, we performed an EMSA using nuclear extracts of TGF-β1–stimulated Jurkat T cells and radiolabeled oligo with the SBE variant sequence. As shown in Fig. 4 a, we found that the nuclear extracts contained proteins that bound to the variant sequence and that such binding was blocked by an unlabeled competitor sequence, but not by a mutated variant sequence. Furthermore, we showed that the addition of anti-Smad4 to the mixture containing the extract plus variant sequence causes a supershift. Thus, activated Smad4 does in fact bind to an SBE variant sequence in the IL-10 promoter.

To assess whether the IL-10 promoter not only binds Smad but also responds to Smad signaling, we constructed self-inactivating retroviral reporter vectors that express luciferase under the control of the IL-10 promoter rather than the retroviral promoter (19, 26). These included a vector containing an IL-10 promoter with a mutated SBE variant. The retroviruses also expressed GFP, which al-

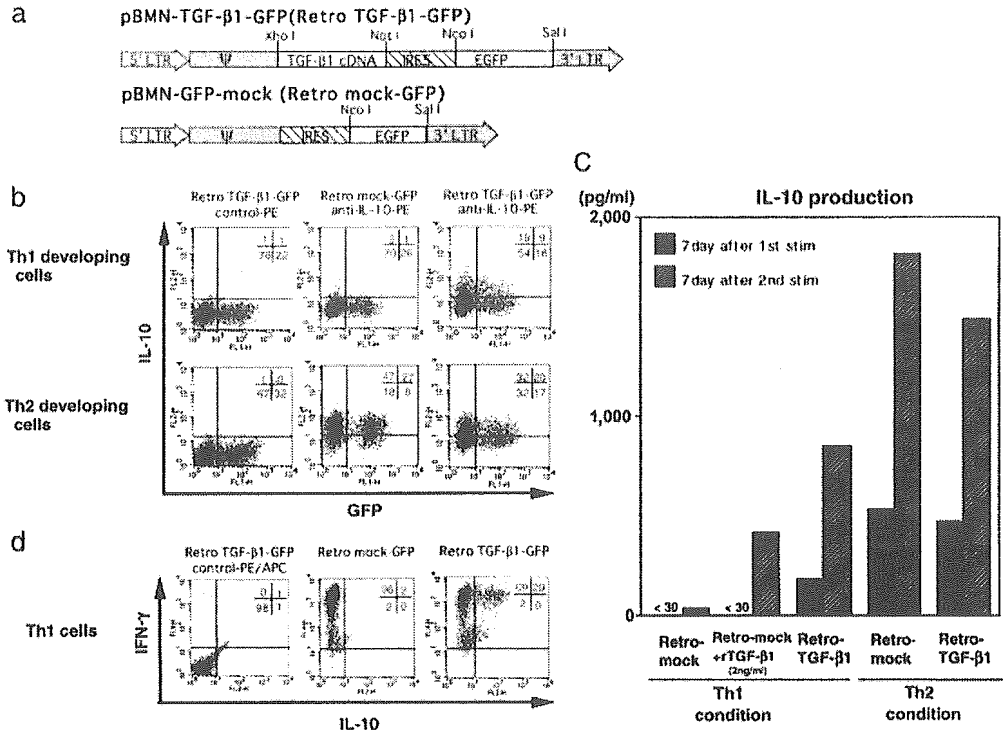

Figure 3. Production of IL-10 by Th1 cells infected with a TGF-β1–expressing retrovirus. (a) Schematic map of the retroviruses used. (b) Intracellular IL-10 staining of developing Th1 and Th2 cells on day 6 after stimulation with αCD3/CD28 with retroviral infection by retro-TGF-β1-GFP or retro-mock-GFP. IL-10 is produced by both GFP^- and GFP^+ Th1 developing cells. Th2 cells produce IL-10 in the absence of retro-TGF-β1-GFP infection. (c) Secretion of IL-10 by $CD4^+$ T cells infected with retro-TGF-β1-GFP or retro-mock-GFP under Th1 or Th2 priming conditions. IL-10 production by cells infected with retro-mock-GFP under Th1 condition with the addition of rTGF-β1 was also studied. Retro-TGF-β1–infected Th1 and Th2 cells produced more amounts of IL-10 after the second stimulation, however, although Th2 cells produce IL-10 with the infection of retro-mock, Th1 cells only do so with the addition of rTGF-β1 or infection of retro-TGF-β1. (d) Intracellular staining of IL-10 and IFN-γ of Th1 cells after the third round stimulation with αCD3/CD28.

lowed assessment of infection efficiency of the retrovirus and normalization. We then coinfected polarized Th1 and Th2 cells with these retroviruses as well as with retroviruses expressing either pBMN-TGF-β1 or pBMN-mock (in which the GFP cassette has been deleted) and monitored TGF-β1 production by the coinfected cells by ELISA. As shown in Fig. 4 b, in Th1 cells, the wild-type IL-10 promoter gene generated a strong luciferase signal in cells ex-

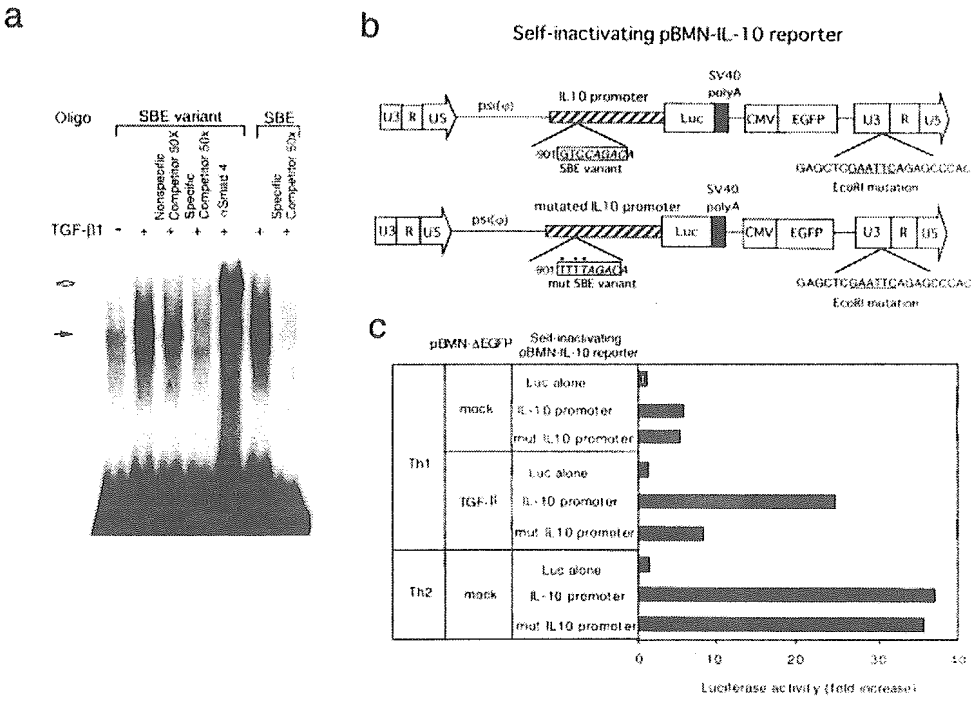

Figure 4. TGF-β1 activates IL-10 promoter transcription through Smad4 in Th1 cells. (a) EMSA shows that TGF-β1–induced nuclear extracts exhibited binding activity to an SBE variant oligo as well as native SBE oligo. Anti-Smad4 antibody causes supershift of the complex with the SBE variant. (b) Schematic map of self-inactivating pBMN-IL-10 luciferase reporter genes. (c) Luciferase activity of IL-10 promoter was assayed by infection with self-inactivating retroviruses. Retrovirally expressed TGF-β1 induces IL-10 promoter activity in developing Th1 cells signaling through SBE variant sequence, but TGF-β1 is not required for activation of IL-10 promoter in developing Th2 cells.

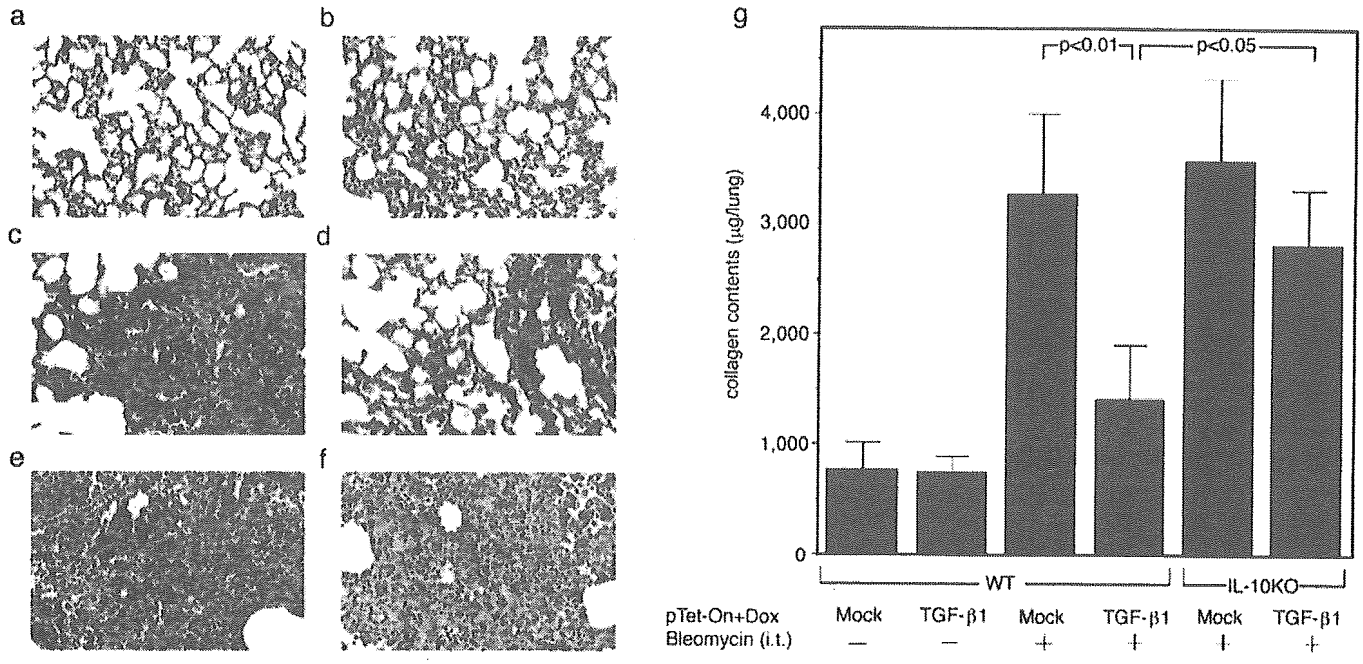

Figure 5. Amelioration of bleomycin-induced lung fibrosis by intranasal administration of pTet-On-TGF-β1. (a–f) Masson's trichrome stain of lung sections from bleomycin-treated and control mice including B6 wild-type (a–d) and IL-10 KO (e and f). pTet-On-mock and Dox-treated mouse lungs (a, c, and e) and pTet-On-TGF-β1 and Dox-treated mouse lungs (b, d, and f) without (a and b) or with bleomycin induction (c–f). Representative pictures are shown. ×400. (g) Collagen content (n = 9 in each group) in the bleomycin-induced fibrotic lungs evaluated by Sircol dye assay. Administration of pTet-On-TGF-β effectively reduces fibrotic changes in both assays.

pressing TGF-β1, whereas only a basal luciferase signal was seen in cells with a mutated promoter. In contrast, in Th2 cells both the wild-type and mutated promoters were activated even in the absence of TGF-β1, indicating that IL-10 production is independent of TGF-β1-Smad signaling and the transcriptional activation of IL-10 in Th2 cells does not depend on the SBE variant sequence. These data provide strong support for the view that TGF-β1 induces IL-10 via transcriptional activation of the IL-10 promoter by activated Smad protein(s).

IL-10 Induced by Intranasal pTet-On-TGF-β1 Ameliorates Bleomycin-induced Lung Fibrosis. The above evidence that TGF-β1 production by T cells is associated with IL-10 induction whereas such production in other non–T cells is not, implied that the IL-10 production has effects on TGF-β function during lymphoid cell interactions. We investigated this possibility in relation to the induction of fibrosis, a notorious side effect of TGF-β1 secretion (27–29). In particular, we examined the effect of intranasal administration of pTet-On-TGF-β1 on bleomycin-induced fibrosis in the lung, as quantified by Masson trichrome staining and by collagen content. In control studies shown in Fig. 5, a and b, we established that intranasal administration of either pTet-mock or pTet-On-TGF-β1 did not induce fibrotic changes. Then, as shown in Fig. 5, c and d, we established that although bleomycin induced severe fibrosis when accompanied by the administration of pTet-mock, such fibrosis was significantly decreased when accompanied by the administration of pTet-On-TGF-β1 plus Dox. Thus, rather than itself inducing fibrosis, pTet-On-TGF-β1 inhibited fibrosis induced by bleomycin. Finally, as shown in Fig. 5, e and f, the same study performed in IL-10–deficient mice showed that in the absence of IL-10 induction, the protective effect of the pTet-On-TGF-β1 plasmid was lost and in this case bleomycin did induce fibrosis in mice administered pTet-On TGF-β1 plus Dox. As shown in Fig. 5 g, confirmation of this result was obtained by measurement of the collagen content of the lungs studied under the various conditions outlined above. This provides strong evidence that the ability of pTet-On-TGF-β1 to inhibit bleomycin-induced fibrosis is in fact due to its ability to induce IL-10.

Discussion

In these studies, using a regulatable TGF-β1–expressing plasmid (termed pTet-On-TGF-β1), we show that in vivo TGF-β1 secretion rapidly induces IL-10 secretion and that such induction occurs in Th1 cells via Smad4 binding to and activation of the IL-10 promoter. The close association of these two major "regulatory" cytokines thus revealed mirrors their association during suppressor function occurring in association with Th1-mediated inflammation (30). Powrie et al. (31) for instance, has shown that in the SCID-transfer model of colitis, the regulatory effect of the subpopulation of cells in the memory CD45RB^{low} cells is abrogated by administration of anti–TGF-β1 or anti–IL-10R and that such cells either produce or induce TGF-β1 and/or IL-10 (8, 9). In addition, we have shown that the regulation of TNBS colitis by feeding TNP-substituted proteins

requires both TGF-β1 and IL-10 (7). Finally, we and others have also shown that both "self-MHC-reactive" and $CD4^+$ $CD25^+$ regulatory T cells produce both TGF-β1 and IL-10 upon appropriate stimulation (32–34).

The fact that TGF-β induces IL-10 as shown here whereas IL-10 does not induce TGF-β1 strongly suggests that IL-10 facilitates TGF-β1 regulatory function and not the other way around (4, 7). One way such facilitation could occur is through IL-10 enhancement of TGF-β1 signaling. This possibility is supported by Cottrez and Groux (35) who showed that IL-10 maintains TGF-β1RII expression on activated cells that would otherwise down-regulate this receptor. In addition, it is known that Th1 cytokines such as IFN-γ and TNF-α up-regulate Smad7, a cytosolic intermediate that inhibits TGF-β1 signaling via other Smads (36, 37) and it is thus possible (but not yet proven) that IL-10 inhibits such Smad7 up-regulation.

Another way that IL-10 could facilitate TGF-β1 regulatory activity is via an ability to sustain TGF-β1 secretion or allow the expansion of TGF-β1–producing cells. Support for this possibility comes from our recent observation that TGF-β1–mediated regulatory function induced by feeding antigen is not only abrogated by anti–TGF-β1 administration, but also by anti–IL-10 administration and that in fact no TGF-β1 production occurs in such fed mice given anti–IL-10 (7). Further work showed that the effect of anti–IL-10 does not occur during the induction of TGF-β1–producing cells (in the absence of Th1 cytokines), but rather during the expansion of these cells (in the presence of Th1 cytokines) when they might be expected to undergo antigen-induced proliferation. These findings suggest that either nascent TGF-β1–producing regulatory cells undergo a turn-off of TGF-β1 production or fail to expand in the face of Th1 cytokine production. Thus, it is reasonable to suggest that IL-10 sustains TGF-β1 secretion directly by counteracting the negative effect of Th1 cytokines on TGF-β1 production or indirectly by down-regulating Th1 cytokine secretion. Both of these possibilities would explain the observation that mice expressing large amount of IL-10 in epithelial cells as a result of an epithelial cell–specific IL-10 transgene exhibit an increase in TGF-β1 expression in the underlying LP (38). In addition, it accords with the observation that T cell TGF-β1 secretion is not seen during the Th1 response observed when TNBS colitis is induced in SJL/J mice but is seen during the Th2 response observed in the oxazolone colitis occurring in the same strain (39). Taken together, these considerations strongly suggest that TGF-β1 induction of IL-10 fulfills an important requirement of regulatory T cell activity.

Although TGF-β1 and IL-10 as discussed above might be interdependent during a regulatory response, it is possible that their cosecretion also occurs because these cytokines regulate different aspects of an inflammatory response and are thus synergistic in their activity. This is supported by the fact that in Th1-driven inflammation, although TGF-β1 regulates Th1 cells via its ability to down-regulate T-bet and the IL-12Rβ2 chain (4, 40), IL-10 regulates via down-regulation of IL-12 and TNF-α secretion (4, 41, 42). Thus, the regulatory activity of TGF-β1 and IL-10 acting together is greater than either of these cytokines acting alone.

An important and interesting aspect of TGF-β1 induction of IL-10 relates to our observation that such induction affects Th1 cells rather than Th2 cells (which exhibit IL-10 secretion in the absence of TGF-β1 and which are not induced to produce additional IL-10 by the presence of TGF-β). In a previous study by Blokzijl et al. (43) using the HT-2 cell line (a cell line that produces IL-10 but not IL-4 or IL-5), it was found that TGF-β1–induced Smad3 interacts directly with GATA-3 and the complex formed acts cooperatively to up-regulate IL-10 expression (by ~1.8-fold) in a Smad3- and GATA-3–dependent fashion. This result is somewhat at odds with our data showing that IL-10 production by freshly prepared Th2 cells is not enhanced by TGF-β1 and is best explained by differences in the cells being studied. Blokzijl et al. (43) used an established cell line of uncertain lineage, whereas we used a freshly stimulated Th2 cell line. Finally, we observed that $CD4^+$ T cells exposed to TGF-β1 during induction of T cell differentiation produced much lower amounts of IL-10 when stimulated under neutral priming conditions than under Th1 priming conditions (unpublished data). This indicates that activation of the IL-10 promoter is not solely dependent on TGF-β1, but also involves other unknown factors induced during the differentiation of Th1 cells.

In addition to its role as a suppressor of inflammation, TGF-β1 has a known capacity to induce collagen and fibronectin synthesis and thus to promote fibrosis. In contrast, IL-10 has been shown to inhibit collagen synthesis and prevent fibrosis (44, 45), not only that caused by TGF-β1, but also by TNF-α (46, 47) and IL-13 (14). On this basis we explored the possibility that the fibrogenic properties of TGF-β1 can be brought under control by IL-10 cosecretion. In these studies we showed that intranasal administration of pTet-On-TGF-β1 did not induce fibrosis of the lung, the organ most exposed to TGF-β1 as a result of intranasal plasmid administration. In contrast, such administration prevented bleomycin-induced pulmonary fibrosis, a fibrosis that is at least in part mediated by endogenous TGF-β production (27, 48). That this seemingly paradoxical effect was due to the cosecretion of IL-10 was shown by the fact that TGF-β1 plasmid administration did not prevent bleomycin-induced fibrosis in IL-10–deficient mice (in whom IL-10 cosecretion cannot occur). These findings strongly suggest that the IL-10 antifibrotic effect is dominant over the TGF-β1 profibrotic effect, even in the face of endogenous (nonlymphoid origin) TGF-β production and that IL-10 protects the animal from TGF-β1–mediated fibrosis. By extension, they suggest that induction of TGF-β1–secreting cells by administration of a plasmid such as pTet-On-TGF-β1 that leads to superphysiologic IL-10 secretion may have an unexpected antifibrotic effect that bodes well for its use as a therapy for autoimmune diseases.

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TGF-β1 Plays an Important Role in the Mechanism of $CD4^+CD25^+$ Regulatory T Cell Activity in Both Humans and Mice

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In previous studies, we have shown that murine $CD4^+CD25^+$ regulatory T cells produce high levels of TGF-β1 in a cell surface and/or secreted form, and blockade of such TGF-β1 by anti-TGF-β curtails the ability of these cells to suppress $CD25^-$ T cell proliferation and B cell Ig production in in vitro suppressor assays. In further support for the role of TGF-β1 in suppression by $CD4^+CD25^+$ T cells, we show in this study that another TGF-β1-blocking molecule, recombinant latency-associated peptide of TGF-β1 (rLAP), also reverses suppression by mouse $CD4^+CD25^+$ T cells as well as their human counterparts, $CD4^+CD25^{high}$ T cells. In addition, we show that $CD25^-$ T cells exposed to $CD4^+CD25^+$ T cells in vitro manifest activation of Smad-2 and induction of CD103, the latter a TGF-β-inducible surface integrin. In further studies, we show that while $CD4^+CD25^+$ T cells from TGF-β1-deficient mice can suppress $CD25^-$ T cell proliferation in vitro, these cells do not protect recipient mice from colitis in the SCID transfer model in vivo, and, in addition, $CD4^+LAP^+$, but not $CD4^+LAP^-$ T cells from normal mice protect recipient mice from colitis in this model. Together, these studies demonstrate that TGF-β1 produced by $CD4^+CD25^+$ T cells is involved in the suppressor activity of these cells, particularly in their ability to regulate intestinal inflammation. *The Journal of Immunology,* **2004, 172: 834–842.**

There is accumulating evidence that the generation of regulatory T cells is necessary to maintain self-tolerance and prevent the onset of autoimmune disease. One such regulatory cell is a $CD4^+CD25^+$ T cell population that comprises 5–10% of the total peripheral $CD4^+$ T cells in normal adult mice and expresses surface CD25 (IL-2Rα) before activation (1). In certain strains of mice, elimination of this $CD4^+CD25^+$ regulatory T cell population by early thymectomy results in various autoimmune diseases, which, in turn, are preventable by its restoration by adaptive transfer (2). Similarly, transfer of $CD25^+$-depleted splenocytes into nude mice results in various autoimmune diseases that are prevented by cotransfer of $CD4^+CD25^+$ T cells (2). In more recent studies, $CD4^+CD25^+$ regulatory T cells very similar to the murine regulatory cell have also been shown to be present in humans (3–7).

In a series of in vitro studies, it has been shown that murine $CD4^+CD25^+$ T cells are relatively unresponsive to TCR-mediated stimulation and suppress T cell proliferation of cocultured $CD4^+CD25^-$ T cells (8, 9). In addition, it has been shown that such suppression acted via a cell-cell contact mechanism rather than through the secretion of suppressor cytokines such as TGF-β1 or IL-10 (8, 9). However, in a previous report, we have shown that $CD4^+CD25^+$ T cells are in fact capable of producing TGF-β1 either as a secreted or a cell surface protein, depending on the type and/or strength of TCR stimulation (10). Thus, when $CD4^+CD25^+$ T cells are stimulated with plate-bound anti-CD3 (under cross-linking conditions), they both secrete TGF-β1 and also express cell surface-bound TGF-β1; in contrast, when the same cells are stimulated by Ag plus APCs, they mainly express cell surface TGF-β1 and secrete little, if any, of this cytokine. On this basis, it seems likely that under the stimulation conditions in which $CD4^+CD25^+$ T cell suppressor function is generally measured in vitro $CD4^+CD25^+$ T cells would express cell surface TGF-β1, but not secrete TGF-β1. Thus, we postulated that in vitro $CD4^+CD25^+$ T cell suppressor function is mediated by contact with cell surface TGF-β1. In studies to support this concept, we showed that high concentrations of anti-TGF-β1 inhibit the suppressor function of $CD4^+CD25^+$ T cells in in vitro coculture assays (10). In addition, other investigators reported that suppressor function of both mouse lymphocytes and human $CD4^+CD25^+$ thymocytes are at least partially reversed by neutralization of TGF-β (11, 12).

Despite these findings, the involvement of TGF-β1 in $CD4^+CD25^+$ T cell immunoregulatory function remains controversial because in previous studies neutralization of TGF-β by anti-TGF-β failed to reverse $CD4^+CD25^+$ suppressor function in vitro (8, 9). In addition, it has recently been shown that $CD4^+CD25^+$ T cells from normal mice could suppress the proliferation of cocultured $CD4^+CD25^-$ T cells from TGF-βRII dominant-negative transgenic mice and Smad-3-deficient mice and, in addition, $CD4^+CD25^+$ T cells from TGF-β1-deficient mice could suppress cocultured $CD25^-$ T cells from normal mice, suggesting that TGF-β1 is not necessary for suppressor function in in vitro coculture system (13). These contradictory studies did not, however, lead to an alternative suppressor mechanism, and led us to again examine the role of TGF-β1 in $CD4^+CD25^+$ T cell regulatory function.

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In the current series of studies, we showed first that in vitro suppressor function of both murine and human $CD4^+CD25^+$ regulatory T cells is inhibited by another TGF-β1-blocking agent, recombinant latency-associated peptide of TGF-β1 (rLAP),[2] in a dose-dependent fashion. We then showed that $CD4^+CD25^+$ T cells induce the expression of CD103 (α_E integrin) and activated Smad-2 in cocultured $CD25^-$ T cells, thus providing strong evidence that cell surface TGF-β1 acts as a signaling molecule in the coculture situation. Moving then to in vivo studies, we showed that while $CD4^+CD25^+$ T cells from TGF-β1-deficient mice do exhibit suppressor activity in vitro, such cells failed to prevent the development of colitis in the SCID transfer colitis model, indicating that cells from these mice do exhibit impaired suppressor function. In addition, these data show that $CD4^+CD25^+$ T cells exert suppressor activity in vivo by production of TGF-β1 rather than by inducing other cells to do so. Finally, we showed that $CD4^+LAP^+$ T cells, but not $CD4^+LAP^-$ T cells, prevent colitis in the transfer model, showing that cell surface LAP could serve as a marker for regulatory T cells.

Materials and Methods

Mice

Specific pathogen-free, 8-wk-old female BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). TGF-β1-deficient mice (14) were a kind gift from J. Letterio (National Cancer Institute, National Institutes of Health, Bethesda, MD). Splenocytes were purified from TGF-$\beta1^{+/+}$, TGF-$\beta1^{+/-}$, and TGF-$\beta1^{-/-}$ mice at the age of 1–2 wk, as TGF-$\beta1^{-/-}$ mice die before age 3 wk due to systemic autoimmune inflammation. C57BL/6 Rag-2 knockout mice and C.B-17 SCID mice were purchased from Taconic Farms (Germantown, NY). Thy-1.1^+ congenic BALB/c mice were a kind gift from J. Sprent (The Scripps Research Institute, La Jolla, CA). Animal use adhered to National Institutes of Health Animal Care Guidelines.

Reagents

Anti-mouse CD3 (145-2C11), anti-mouse CD28 (37.51), anti-mouse CTLA-4 (UC10-4F10-11), biotin-conjugated anti-mouse CD25 (7D4), FITC-conjugated anti-mouse CD4 (GK1.5 and RM4-5), PE-conjugated anti-mouse CD45RB (16A), biotin-conjugated anti-mouse IgG1, PE-conjugated streptavidin, CyChrome-conjugated streptavidin, FITC-conjugated anti-human CD4 (RPA-T4), PE-conjugated anti-human CD25 (M-A251), and 7-amino actinomycin D were purchased from BD PharMingen (San Diego, CA). Mouse anti-LAP (TGF-β1) mAb (clone 27232.11, mouse IgG1) was a kind gift from R&D Systems (Minneapolis, MN). rLAP, anti-TGF-β mAb (clone 1D11), biotin-conjugated goat anti-LAP Ab, and biotin-conjugated anti-LAP mAb (clone 27240) were purchased from R&D Systems. Streptavidin microbeads were purchased from Miltenyi Biotec (Auburn, CA). Anti-human CD3 mAb (OKT3) was purchased from Ortho Biotech (Raritan, NJ). Anti-phospho-Smad-2 (Ser^{465}, Ser^{467}) Ab was purchased from Upstate Biotechnology (Waltham, MA). Anti-Smad-2 mAb was purchased from Zymed Laboratories (South San Francisco, CA). Human rIL-2 was purchased from Life Technologies (Rockville, MD).

Cell purification

Mouse $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified, as described previously (10). Human $CD4^+CD25^+$ regulatory T cells were isolated from leukapheresis packs obtained from healthy donors after informed consent was given. In brief, PBMC isolated by Ficoll density-gradient centrifugation were passed over $CD4^+$ T cell enrichment columns (R&D Systems) to obtain $CD4^+$ T cells. The latter were then stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 and sorted by FACSVantage SE II (BD Biosciences, San Jose, CA) to obtain highly purified $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cell populations. The purity of the $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells was >85 and >95%, respectively.

Flow cytometry

Splenocytes from TGF-$\beta1^{+/+}$, TGF-$\beta1^{+/-}$, and TGF-$\beta1^{-/-}$ mice were stimulated with soluble anti-CD3 (10 μg/ml) and rIL-2 (50 U/ml) for 3 days. Cells were stained with anti-LAP mAb (clone 27232.11, mouse IgG1); washed, biotin-conjugated anti-mouse IgG1; and washed. Cells were then incubated with FITC-conjugated anti-CD4 and PE-conjugated streptavidin and washed. For exclusion of dead cells, cells were stained with 7-amino actinomycin D before analysis.

Purified human $CD4^+CD25^{high}$ and $CD25^-$ T cells were stimulated with anti-CD3 (1 μg/ml), irradiated APC, and rIL-2 (50 U/ml) for 5 days. Cells were stained with biotin-conjugated goat anti-LAP Ab, washed, and stained with FITC-conjugated anti-CD4 and PE-streptavidin. Dead cells were excluded by staining with 7-amino actinomycin D.

The analysis was performed on FACScan (BD Biosciences) flow cytometer with CellQuest II software (BD Biosciences). In some experiments, 7-amino actinomycin D staining was necessary to exclude nonspecific high background on dead cells.

Cell culture

Cell cultures of mouse $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells for in vitro suppression assays using [^{3}H]thymidine incorporation readouts were performed, as described previously (10); for this purpose, indicated cell numbers of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were placed in wells of 96-well plates. In some studies, rLAP was added to the cell cultures at various indicated concentrations.

For human $CD4^+$ T cells, 2.5×10^4 $CD4^+CD25^-$ T cells with or without the same number of $CD4^+CD25^{high}$ T cells were cultured in 96-well plates and stimulated with 1 μg/ml anti-CD3 (OKT3) and 5×10^4 irradiated syngeneic PBMC for 4 days at 37°C and pulsed with 1 μCi of [^{3}H]thymidine for the last 6 h of culture. RPMI 1640 supplemented with 3% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES (pH 7.0) was used as culture medium for human cells.

FACS analysis of CD103 expression on $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from Thy-1.1^+ congenic BALB/c mice. In parallel, $CD4^+CD25^+$ T cells were purified from normal (Thy-1.2^+) BALB/c mice. Thy-1.1^+ $CD25^-$ T cells were stimulated in the presence or absence of Thy-1.2^+ $CD25^+$ T cells with soluble anti-CD3 (10 μg/ml), irradiated APC, and rIL-2 (50 U/ml) for 5 days. For positive control, Thy-1.1^+ $CD25^-$ T cells were stimulated in the presence of rTGF-β1 (5 ng/ml). In parallel, Thy-1.1^+ $CD25^+$ T cells were stimulated for comparison. After cultivation, cells were stained with FITC-conjugated anti-Thy-1.1, PE-conjugated anti-CD103, or isotype-matched control Ab, and CyChrome-conjugated anti-CD4. Expression of CD103 on Thy-1.1^+ $CD4^+$ cells was analyzed by FACScan (BD Biosciences) flow cytometer.

Detection of Smad-2 phosphorylation

To detect induction of Smad-2 phosphorylation in $CD4^+CD25^-$ T cells cocultured with $CD4^+CD25^+$ T cells, murine $CD4^+$ T cells were purified by CD4 purification column (R&D Systems), and then separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells using PE-conjugated anti-CD25 and anti-PE microbeads ($CD4^+CD25^+$ regulatory T cell isolation kit; Miltenyi Biotec). $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were then stimulated with soluble anti-CD3 (10 μg/ml) plus irradiated adherent APCs (non-CD4 fraction) for 3 h. The nonadherent $CD4^+$ T cells were then collected, washed three times, and mixed with freshly isolated resting $CD4^+CD25^-$ T cells. The cell mixtures thus obtained were added back to the washed original wells, briefly spun down to ensure cell-cell contact, and incubated at 37°C for 90 min. Negative controls consisted of $CD4^+CD25^-$ T cells incubated with irradiated APCs in the absence of $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells. After incubation, $CD4^+CD25^-$ T cells were negatively repurified with biotin non-CD4 T cell Ab mixture, followed by the treatment of PE anti-mouse CD25, and solubilized in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 7.5, 2 mM EDTA, 10 mM NaF, and 1 mM Na_3VO_4 with protease inhibitors). Finally, a Western blot was performed, as described previously (10), using anti-phospho-Smad-2 Ab to detect phosphorylated Smad-2 and anti-Smad-2 mAb to detect total Smad-2 protein (on the stripped membrane).

Transfer of wild-type and TGF-β1-deficient $CD4^+CD25^+$ T cells with $CD45RB^{high}$ T cells into Rag-2 knockout recipients

Splenocytes from C57BL/6 mice were stained with FITC-conjugated anti-mouse CD4 (RM4-5) and PE-conjugated anti-mouse CD45RB, after which $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells were purified by FACS sorting. Wild-type and TGF-β1-deficient $CD4^+CD25^+$ T cells were purified, as described previously (10). $CD4^+CD45RB^{high}$ T cells (3×10^5)

[2] Abbreviation used in this paper: LAP, latency-associated peptide of TGF-β.

were injected i.p. into Rag-2-deficient mice, accompanied, in some recipients, by CD4$^+$CD45RB^{low} T cells (1.5×10^5) from normal C57BL/6 or CD4$^+$CD25$^+$ T cells (1.2×10^5) from wild-type or TGF-β1-deficient mice. After transfer, recipient mice were weighted weekly until they were euthanized for histopathologic analysis.

Transfer of LAP$^+$ and LAP$^-$CD4$^+$ T cells with CD45RB^{high} T cells into C.B-17 SCID recipients

Splenocytes from BALB/c mice were stained with FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD45RB, after which CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} T cells were purified by FACS sorting. In parallel, CD4$^+$ T cells stimulated with plate-bound anti-CD3 (10 μg/ml), soluble anti-CD28 (2 μg/ml), and rIL-2 (50 U/ml) for 3 days were collected and stained with FITC-conjugated anti-mouse CD4 (RM4-5) and anti-LAP mAb (27232.11), biotin-conjugated anti-mouse IgG1, and PE-conjugated streptavidin. CD4$^+$LAP$^+$ and CD4$^+$LAP$^-$ T cells were purified by FACS sorting. Dead cells were excluded by 7-amino actinomycin D staining. CD4$^+$CD45RB^{high} T cells (3×10^5) were injected i.p. in C.B-17 SCID mice, accompanied in some recipients by CD4$^+$CD45RB^{low} (1.5×10^5), CD4$^+$LAP$^+$ (1×10^5), or CD4$^+$LAP$^-$ (1×10^5) T cells. After transfer, recipient mice were weighted weekly until they were euthanized for histopathologic analysis.

Results

rLAP inhibits suppressor function of murine CD4$^+$CD25$^+$ regulatory T cells

To explore further the role of TGF-β in the suppressor function of murine CD4$^+$CD25$^+$ T cells in vitro, we conducted suppressor arrays in the presence and absence of rLAP, a molecule that could block TGF-β activity by capturing active TGF-β or by blocking the association of latent TGF-β with an activating molecule, such as thrombospondin-1 and $\alpha_v\beta_6$ integrin (15–17). Accordingly, we set up cultures in which CD4$^+$CD25$^-$ T cells were stimulated with soluble anti-CD3 and APC in the presence or absence of CD4$^+$CD25$^+$ T cells, as well as with various amounts of rLAP. As shown in Fig. 1, in the absence of rLAP, CD4$^+$CD25$^-$ T cells proliferated well in response to stimulation, while CD4$^+$CD25$^+$ T cells proliferated poorly. In addition, coculture of CD4$^+$CD25$^-$ T cells with CD4$^+$CD25$^+$ T cells resulted in profound suppression of CD4$^+$CD25$^-$ T cell proliferation. However, in the presence of rLAP, proliferation of CD4$^+$CD25$^-$ T cells was further increased and, more importantly, suppression by CD4$^+$CD25$^+$ T cells was significantly reversed in a dose-dependent fashion. Of note, reversal of suppression was obtained with much lower concentrations of rLAP than reversal by anti-TGF-β (10). These results thus lend additional credence to the idea that such reversal involves a TGF-β1-mediated mechanism. The fact that CD4$^+$ CD25$^-$ T cell proliferation increases in the presence of rLAP may be due to the fact that, as shown previously, CD4$^+$CD25$^-$ T cells do produce some TGF-β when stimulated with a variety of conditions (10) or are being suppressed by TGF-β known to be present in the FCS in the culture medium. Such augmentation of proliferation was previously seen when anti-TGF-β was added to the cultures (10). Finally, it should be noted that while rLAP caused substantial inhibition of suppression by CD4$^+$CD25$^+$ T cells, such inhibition was not complete. Several possible explanations for this can be put forward, but we think the most cogent is that CD4$^+$CD25$^+$ T cells inhibit CD25$^-$ T cells via a second mechanism as well under certain stimulation conditions (see discussion below).

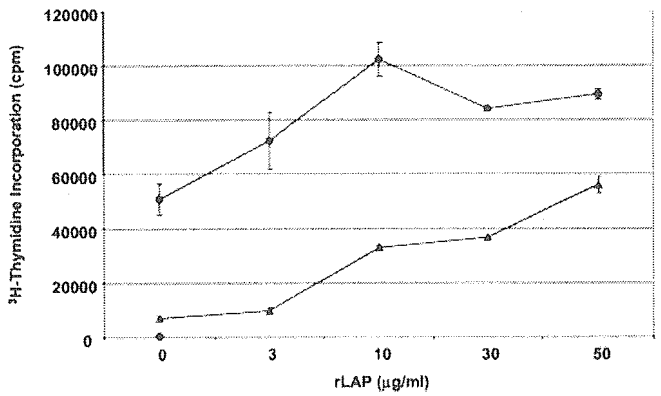

FIGURE 1. rLAP reverses suppressor function of murine CD4$^+$CD25$^+$ T cells. Mouse CD4$^+$CD25$^-$ T cells (4×10^4/well) were stimulated with 10 μg/ml soluble anti-CD3 and irradiated APC in the presence or absence of CD4$^+$CD25$^+$ T cells (3×10^4/well). Various concentrations of rLAP were added to the cell culture. ●, CD4$^+$CD25$^-$ T cell; ▲, CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cell; ◆, CD4$^+$CD25$^+$ T cell. Representative results of three independent experiments are shown.

Expression of cell surface-bound LAP on human CD4$^+$CD25$^+$ T cells and inhibition of human CD4$^+$CD25$^+$ suppressor function by rLAP

Recently, it was reported that human CD4$^+$CD25$^+$ T cells, comprising ~5–15% of CD4$^+$ peripheral blood T cells, contain a subpopulation with suppressor function similar to that exhibited by murine CD4$^+$CD25$^+$ T cells (3–6). However, further work reported previously and corroborated in this study indicated that only CD4$^+$CD25^{high} T cells, comprising 1–2% of circulating CD4$^+$ T cells, possess suppressor capability (7). In view of these findings, we investigated expression of cell surface TGF-β1 on CD4$^+$CD25^{high} and CD4$^+$CD25$^-$ T cells. First, we isolated CD4$^+$CD25^{high} and CD4$^+$CD25$^-$ T cells from human peripheral blood by flow cytometric sorting (Fig. 2*A*). We then stimulated the cells with soluble anti-CD3, APC, and rIL-2, and after 5 days stained the cells thus activated with polyclonal goat anti-LAP Ab. As shown in Fig. 2*B*, the expression level of cell surface LAP was high on CD25^{high} T cells after stimulation, whereas only a small amount of LAP was found on CD25$^-$ T cells. Similar results were obtained with another biotin-conjugated mAb against LAP (clone 27240) (data not shown). However, using an Ab against active TGF-β1, we could detect only marginal amount of TGF-β1 on the cell surface; this contrasts with results obtained in mouse cells.

Next, we conducted studies to determine whether the suppressor function of human CD4$^+$CD25^{high} T cells can also be blocked by rLAP. We therefore set up assays of human cells in the presence or absence of rLAP, similar to those in the previously described studies of mouse cells. As shown in Fig. 3, human CD4$^+$CD25$^-$ T cells proliferated well in response to stimulation with soluble anti-CD3 and APC and human CD4$^+$CD25^{high} T cells profoundly suppressed proliferation of these cells, as reported previously (7). Also in concert with the mouse data, addition of rLAP to the cell cultures caused significant reversal of suppression in a dose-dependent fashion. These results indicate that suppressor function of human CD4$^+$CD25^{high} regulatory T cells also involves a TGF-β-dependent mechanism.

CD4$^+$CD25$^+$ regulatory T cells induce TGF-βR signaling in the target cells

To provide further evidence that cell surface TGF-β on the surface of CD4$^+$CD25$^+$ T cells is involved in the suppression mediated by these cells, we sought evidence that such TGF-β signals the CD25$^-$ target cells. In an initial series of experiments along these lines, we took advantage of the fact that CD103 expression can be induced by TGF-β1, and thus, expression of this integrin on CD4$^+$CD25$^-$ target cells should increase during coculture with CD4$^+$CD25$^+$ T cells in the presence of cell surface-bound TGF-β1. Accordingly, we cocultured Thy-1.2$^+$ CD4$^+$CD25$^+$ T cells with Thy-1.1$^+$ CD4$^+$CD25$^-$ target cells and stimulated with anti-CD3, APC, and

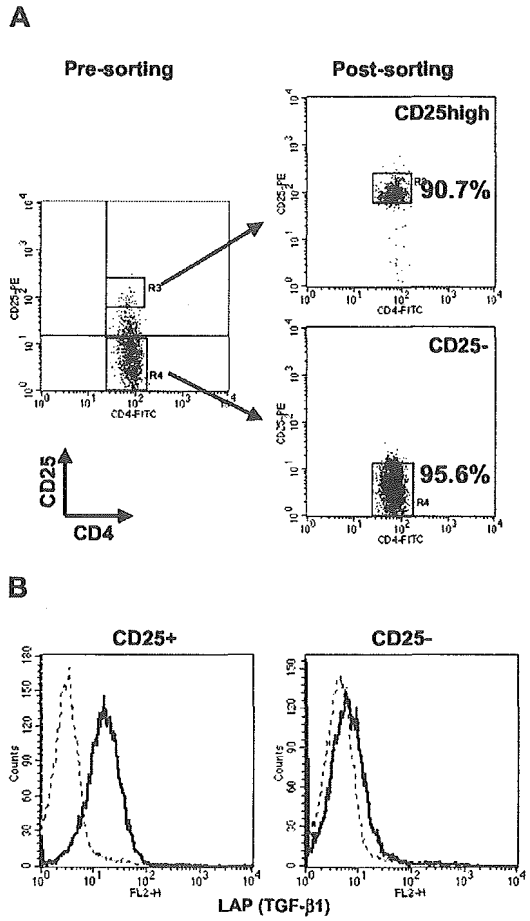

FIGURE 2. Purification of human $CD4^+CD25^{high}$ T cells and expression of LAP on activated $CD4^+CD25^{high}$ T cells. *A*, Purified human $CD4^+$ T cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25. $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were then purified by FACS sorting. *B*, Purified $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were stimulated with soluble anti-CD3 (1 μg/ml), irradiated APC, and rIL-2 (50 U/ml) for 5 days. Cells were then stained with FITC-conjugated anti-CD4, biotin-conjugated anti-LAP, and PE-conjugated streptavidin. Expression of LAP on $CD4^+$ gate is shown. Results are representative of six independent experiments.

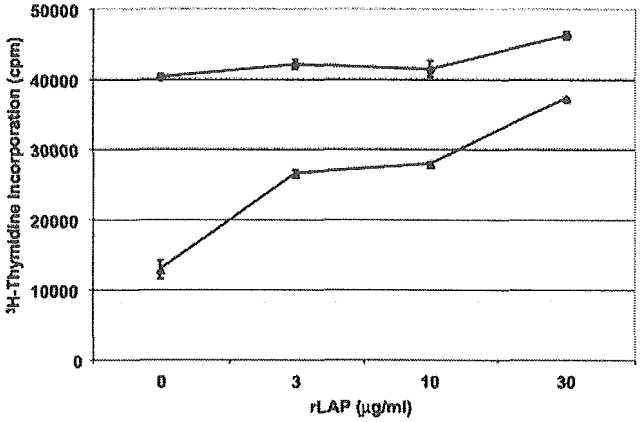

FIGURE 3. rLAP reverses suppressor function of human $CD4^+CD25^{high}$ T cells. Human $CD4^+CD25^-$ T cells were stimulated with 1 μg/ml soluble anti-CD3 and irradiated APC in the presence or absence of $CD4^+CD25^{high}$ T cells. Various concentrations of rLAP were added to the cell culture. ●, $CD4^+CD25^-$ T cell; ▲, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cell. Results are representative of three independent experiments.

rIL-2. We then stained the cells and discriminated suppressors and targets on the basis of Thy-1.1 expression. As shown in Fig. 4, cultured Thy-1.1^+ $CD4^+CD25^-$ T cells contained very few $CD103^+$ cells (~1.1%), whereas the same cells cultured in the presence of $CD4^+CD25^+$ T cells contain as many $CD103^+$ cells as the same cells cultured with rTGF-β1. As reported previously (18), $CD4^+CD25^+$ T cells contained a substantial subpopulation of $CD103^+$ cells, possibly due to autoactivation.

In a second series of experiments, we determined TGF-β1 signaling in $CD25^-$ target T cells, by measuring the appearance of activated Smad-2 in these cells following admixture with $CD4^+CD25^+$ suppressor T cells. In these studies, we mixed activated $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells with resting $CD4^+CD25^-$ T cells (target cells) and then briefly centrifuged the cell mixture to promote cell-cell contact. We then reisolated the $CD4^+CD25^-$ target cells and subjected lysates of these cells to Western blot analysis using anti-phospho-Smad-2 Ab. As shown in Fig. 5, $CD4^+CD25^-$ target cells in contact with $CD4^+CD25^+$ T cells gave rise to a distinctly stronger phosphorylated Smad-2 signal than $CD4^+CD25^-$ T cells in contact with $CD4^+CD25^-$ T cells or $CD4^+CD25^-$ T cells incubated alone. In contrast, total Smad-2 protein was similar in all cultures. Together, these studies show that TGF-β1 on the surface of $CD4^+CD25^+$ T cells signals $CD4^+CD25^-$ T cells: in one case, to induce expression of a TGF-β-dependent integrin, and, in another, to directly stimulate Smad-2.

TGF-β1 produced from $CD4^+CD25^+$ T cells is important in suppression of intestinal inflammation in vivo

The above results strongly support the idea that TGF-β1 is involved in $CD4^+CD25^+$ T cell-mediated suppression of T cell proliferation in vitro. However, the opposite conclusion was drawn from studies in which $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ mice were shown to mediate suppression in vitro (13). To further explore these results, we assessed the suppressor function of $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ mice both in vitro or in vivo. In initial in vitro studies shown in Fig. 6, we found that TGF-β1-deficient $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ could indeed suppress T cell proliferation in vitro to the same changes as $CD4^+CD25^+$ T cells from wild-type mice.

However, a very different result was obtained in in vivo studies of $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ mice. In this case, as shown in Fig. 7*A*, Rag-2-deficient recipients of $CD4^+CD45RB^{high}$ T cells derived from normal mice showed progressive weight loss that was inhibited by cotransfer of $CD4^+CD45RB^{low}$ T cells or $CD4^+CD25^+$ T cells from wild-type mice, whereas, in contrast, such weight loss was not inhibited by cotransfer of $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ mice. In addition, as shown in Figs. 7*B* and 8*A*, this pattern of weight loss corresponded to the macroscopic and microscopic changes present in the colons of the various groups. Finally, perhaps most importantly, these weight curves also corresponded with the pooled histological scores of mice in the various groups, which shows again that $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ did not protect Rag-2 knockout recipients of $CD45RB^{high}$ T cells from the development of colitis (Fig. 8*B*). It should be noted that these results cannot be explained by postulating that $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ have an intrinsic proinflammatory effect because the development of weight loss in recipients of these cells was limited to the colon and did not occur until after a long latent period, presumably due to the long time necessary for the development of colitis in the transfer model. It should also be noted that these results correlate with previous

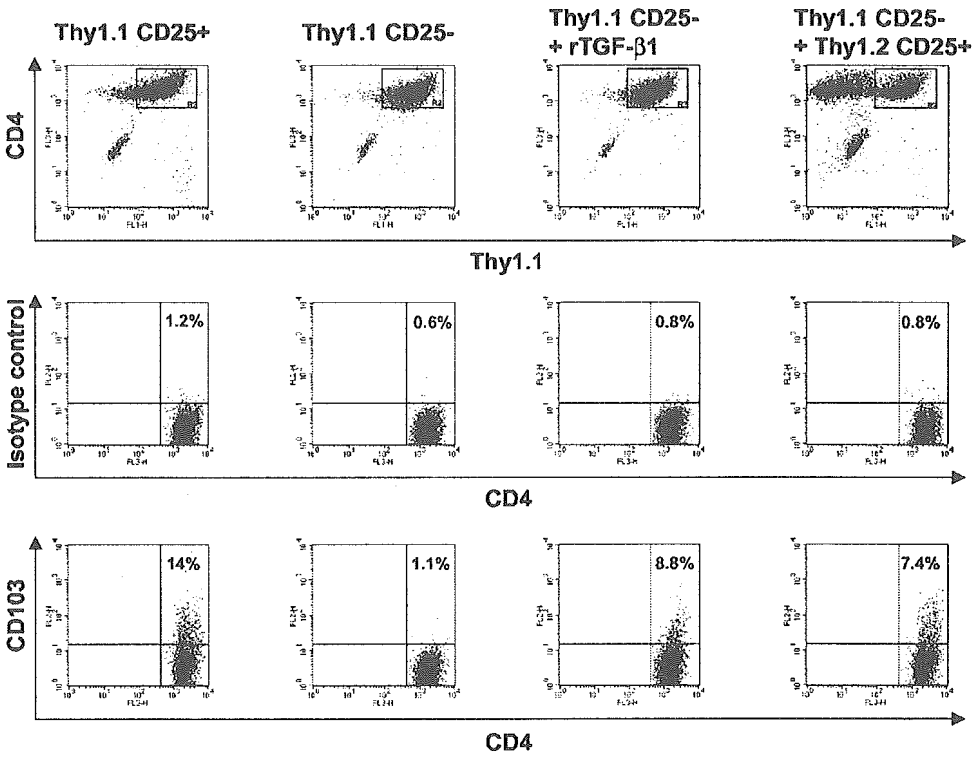

FIGURE 4. $CD4^+CD25^+$ T cells induce CD103 expression on $CD4^+CD25^-$ T cells. Thy-$1.1^+CD4^+CD25^+$ T cells (*left panels*), Thy-$1.1^+CD4^+CD25^-$ T cells in the absence (*second left panels*) and the presence (*second right panels*) of rTGF-β1 (5 ng/ml), and Thy-$1.1^+CD4^+CD25^-$ in the presence of Thy-$1.2^+CD4^+CD25^+$ T cells (*right panels*) were stimulated with soluble anti-CD3 (10 μg/ml), irradiated APC, and IL-2 (50 U/ml) for 5 days. Cells were stained with FITC-conjugated anti-Thy-1.1, PE-conjugated anti-CD103 or isotype control, and CyChrome-conjugated anti-CD4. *Top panels*, The expression of Thy-1.1 and CD4. Staining with anti-CD103 (*bottom panels*) and isotype control Ab (*middle panels*) on Thy-1.1^+CD4^+ gate is shown. These data are representative of three independent experiments.

studies showing that anti-TGF-β administration abrogates the protective effect of $CD4^+CD25^+$ T cells (19).

In vivo regulatory function of $CD4^+LAP^+$ and $CD4^+LAP^-$ T cells

In a final series of studies, we examined the capacity of TGF-β1-bearing T cells from normal mice to mediate regulatory function in vivo, using a SCID transfer model of colitis. In initial studies, we determined the percentage of spleen $CD4^+$ T cells expressing LAP on the cell surface after activation. As shown in Fig. 9*A*, 7.5% of activated $CD4^+$ T cells obtained from wild-type mice were cell surface LAP positive after staining with anti-LAP mAb. To confirm the specificity of the staining, we stained $CD4^+$ T cells obtained from TGF-$\beta1^{-/-}$ mice, and, as expected, they were negative for LAP expression. In addition, 3.5% of $CD4^+$ T cells from TGF-$\beta1^{+/-}$ heterozygous mice expressed cell surface LAP, approximately half as many cells as in wild-type mice.

In further studies, we determined the in vivo regulatory potential of LAP^+ and LAP^- $CD4^+$ T cells from normal mice. As shown in Fig. 9*B*, we purified LAP^+ and LAP^- cells from activated $CD4^+$ T cells by FACS sorting, and then transferred the sorted cells into SCID recipients along with $CD4^+CD45RB^{high}$ T cells. As shown in Fig. 10*A*, SCID recipients of $CD45RB^{high}$ T cells derived from normal mice revealed progressive weight loss that was inhibited by cotransfer of $CD4^+CD45RB^{low}$ T cells or $CD4^+LAP^+$ T cells; in contrast, it was not inhibited by cotransfer of $CD4^+LAP^-$ T cells. As shown in Fig. 10*B*, macroscopic study of colons of the recipients of $CD45RB^{high}$ T cells revealed marked swelling and shortening, which was almost completely suppressed in mice cotransferred $CD45RB^{low}$ cells; cotransfer of LAP^+ T cells also suppressed inflammation, although in this case, slight signs of edema remained. Finally, as shown in Fig. 10*B*, colons of recipients of $CD45RB^{high}$ and LAP^- T cells showed severe swelling and shortening similar to that observed in mice transferred $CD45RB^{high}$ T cell alone. Finally, as shown in Fig. 11, histologic examination and colitis scoring confirmed these results. These findings provide a clear indication that surface LAP is reflecting a

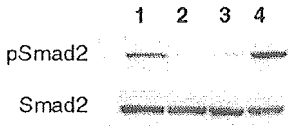

FIGURE 5. $CD4^+CD25^+$ T cells induce Smad-2 phosphorylation in $CD4^+CD25^-$ T cells. Purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were stimulated with soluble anti-CD3 (10 μg/ml) in the presence of irradiated APC. Then freshly isolated $CD4^+CD25^-$ T cells were mixed with or without activated $CD4^+CD25^+$ T cells, $CD4^+CD25^-$ T cells, or rTGF-β1, and briefly spun down and incubated for 90 min. Then $CD4^+CD25^-$ T cells were repurified by magnetic beads and lysed. Phosphorylated Smad-2 (*top panel*) and total Smad-2 protein (*bottom panel*) were detected by Western blot. $CD4^+CD25^-$ T cells were mixed with $CD4^+CD25^+$ T cells and APC (*lane 1*); mixed with $CD4^+CD25^-$ T cells and APC (*lane 2*); incubated with APC (*lane 3*); and incubated with rTGF-β1 (5 ng/ml) and APC (*lane 4*).

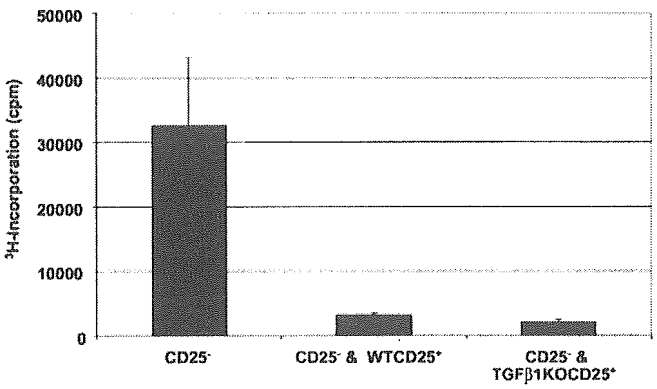

FIGURE 6. TGF-β1-deficient $CD4^+CD25^+$ T cells mediate suppression in vitro. $CD4^+CD25^-$ T cells (4×10^4/well) from wild-type mice were stimulated with 10 μg/ml of soluble anti-CD3 and irradiated APC in the presence or absence of $CD4^+CD25^+$ T cells (4×10^4/well) from wild-type or TGF-β1-null mice.

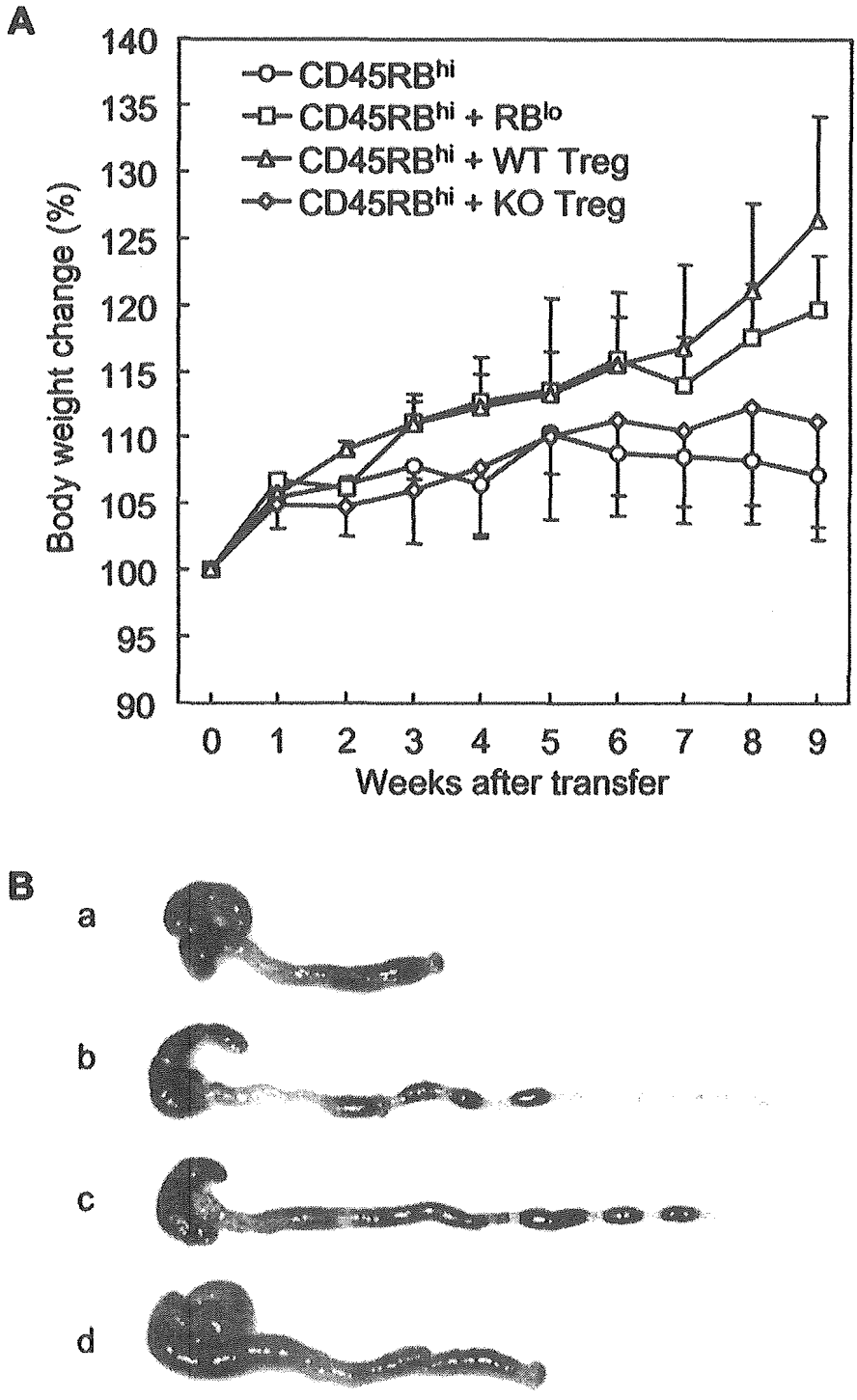

FIGURE 7. In vivo suppressor function of $CD4^+CD25^+$ T cells from TGF-β1-deficient mice in the SCID transfer model of colitis. Weight curves and gross appearance of colons. *A*, Rag-2-deficient mice were reconstituted with $CD4^+CD45RB^{high}$ T cells (○), $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells (□), $CD4^+CD45RB^{high}$ and wild-type $CD4^+CD25^+$ T cells (△), or $CD4^+CD45RB^{high}$ and TGF-β1-deficient $CD4^+CD25^+$ T cells (◇). The change in weight was monitored weekly after the transfer. *B*, The gross appearance of colons in each group. Recipients of *a*, $CD4^+CD45RB^{high}$ T cells; *b*, $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells; *c*, $CD4^+CD45RB^{high}$ and wild-type $CD4^+CD25^+$ T cells; and *d*, $CD4^+CD45RB^{high}$ and TGF-β1-deficient $CD4^+CD25^+$ T cells.

capability of cells with protective regulatory function in intestinal inflammation.

Discussion

In recent years, several molecules have been implicated in the suppressor mechanism underlying the immunoregulatory function of $CD4^+CD25^+$ T cells, including cell surface molecules such as CTLA-4 and glucocorticoid-induced TNFR, cell surface or secreted molecules such as TGF-β1, and intracellular molecules such as FoxP3 (10, 18–22). However, the interrelation of these molecules or their final common pathway has yet to be defined. In a previous study, we showed that $CD4^+CD25^+$ T cells after stimulation express high levels of TGF-β1 in a surface-bound and/or a secreted form, and addition of anti-TGF-β to in vitro cocultures of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells, or $CD4^+CD25^+$, $CD4^+CD25^-$ T cells, and B cells results in reversal of suppression of T cell proliferation and B cell Ig production, respectively (10). These findings have been subsequently corroborated by several other groups that have also found that addition of anti-TGF-β1 or soluble TGF-βR to in vitro cocultures reversed suppression of $CD4^+CD25^+$ T cells, at least in part (11, 12, 23). Nevertheless, the involvement of TGF-β1 in the suppressor function of $CD4^+CD25^+$ T cells remains controversial, mainly because, as already mentioned, $CD4^+CD25^+$ T cells can still suppress $CD25^-$ T cells from mice in whom TGF-β1 signaling is impaired and $CD4^+CD25^+$ T cells from TGF-β1-deficient mice can still suppress $CD4^+CD25^-$ T cells from normal mice in vitro (13). Therefore, in

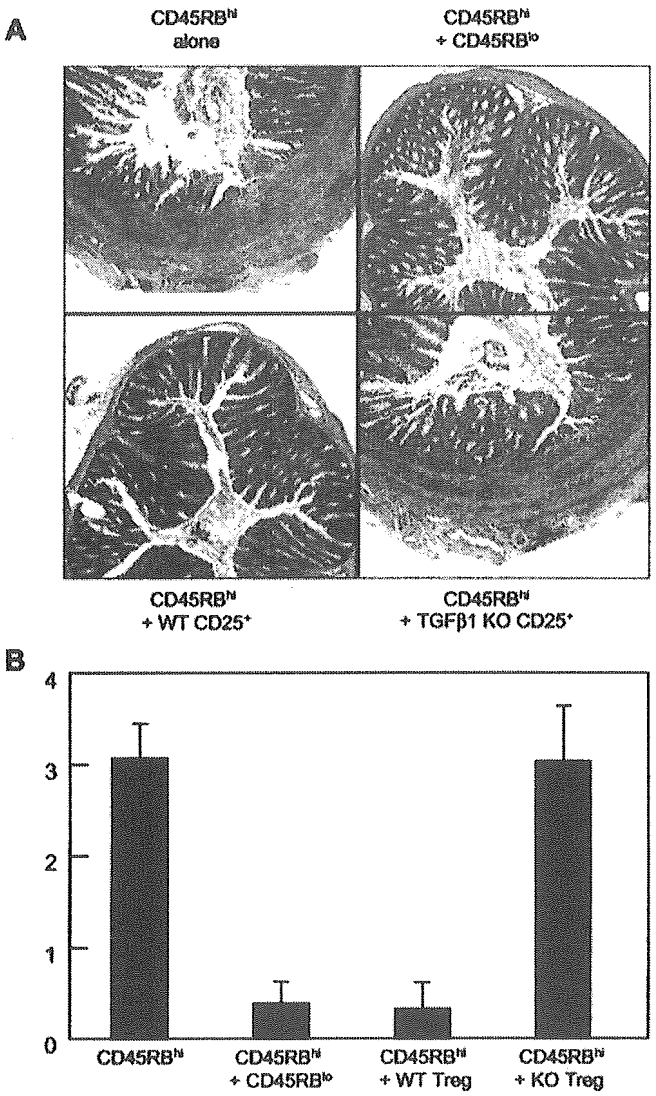

FIGURE 8. In vivo suppressor function of $CD4^+CD25^+$ T cells from TGF-β1-deficient mice in SCID transfer model of colitis. Microscopic appearance and colonic score. *A*, Representative microscopic appearance of colon of the Rag-2-deficient recipients reconstituted with $CD4^+$ $CD45RB^{high}$ T cells, $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells, $CD4^+CD45RB^{high}$ and wild-type $CD4^+CD25^+$ T cells, or $CD4^+$ $CD45RB^{high}$ and TGF-β1-deficient $CD4^+CD25^+$ T cells. *B*, The score of colonic inflammation in each group is shown. Similar results were obtained in two independent experiments.

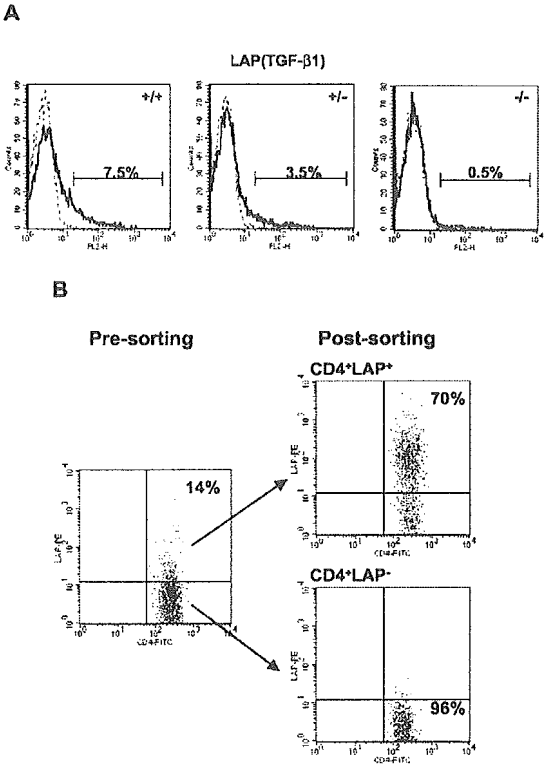

FIGURE 9. Purification of LAP^+ and LAP^- cells from activated mouse $CD4^+$ T cells. *A*, Expression of cell surface LAP on wild-type, but not on TGF-β1-deficient $CD4^+$ T cells. Total splenocytes purified from TGF-$\beta1^{+/+}$, TGF-$\beta1^{+/-}$, and TGF-$\beta1^{-/-}$ mice were stimulated with soluble anti-CD3 (10 μg/ml) and rIL-2 (50 U/ml) for 3 days. Cells were stained with FITC-conjugated anti-CD4 and anti-LAP mAb (clone 27232.11, mouse IgG1), biotin-conjugated anti-mouse IgG1, and PE-conjugated streptavidin. Cells were stained with 7-amino actinomycin D before analysis for dead cell exclusion. $CD4^+$ cells were gated, and the expression of LAP was shown. Results shown are representative of three independent experiments. *B*, LAP^+ and LAP^- $CD4^+$ T cells were purified by FACS sorting.

this study, we re-examined the involvement of TGF-β1 in $CD4^+CD25^+$ T cell suppressor function and present new evidence buttressing the role of this molecule in the suppressor process.

Recognizing that the results of studies with anti-TGF-β have been variable in their ability to demonstrate inhibition of $CD4^+CD25^+$ T cell-mediated suppression, we sought another inhibiting agent. We ultimately chose to evaluate rLAP as a possible inhibitor because this molecule is as TGF-β1 specific as anti-TGF-β1 and binds to TGF-β1 with at least as high an affinity as anti-TGF-β1 (24). This substance could conceivably block TGF-β1 suppressor activity by either of two mechanisms: 1) by combining with newly released active TGF-β1 and converting it back into a latent form; and 2) by competitive inhibition of latent TGF-β1 (LAP) for potential binding sites at which conversion of latent TGF-β1 to an active form normally occurs. Indeed, we found that rLAP inhibited $CD4^+CD25^+$ T cell-mediated suppression in the in vitro assay in a dose-dependent fashion and was able to do so in assays of either murine or human cells. These results provide independent confirmation that TGF-β1 is at least partially involved in the in vitro suppressor activity of $CD4^+CD25^+$ T cells. In retrospect, we would suggest that the failure of some investigators to obtain inhibition of suppression with anti-TGF-β1 can be attributed to either the use of too low a concentration of Ab or an Ab that does not block the ability of latent TGF-β1 to bind to sites where conversion to an active form must occur or alternatively to block the ability of TGF-β1 to bind to the TGF-βR after conversion to an active form. In addition, we have noted that blockade by anti-TGF-β varies with the degree of suppression and that at higher levels of suppression one observes less blockade. This may relate to the fact that at higher levels of suppression there is sufficient redundancy in the system to preclude effective blockade at reasonable Ab doses. Thus, the study of inhibition by anti-TGF-β should be done at a variety of E:T cell ratios. Finally, because TGF-β1-mediated suppression would necessarily involve both an activation step (resulting in dissociation from LAP) and a receptor interaction step and because these steps may be interrelated, it may be difficult to inhibit with Ab, unless one uses a great excess of Ab. LAP would be less subject to this difficulty because it can act before activation of TGF-β1.

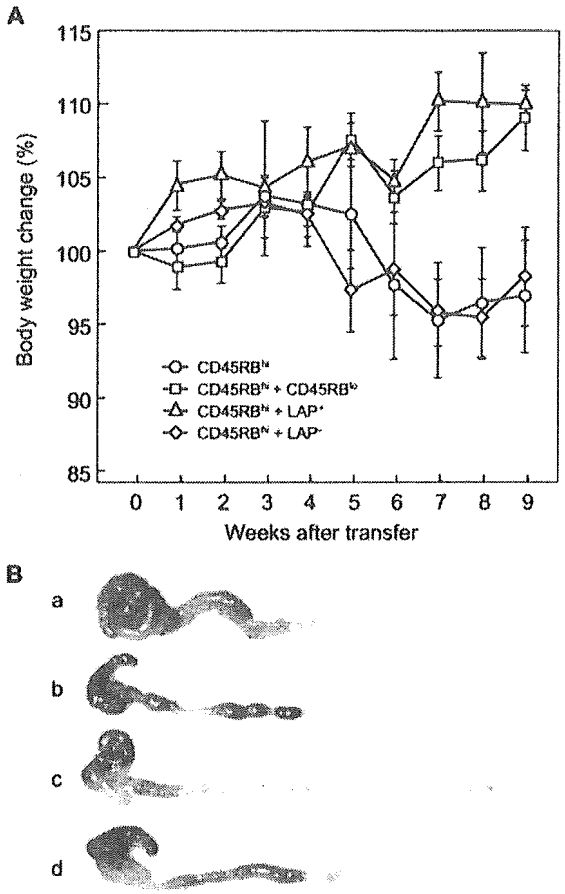

FIGURE 10. In vivo suppressor function of LAP^+ and LAP^- T cells in the SCID transfer colitis. Weight curves and gross appearance of colons. *A*, C.B-17 SCID mice were reconstituted with $CD4^+CD45RB^{high}$ T cells (○), $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells (□), $CD4^+CD45RB^{high}$ and $CD4^+LAP^+$ T cells (△), or $CD4^+CD45RB^{high}$ and $CD4^+LAP^-$ T cells (◇). The change in weight was monitored weekly after the transfer. *B*, The gross appearance of colons in each group. Recipients of *a*, $CD4^+CD45RB^{high}$ T cells; *b*, $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells; *c*, $CD4^+CD45RB^{high}$ and $CD4^+LAP^+$ T cells; and *d*, $CD4^+CD45RB^{high}$ and $CD4^+LAP^-$ T cells.

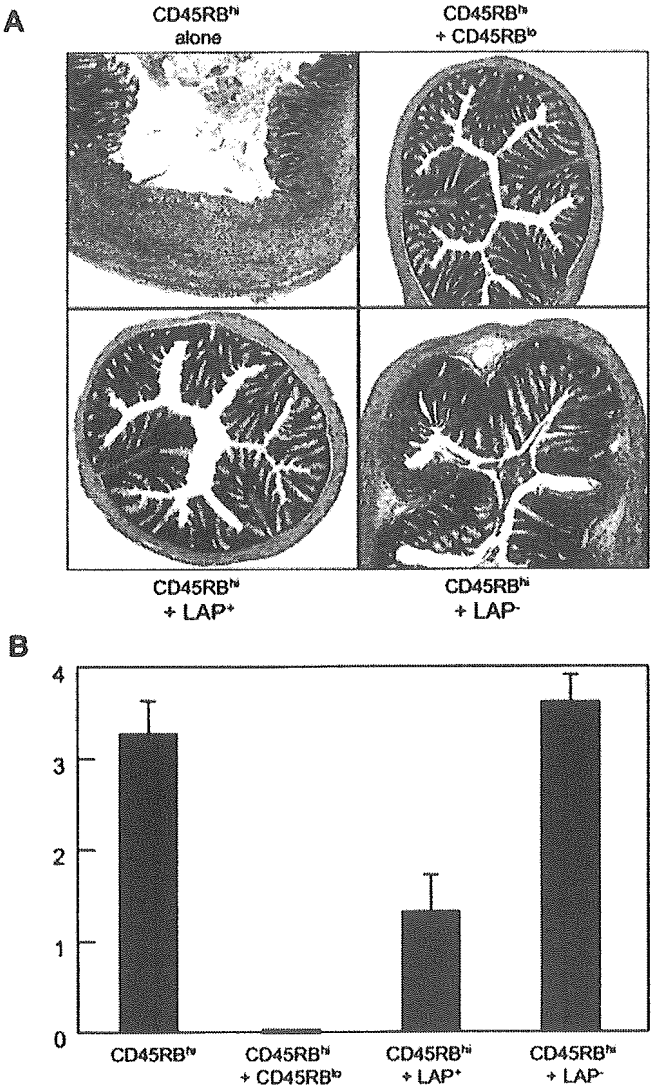

FIGURE 11. In vivo suppressor function of LAP^+ and LAP^- T cells in SCID transfer colitis. Microscopic appearance and colonic score. *A*, Representative microscopic appearance of colon of the C.B-17 SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells, $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells, $CD4^+CD45RB^{high}$ and$CD4^+LAP^+$ T cells, or $CD4^+CD45RB^{high}$ and $CD4^+LAP^-$ T cells. *B*, The score of colonic inflammation in each group is shown. Similar results were obtained in another independent experiment.

In a second approach to investigating whether TGF-β1 is acting as a suppressor factor in $CD4^+CD25^+$ T cell-mediated suppression, we sought evidence that TGF-β1 could signal $CD25^-$ T cells via TGF-βR. We showed that this was indeed true in that $CD25^-$ target cells in the in vitro assay when mixed with $CD4^+CD25^+$ T cells (but not with $CD4^+CD25^-$ T cells) undergo Smad-2 phosphorylation. Such activation indicates that the target cells have been signaled via TGF-βR. TGF-β1 signaling of target cells was also demonstrated somewhat more indirectly by the fact that $CD25^-$ target cells cocultured with $CD4^+CD25^+$ T cells were induced to express CD103 (α_E integrin), an integrin previously shown to be regulated by TGF-β1 (25). This finding has added weight in the light of the recent finding that stimulated $CD4^+$ T cells in which TGF-β1 signaling is blocked by overexpression of Smad-7 do not express $\alpha_E\beta_7$, indicating that TGF-β1 is necessary for $\alpha_E\beta_7$ expression, at least in vitro (26). Finally, it is interesting that a significant fraction of $CD4^+CD25^+$ T cells expresses CD103, presumably because they are autostimulated by TGF-β1.

A finding that is seemingly at odds with the TGF-β1 blocking and signaling studies reported in this work concerns the fact that, as reported previously, $CD4^+CD25^+$ T cells from TGF-β1-deficient mice were able to mediate suppression of $CD4^+CD25^-$ T cells in the in vitro assay system. There are a number of possible explanations of these discordant results, but the one that appears most likely to us is that in the absence of TGF-β1, $CD4^+CD25^+$ T cells express and/or up-regulate alternative suppressor mechanisms that ordinarily play a secondary role in the mechanism of suppression. This possibility would accord with the fact mentioned above that anti-TGF-β blockade is more effective at lower levels of suppression (when secondary mechanisms may be less evident) and the finding of Chen and Wahl (23) that $CD4^+CD25^+$ T cells from TGF-β1-deficient mice suppressed less well at the usual ratio of regulatory cells to target cells, but could exert suppression at high ratios. This explanation notwithstanding, further work will be necessary to resolve this discrepancy. Finally, the finding that $CD4^+CD25^+$ T cells are capable of suppressing $CD25^-$ T cells from mice with TGF-β1 signaling defects (13) is not persuasive to us because these cells had leaky defects. Thus, in our hands, cells from either Smad3-deficient mice or mice bearing a dominant-negative TGF-βR were still subject to inhibition of proliferation by

the addition of rTGF-β to the cultures (data not shown). Thus, these cells do not allow a rigorous test of TGF-β-mediated suppressor function.

Additional support for the involvement of TGF-β1 in $CD4^+$ $CD25^+$ T cell regulatory function came from in vivo studies in which we showed that while $CD4^+CD25^+$ T cells from TGF-β1-deficient mice manifest suppressor function in the in vitro suppressor assay, they cannot prevent colitis mediated by $CD45RB^{high}$ T cells transferred to Rag-2-deficient recipients, whereas as shown previously and again in this study, such cells from wild-type mice can prevent colitis. We believe this is a more robust test of the role of TGF-β1 in the $CD4^+CD25^+$ T cell regulatory cell function of cells from TGF-β1-deficient mice than the in vitro study, because in this case the regulatory cells are presumably acting via surface expression or secretion of TGF-β1, which cannot be compensated by another suppressor mechanism.

Previous studies have shown that anti-TGF-β administration to SCID or Rag-2-deficient mice transferred $CD45RB^{high}$ T cells and protective $CD45RB^{low}$ abrogates the protective effect and that $CD4^+CD25^+$ T cells are the subpopulation that mediate protection (19, 27). These studies left unanswered the question of whether the $CD4^+CD25^+$ T cells were producing the TGF-β1 themselves or inducing other cells to produce this cytokine. This question is answered by the present findings showing that the TGF-β1 must in fact come from the $CD4^+CD25^+$ T cells themselves.

In related studies, we showed that $CD4^+LAP^+$ T cells, but not $CD4^+LAP^-$ T cells, from normal mice prevent colitis in the transfer model. This again provides yet additional evidence for the link between cell surface TGF-β1 expression and regulatory function. Recently, Oida et al. (28) reported that $CD25^-LAP^{high}$ cells also prevent colitis in the transfer model and such prevention is also TGF-β dependent. This cell population may represent a population in a different state of activation or differentiation than the cells investigated in this study, but nevertheless, the results are consistent with our finding that LAP^+ cells (cells bearing cell surface TGF-β1) are suppressor cells whether or not they express $CD25^+$.

Taken together, these studies show that TGF-β1 produced by $CD4^+CD25^+$ regulatory T cells are an important mechanism by which these cells exert suppression in vitro and more, particularly, in vivo. However, they leave open the possibility that other suppressor mechanisms may also exist and may come to the fore under some circumstances. Finally, studies presented in this work and previously strongly suggest that the presence of LAP on the cell surface is an excellent marker of regulatory cells, perhaps better than CD25 itself.

Acknowledgments

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IκBNS Inhibits Induction of a Subset of Toll-like Receptor-Dependent Genes and Limits Inflammation

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Summary

Toll-like receptor (TLR)-mediated immune responses are downregulated by several mechanisms that affect signaling pathways. However, it remains elusive how TLR-mediated gene expression is differentially modulated. Here, we show that IκBNS, a TLR-inducible nuclear IκB protein, negatively regulates induction of a subset of TLR-dependent genes through inhibition of NF-κB activity. IκBNS-deficient macrophages and dendritic cells show increased TLR-mediated expression of genes such as IL-6 and IL-12p40, which are induced late after TLR stimulation. In contrast, IκBNS-deficient cells showed normal induction of genes that are induced early or induced via IRF-3 activation. LPS stimulation of IκBNS-deficient macrophages prolonged NF-κB activity at the specific promoters, indicating that IκBNS mediates termination of NF-κB activity at selective gene promoters. Moreover, IκBNS-deficient mice are highly susceptible to LPS-induced endotoxin shock and intestinal inflammation. Thus, IκBNS regulates inflammatory responses by inhibiting the induction of a subset of TLR-dependent genes through modulation of NF-κB activity.

Introduction

Toll-like receptors (TLRs) are implicated in the recognition of specific patterns of microbial components and subsequent induction of gene expression. TLR-dependent gene expression is induced through activation of two distinct signaling pathways mediated by the Toll/IL-1 receptor (TIR) domain-containing adaptors MyD88 and TRIF. These signaling pathways finally culminate in the activation of several transcription factors, such as NF-κB and IRF families (Akira and Takeda, 2004). The MyD88-dependent gene induction is achieved by an early phase of NF-κB and IRF-5 activation in macrophages (Kawai et al., 1999; Takaoka et al., 2005). The TRIF-dependent gene induction is mainly regulated by IRF-3 (Sakaguchi et al., 2003; Yamamoto et al., 2003).

TLR-mediated gene expression regulates activation of not only innate immunity but also adaptive immunity, which provides antigen-specific responses against harmful pathogens (Iwasaki and Medzhitov, 2004; Pasare and Medzhitov, 2004). However, TLR-mediated activation of innate immunity, when in excess, triggers development of autoimmune disorders and inflammatory diseases, such as SLE, cardiomyopathy, atherosclerosis, diabetes mellitus, and inflammatory bowel diseases (Bjorkbacka et al., 2004; Eriksson et al., 2003; Kobayashi et al., 2003; Lang et al., 2005; Leadbetter et al., 2002; Michelsen et al., 2004). Excessive activation of TLR4 by LPS induces endotoxin shock, a serious systemic disorder with a high mortality rate. Therefore, TLR-dependent innate immune responses must be finely regulated, and underlying mechanisms are now being examined extensively (Liew et al., 2005). Several negative regulators of TLR-mediated signaling pathways have been proposed. Cytoplasmic molecules, such as an alternatively spliced short form of MyD88 (MyD88s), IRAK-M, SOCS1, A20, PI3-kinase, and TRIAD3A, are all involved in negative regulation of TLR pathways (Boone et al., 2004; Burns et al., 2003; Chuang and Ulevitch, 2004; Fukao et al., 2002; Kinjyo et al., 2002; Kobayashi et al., 2002; Nakagawa et al., 2002). Membrane bound SIGIRR, ST2, TRAILR, and RP105 are also implicated in these processes (Brint et al., 2004; Diehl et al., 2004; Divanovic et al., 2005; Wald et al., 2003).

TLR-dependent gene induction is also regulated by nuclear IκB proteins, such as IκBζ, Bcl-3, and IκBNS. IκBζ is indispensable for positive regulation of a subset of TLR-dependent genes, such as IL-6 and IL-12p40 (Yamamoto et al., 2004). In contrast, Bcl-3 and IκBNS seem to be involved in negative regulation of TLR-dependent gene induction. Bcl-3 was shown to be involved in selective inhibition of TLR-dependent TNF-α production (Kuwata et al., 2003; Wessells et al., 2004). An in vitro study indicated that IκBNS is induced by IL-10 or LPS and selectively inhibits IL-6 production in macrophages (Hirotani et al., 2005). Thus, nuclear IκB proteins differentially regulate TLR-dependent gene expression. However, the physiological role of IκBNS is still unclear.

In this study, we analyzed TLR-dependent inflammatory responses in IκBNS-deficient mice. We found that IκBNS is involved in selective inhibition of a subset of MyD88-dependent genes, including IL-6, IL-12p40, and IL-18. In IκBNS-deficient macrophages, LPS-induced activation of NF-κB was prolonged. Accordingly, IκBNS-deficient mice showed increased production of these cytokines accompanied by high sensitivity to LPS-induced endotoxin shock. Furthermore, IκBNS-deficient mice were highly susceptible to intestinal inflammation caused by disruption of the epithelial barrier. These findings indicate that IκBNS inhibits the induction of a group of TLR-dependent genes, thereby preventing excessive inflammation.

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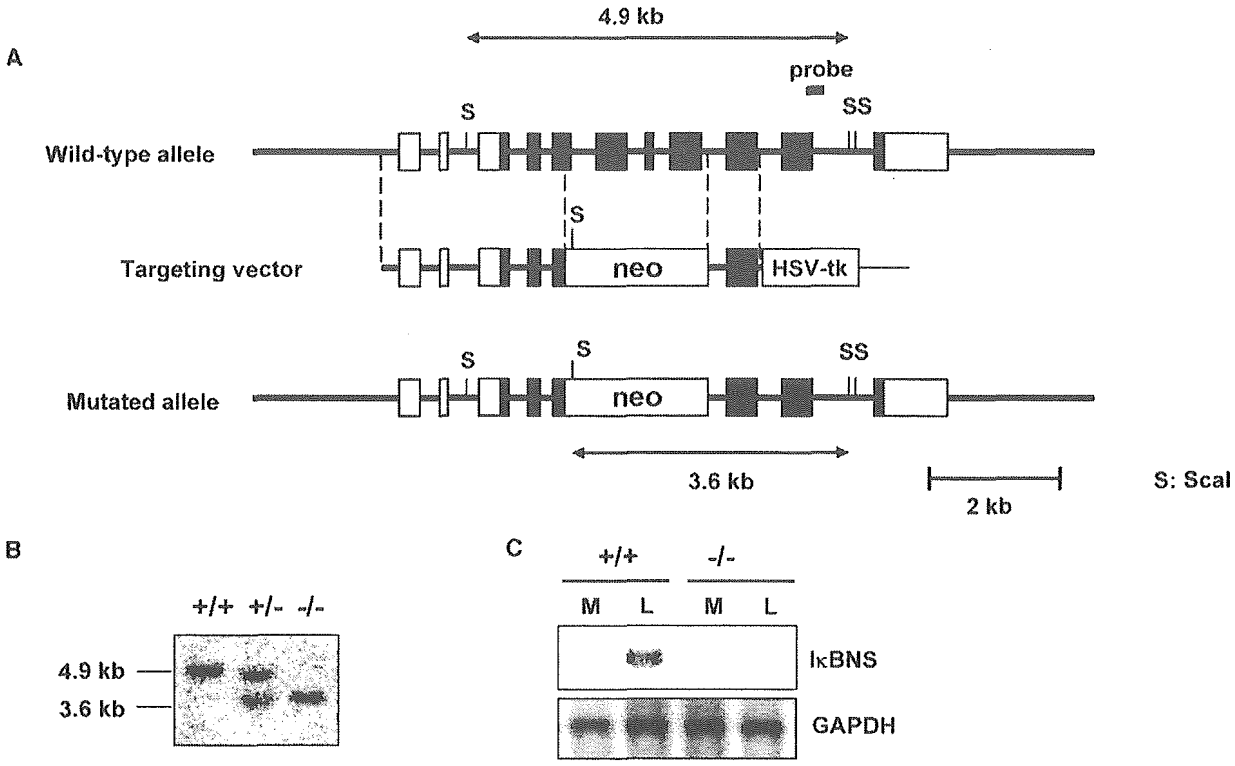

Figure 1. Targeted Disruption of the Mouse *Ikbns* Gene

(A) Maps of the IκBNS wild-type genome, targeting vector, and predicted targeted gene. Open and closed boxes denote the noncoding and coding exons, respectively. Restriction enzymes: S, ScaI.

(B) Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with ScaI, electrophoresed, and hybridized with the probe indicated in (A). The approximate size of the wild-type band is 4.9 kb, and the mutated band is 3.6 kb.

(C) Peritoneal macrophages were cultured with or without 100 ng/ml LPS for 1 hr (L and M, respectively), and total RNA was extracted, electrophoresed, transferred to nylon membrane, and hybridized with the mouse IκBNS full-length cDNA probe. The same membrane was rehybridized with a GAPDH probe.

Results

Targeted Disruption of the IκBNS Gene

To study the functional role of IκBNS in TLR-dependent responses, a null mutation in the *Ikbns* allele was introduced through homologous recombination in embryonic stem (ES) cells (Figures 1A and 1B). IκBNS$^{-/-}$ mice were born alive and grew healthy until 20 weeks of age. We performed Northern blot analysis to confirm that the mutation causes inactivation of the *Ikbns* gene. LPS robustly induced IκBNS mRNA in wild-type macrophages, but not in IκBNS$^{-/-}$ macrophages (Figure 1C).

A previous report indicated that IκBNS is involved in negative selection of thymocytes (Fiorini et al., 2002). Therefore, we first analyzed lymphocyte composition in lymphoid organs such as thymus and spleen by flow cytometry (Figures S1A and S1B). Total cell number and CD4/CD8 or CD3/B220 populations in thymus and spleen were not altered in IκBNS$^{-/-}$ mice. Splenic T cells from IκBNS$^{-/-}$ mice showed similar levels of proliferative responses to IL-2 and IL-7 as did wild-type T cells. Moreover, IκBNS$^{-/-}$ T cells proliferated to almost equal degrees in response to anti-CD3 antibody compared to wild-type T cells (Figure S1C). These results indicate that T cell development and functions were generally unaffected in IκBNS$^{-/-}$ mice.

Increased IL-6 and IL-12p40 Production in IκBNS-Deficient Cells

Since IκBNS expression was induced within 1 hr of LPS stimulation in macrophages (Figure 1B), we stimulated peritoneal macrophages with various concentrations of LPS and analyzed for production of TNF-α and IL-6 (Figure 2A). In macrophages from IκBNS$^{-/-}$ mice, LPS-induced TNF-α production was comparable to wild-type cells, but IL-6 production was significantly increased. We then analyzed whether IκBNS$^{-/-}$ macrophages produce increased amounts of IL-6 in response to other TLR ligands, since IκBNS mRNA was induced by several TLR ligands as well as the TLR4 ligand LPS in a MyD88-dependent manner (Figure S2A). Peritoneal macrophages were stimulated with mycoplasmal lipopeptides (TLR6 ligand), Pam_3CSK_4 (TLR1 ligand), peptidoglycan (TLR2 ligand), and imiquimod (TLR7 ligand), and analyzed for production of TNF-α and IL-6 (Figure 2B). In response to these TLR ligands, the production of IL-6, but not TNF-α, was increased in IκBNS$^{-/-}$ mice. We next analyzed the response of bone marrow-derived dendritic cells (DCs). DCs from IκBNS$^{-/-}$ mice produced similar amounts of TNF-α and increased amounts of IL-6 in response to LPS compared to wild-type DCs (Figure 2C). In addition, DCs showed LPS-induced production of IL-12p40 and IL-12p70, and production of these

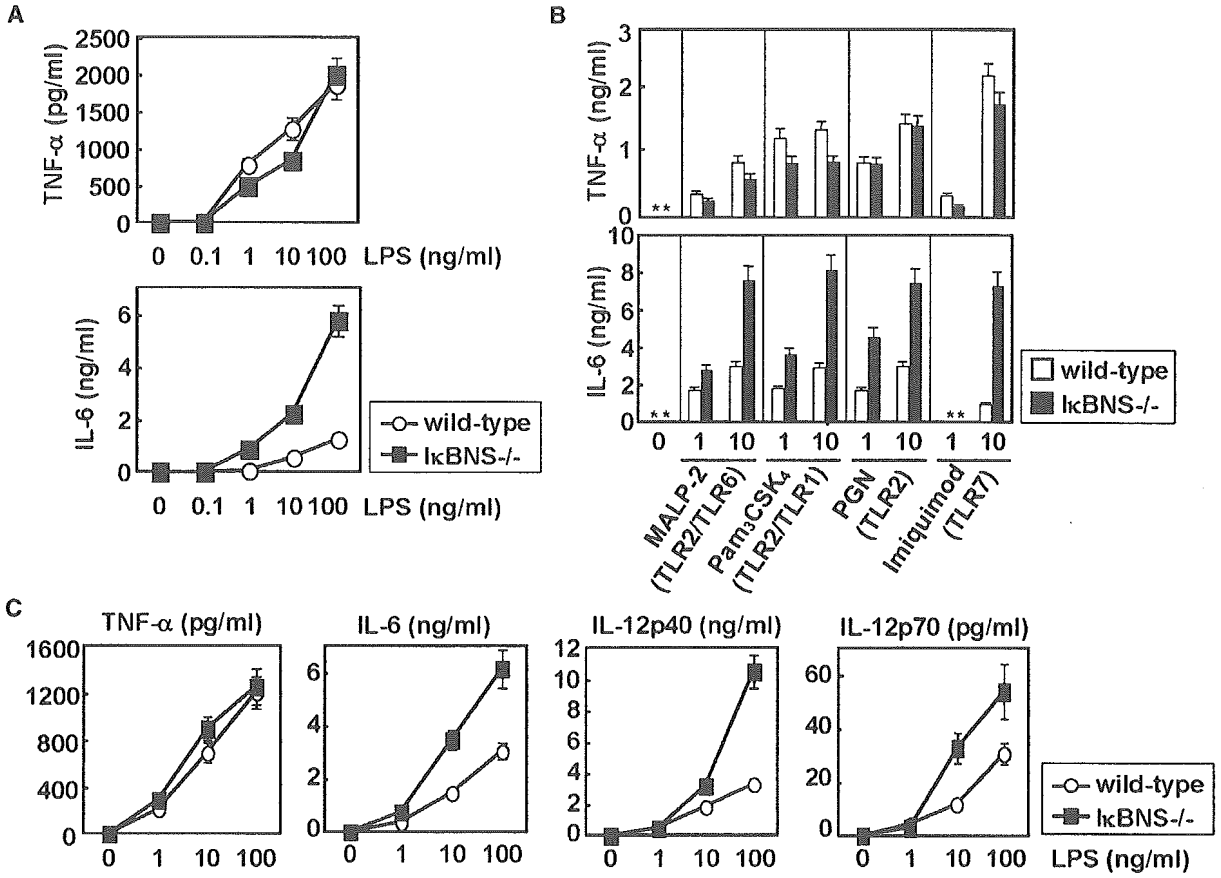

Figure 2. Increased Production of IL-6 and IL-12p40 in IκBNS$^{-/-}$ Macrophages and Dendritic Cells

(A) Peritoneal macrophages were stimulated with the indicated concentration of LPS for 24 hr. Concentrations of TNF-α and IL-6 in the culture supernantants were analyzed by ELISA. Data are mean ± SD of triplicate cultures in a single experiment, representative of three independent experiments.

(B) Peritoneal macrophages were cultured with 1 or 10 ng/ml of TLR6 ligand (MALP-2), 1 or 10 ng/ml TLR1 ligand (Pam_3CSK_4), 1 or 10 μg/ml TLR2 ligand (peptidoglycan; PGN), or 1 or 10 μg/ml TLR7 ligand (imiquimod) for 24 hr. Concentrations of TNF-α and IL-6 in the culture supernatants were analyzed by ELISA. *; not detected.

(C) Bone marrow-derived DCs were stimulated with the indicated concentration of LPS for 24 hr. Concentrations of TNF-α, IL-6, IL-12p40, and IL-12p70 in the culture supernatants were analyzed by ELISA. Data are mean ± SD of triplicate cultures in a single experiment, representative of three independent experiments.

cytokines was significantly increased in IκBNS$^{-/-}$ DCs. Bone marrow-derived DCs and splenic B cells were analyzed for LPS-induced surface expression of CD86 or MHC class II (Figure S2B). LPS-induced augmentation of surface expression of these molecules was not altered in IκBNS$^{-/-}$ mice. Thus, macrophages and DCs from IκBNS$^{-/-}$ mice showed selective increases in TLR-dependent production of IL-6 and IL-12p40.

Enhanced Induction of a Subset of TLR-Dependent Genes in IκBNS-Deficient Macrophages

We further analyzed LPS-induced mRNA expression of TLR-dependent genes in IκBNS$^{-/-}$ macrophages. Peritoneal macrophages were stimulated with LPS for 1, 3, or 5 hr, and total RNA was extracted. Then, mRNA expression of TNF-α and IL-6 was first analyzed by quantitative real-time RT-PCR (Figures 3A and 3B). LPS-induced TNF-α mRNA expression in IκBNS$^{-/-}$ macrophages was similar to wild-type cells. In the case of IL-6 mRNA, expression levels were comparable between wild-type and IκBNS$^{-/-}$ macrophages until 3 hr of LPS stimulation. After 3 hr, IL-6 mRNA levels decreased in wild-type cells. However, IκBNS$^{-/-}$ cells displayed further enhanced expression of IL-6 mRNA. TNF-α mRNA was robustly induced within 1 hr of LPS stimulation, and its expression promptly ceased in wild-type cells. In contrast, IL-6 mRNA expression was induced late compared to TNF-α. Because LPS-induced IκBNS mRNA expression showed similar patterns as TNF-α mRNA, we hypothesized that LPS-inducible IκBNS blocks mRNA expression of genes that are induced late (Figure 3C). Accordingly, we analyzed mRNA expression of other genes that are induced early (*Il-1β*, *Il-23p19*, or *Ikbz*) or late (*Il-12p40*, *Il-18*, or *Csf3*) in response to LPS. LPS-induced mRNA expression of *Il-1β* (IL-1β), *Il-23p19* (IL-23p19), and *Ikbz* (IκBζ) was similarly observed between wild-type and IκBNS$^{-/-}$ macrophages (Figure 3A). LPS-induced expression of *Il-12p40* (IL-12p40), *Il-18* (IL-18) and *Csf3* (G-CSF) was observed at normal levels in IκBNS$^{-/-}$ macrophages at the early phase of LPS stimulation (within 3 hr of LPS stimulation) (Figure 3B). However, at the late phase of LPS stimulation (after 3 hr of LPS stimulation), mRNA expression of these genes was significantly enhanced in

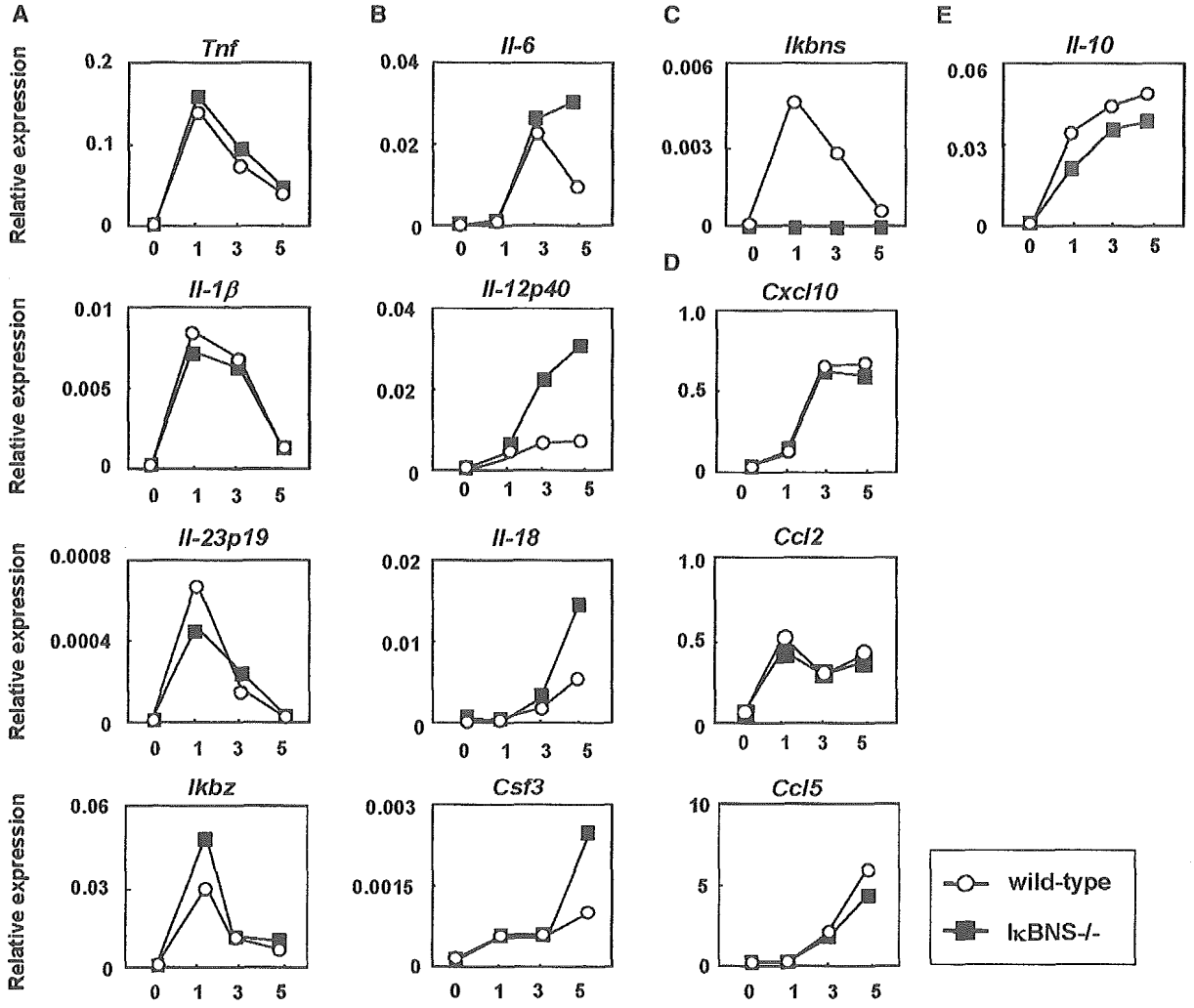

Figure 3. LPS-Induced Expression of Several TLR-Dependent Genes in IκBNS$^{-/-}$ Macrophages

Peritoneal macrophages from wild-type and IκBNS$^{-/-}$ mice were stimulated with 100 ng/ml LPS for the indicated periods. Total RNA was extracted, and then subjected to quantitative real-time RT-PCR analysis using primers specific for *Tnf, Il-1β, Il-23p19, Ikbz* (A), *Il-6, Il-12p40, Il-18, Csf3* (B), *Ikbns* (C), *Cxcl10, Ccl2, Ccl5* (D), and *Il-10* (E). The fold difference of each sample relative to EF-1α levels is shown. Representative of three independent experiments.

IκBNS$^{-/-}$ cells. We also analyzed LPS-induced expression of *Cxcl10* (IP-10), *Ccl2* (MCP-1), and *Ccl5* (RANTES), which are induced by the TRIF-dependent activation of IRF-3 (Figure 3D). LPS-induced expression of these genes was not altered in IκBNS$^{-/-}$ macrophages. An anti-inflammatory cytokine IL-10 is induced by TLR stimulation and thereby inhibits TLR-dependent gene induction (Moore et al., 2001). Therefore, we next addressed LPS-induced IL-10 mRNA expression (Figure 3E). LPS-induced IL-10 mRNA expression was comparable between wild-type and IκBNS$^{-/-}$ macrophages. In addition, LPS-induced production of IL-10 protein was not compromised in IκBNS$^{-/-}$ DCs (Figure S2C). These findings indicate that the enhanced LPS-induced expression of a subset of TLR-dependent genes was not due to the impaired IL-10 production in IκBNS$^{-/-}$ mice.

Prolonged NF-κB Activity in IκBNS-Deficient Cells

Gene expression of *Cxcl10* (IP-10), *Ccl2* (MCP-1), and *Ccl5* (RANTES) was mainly regulated by the transcription factor IRF-3 in the TRIF-dependent pathway, whereas TNF-α, IL-6, and IL-12p40 gene expression was mainly regulated by the MyD88-dependent activation of NF-κB (Akira and Takeda, 2004; Yamamoto et al., 2003). In addition, previous in vitro studies indicated that overexpression of IκBNS leads to compromised NF-κB activity through selective association of IκBNS with p50 subunit of NF-κB (Fiorini et al., 2002; Hirotani et al., 2005). Therefore, we next analyzed LPS-induced activation of NF-κB. LPS-induced degradation of IκBα was not compromised in IκBNS$^{-/-}$ macrophages (Figure S3A). Next, peritoneal macrophages or bone marrow-derived macrophages were stimulated with LPS and DNA binding activity was analyzed by EMSA (Figure 4A; Figure S3B). LPS stimulation resulted in enhanced DNA binding activity of NF-κB in both wild-type and IκBNS$^{-/-}$ macrophages to similar extents within 1 hr. After 1 hr of LPS stimulation, NF-κB activity decreased in wild-type cells. However, NF-κB activity sustained and even at 3 hr of LPS stimulation significant DNA binding activity was still observed in

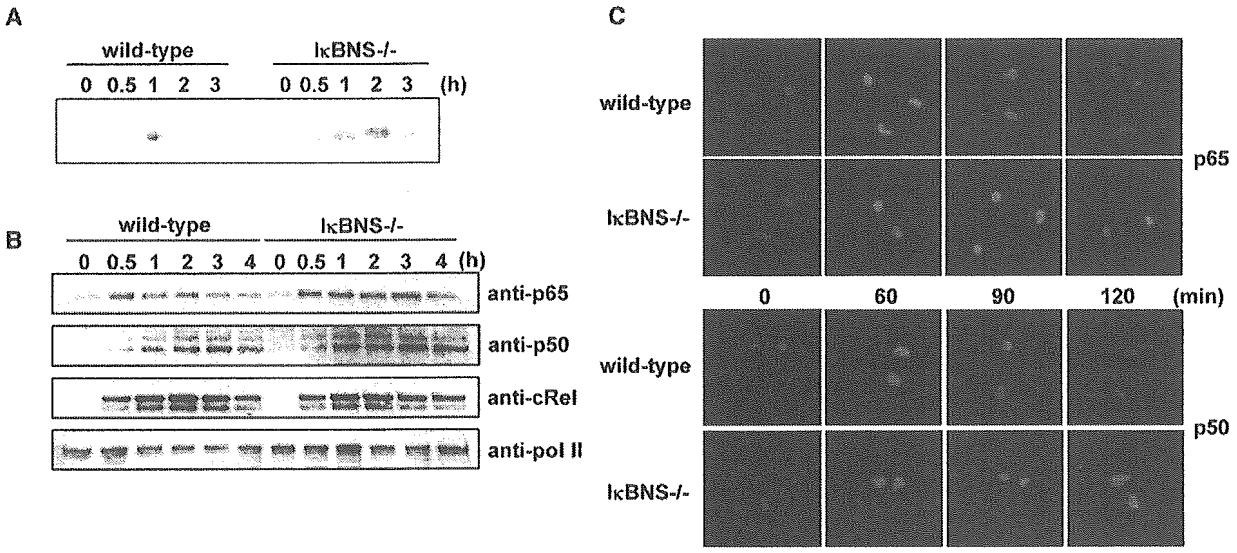

Figure 4. Persistent LPS-Induced Activation of NF-κB in IκBNS$^{-/-}$ Macrophages

(A) Peritoneal macrophages from wild-type and IκBNS$^{-/-}$ mice were stimulated with 100 ng/ml LPS. At the indicated time points, nuclear extracts were prepared, and NF-κB activation was analyzed by EMSA using a NF-κB specific probe.

(B) Peritoneal macrophages were stimulated with LPS. At the indicated time points, nuclear fractions were isolated and subjected to Western blotting using anti-p65 Ab, anti-p50 Ab, anti-cRel Ab, or anti-polII Ab.

(C) Macrophages were stimulated with LPS for the indicated periods. Then, cells were stained with anti-p65 Ab or anti-p50 Ab (red) as well as DAPI (blue), and analyzed by confocal microscopy. Merged images are shown.

IκBNS$^{-/-}$ cells. We next analyzed nuclear localization of NF-κB subunits. Peritoneal macrophages were stimulated with LPS for the indicated periods, and nuclear fractions were analyzed for expression of p65, p50, and c-Rel by immunoblotting (Figure 4B). In wild-type macrophages, nuclear translocation of p65 was observed within 30 min of LPS stimulation, and nuclear localized p65 gradually decreased thereafter. In contrast, nuclear localized p65 was still significantly observed even at 3 hr of LPS stimulation in IκBNS$^{-/-}$ cells. In addition, sustained nuclear localization of p50, but not c-Rel, was observed in IκBNS$^{-/-}$ macrophages (Figure 4B). Nuclear localization of NF-κB subunits was also analyzed by immunofluorescent staining of macrophages (Figure 4C). Without stimulation, p65 and p50 were localized in the cytoplasm, but not in the nucleus, in both wild-type and IκBNS$^{-/-}$ macrophages. LPS stimulation resulted in nuclear staining of both p65 and p50 at 1 hr. Nuclear staining of p65 and p50 gradually decreased after 1 hr of LPS stimulation and was only faintly observed at 2 hr of stimulation in wild-type cells. However, nuclear localization of p65 and p50 was still evident at 2 hr of LPS stimulation in IκBNS$^{-/-}$ cells. These findings indicate that LPS-induced NF-κB activity was prolonged in IκBNS$^{-/-}$ macrophages. NF-κB activity is terminated by degradation of promoter-bound p65 (Natoli et al., 2005; Saccani et al., 2004). We used RAW264.7 macrophage cell line and performed pulse-chase experiments with ^{35}S-labeled amino acids to analyze p65 turnover (Figure S3C). In these cells, labeled p65 was accumulated into the nucleus until 2 hr of LPS stimulation, and then p65 was degraded. In RAW cells constitutively expressing IκBNS, nuclear accumulation of labeled p65 was similarly observed until 1 hr of LPS stimulation. However, the p65 turnover was observed more rapidly and labeled p65 disappeared at 2 hr after LPS stimulation (Figure S3C). These findings indicate that IκBNS mediates the degradation of p65. The MyD88-dependent pathway mediates activation of MAP kinase cascades as well as NF-κB activation. Therefore, LPS-induced phosphorylation of p38, ERK1, ERK2, and JNK was analyzed by Western blotting (Figure S3D). LPS-induced activation of these MAP kinases was not compromised in IκBNS$^{-/-}$ macrophages.

Regulation of p65 Activity at the IL-6 Promoter by IκBNS

We next addressed how IκBNS selectively downregulates induction of genes that are induced late. We utilized the IL-6 and TNF-α promoters, which are representatives of genes activated late and early, respectively. Wild-type macrophages were stimulated with LPS and analyzed for recruitment of endogenous IκBNS to the promoters by chromatin immunoprecipitation (ChIP) assay (Figure 5A). Consistent with previous findings using IκBNS overexpressing macrophage cell lines (Hirotani et al., 2005), endogenous IκBNS was recruited to the IL-6 promoter, but not the TNF-α promoter, in LPS-stimulated macrophages. We next addressed LPS-induced recruitment of p65 to the promoters in wild-type and IκBNS$^{-/-}$ macrophages (Figure 5B). Recruitment of p65 to the TNF-α promoter peaked at 1 hr of LPS stimulation and gradually decreased thereafter in a similar manner in both wild-type and IκBNS$^{-/-}$ cells. Recruitment of p65 to the IL-6 promoter was observed to similar extents until 3 hr of LPS stimulation in wild-type and IκBNS$^{-/-}$ macrophages. After that, it decreased in wild-type macrophages. In contrast, p65 recruitment was still evident, rather enhanced, even after 5 hr of LPS stimulation in IκBNS$^{-/-}$ macrophages. Thus, p65 activity at