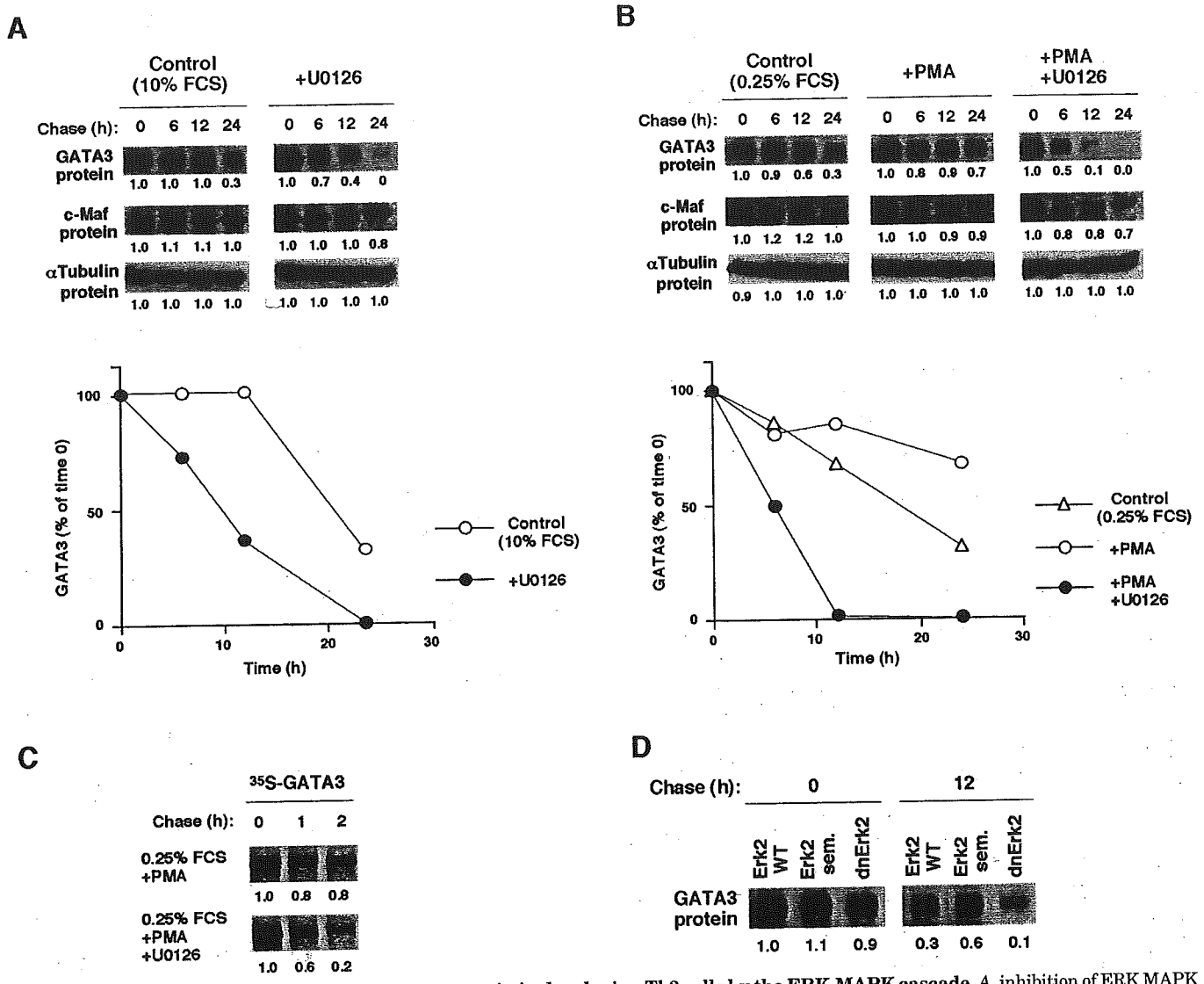

FIG. 3. **Regulation of the expression of GATA3 protein in developing Th2 cells by the ERK-MAPK cascade.** *A*, inhibition of ERK MAPK activation induces GATA3 protein degradation. Splenic CD4 T cells were stimulated under Th2− conditions for 2 days. The cells were then cultured for 6 and 12 h without cytokines in the presence or absence of MEK inhibitor U0126 (20 μM) with 10% FCS in the medium. The expression of GATA3 protein was assessed as in Fig. 2*A*. The expression of c-Maf and α-tubulin protein was also determined as controls. Arbitrary densitometric units are shown below each band, and the percentages of each point are shown in a *graph*. Three independent experiments with different time courses were performed with similar results. *B*, GATA3 protein degradation in the presence of PMA and U0126. Splenic CD4 T cells were stimulated for 2 days as described in *A*. Then the cells were cultured in the presence of PMA (3 ng/ml) and U0126 (20 μM) with just 0.25% FCS in the medium. The protein expression levels of GATA3, c-Maf, and α-tubulin protein were examined. Three independent experiments were performed, and a representative result is shown. *C*, degradation of GATA3 determined by pulse-chase analysis. Splenic CD4 T cells were stimulated as described in *A*. Then the cultured cells were labeled with [^{35}S]methionine and [^{35}S]cysteine and chased in a medium containing 0.25% FCS, nonradioactive methionine and cysteine in the presence or absence PMA (3 ng/ml). The effect of U0126 was examined. ^{35}S-Labeled GATA3 protein was visualized by autoradiography. Arbitrary densitometric units are shown below each band. Three independent experiments were performed, and a representative result is shown. *D*, effect of an active form of ERK2 (Erk2 sem) and a dominant-negative form of ERK2 (dnErk2) on the expression of GATA3. Splenic CD4 T cells were stimulated as described in *A*, and the cells were infected with retrovirus encoding ERK2 mutant bicistronically with human coxsackievirus and adenovirus receptor (*CAR*). Three days after infection, the coxsackievirus and adenovirus receptor-positive population was enriched by MACS and cultured at 37 °C for 12 h without cytokines. Then the expression levels of GATA3 protein were assessed. Arbitrary densitometric units are shown below each band. Four independent experiments were performed, and a representative result is shown.

the cytosol, and treatment with either CHX or MG132 did not have a significant effect on the levels of the mutant protein. Small amounts of dZF protein were detected in the nuclear fraction, and a modest increase was detected in the presence of MG132. Thus it would appear the C-terminal region of GATA3, including the zinc finger region (residues 261–315), is critical for proteasome-dependent degradation.

To visualize the dynamics of localization and accumulation, green fluorescence protein (GFP)-fused wild type GATA3 and the dZF mutant were expressed in NIH3T3 cells. As expected, wild type GATA3 showed decreased fluorescence following CHX treatment, and the decrease in fluorescence was prevented to some extent in the presence of MG132 (supplemental Fig. 1). The dZF mutant was expressed in both nuclear and cytosolic fractions, and the fluorescence intensity was not affected by treatment with CHX or MG132. These results are consistent with the results shown in Fig. 4*C*.

The ERK MAPK Cascade Regulates GATA3 Ubiquitination—In order to assess the involvement of multiubiquitination (Ub) in GATA3 degradation, FLAG-tagged wild type GATA3, dCT, dCF, and dZF mutants were each transfected into 293T cells, and transfectants were treated with MG132. Immuno-

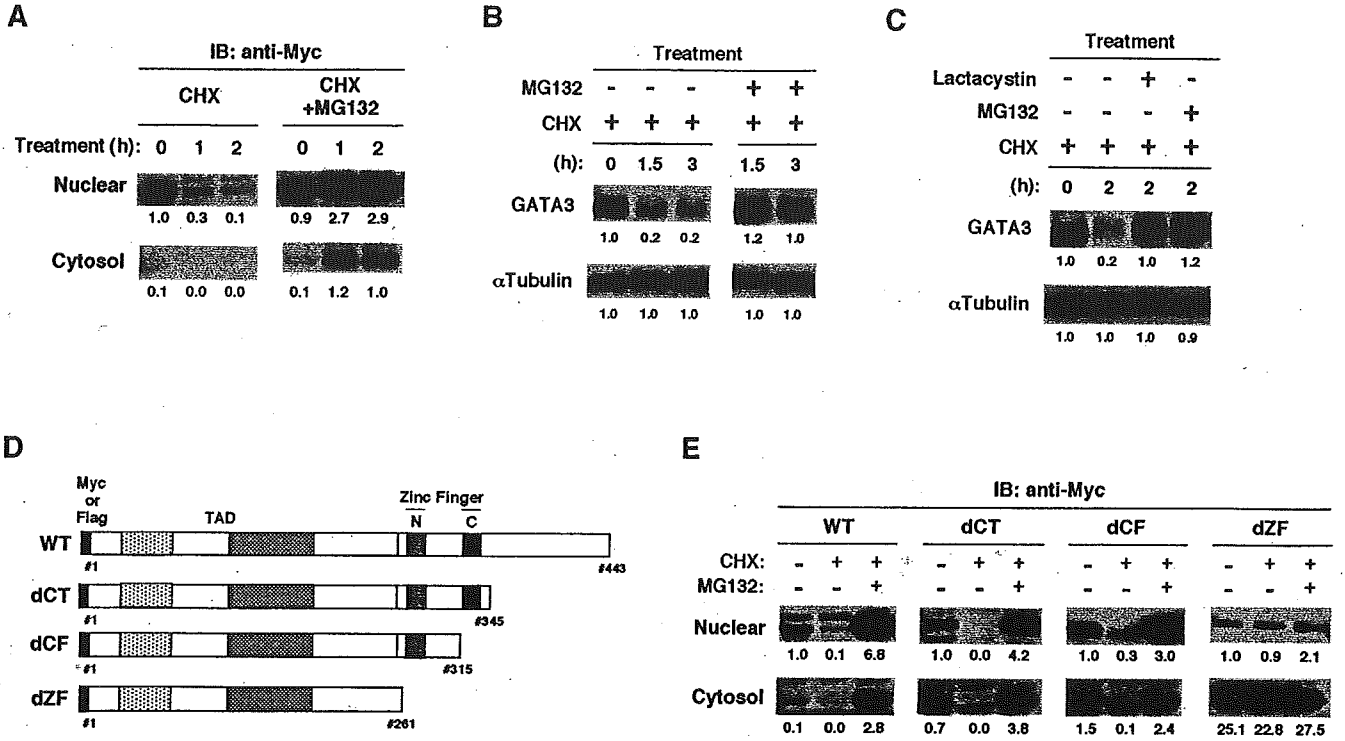

FIG. 4. **GATA3 is rapidly degraded via a 26 S proteasome-dependent pathway.** *A*, GATA3 is degraded in a 26 S proteasome-dependent manner. Myc-tagged GATA3 was introduced into COS cells. The transfected cells were harvested at the indicated time after treatment with cycloheximide (100 μM) and proteasome inhibitor MG132 (50 μM). Nuclear and cytoplasmic extracts were prepared, and the amount of Myc-tagged GATA3 was assessed by immunoblotting (*IB*) with anti-Myc mAb. Arbitrary densitometric units are shown below each band. *B*, splenic CD4 T cells were stimulated under Th2− conditions for 4 days. The cells were then treated with CHX (100 μM) in the presence or absence of MG132 (20 μM) for the indicated times. Total cell extracts were prepared using RIPA lysis buffer. The amount of GATA3 protein was assessed by immunoblotting with anti-GATA3 mAb. Arbitrary densitometric units are shown below each band. *C*, developing Th2 cells prepared as in *B* were treated with CHX (100 μM) in the presence or absence of MG132 (20 μM) or lactacystin (20 μM) for 2 h. The amount of GATA3 protein was assessed as in *B*. *D*, schematic representation of Myc-tagged GATA3 mutants. Wild type GATA3 (*WT*) and three mutants (*dCT*, *dCF*, and *dZF*) are shown with the location of the Myc tag (*Myc*), the transactivation domain (*TAD*), and the two zinc finger domains (*N* and *C*). *E*, degradation and MG132-induced rescue of GATA3 mutants. Myc-tagged GATA3 mutants were transfected into COS7 cells, and the transfectants were treated with the indicated inhibitors for 2 h. The amounts of Myc-tagged GATA3 were assessed as in *A*. Arbitrary densitometric units are shown below each band. Four independent experiments were performed with similar results.

blotting with anti-Ub mAb was performed after anti-FLAG immunoprecipitation (Fig. 5*A*). Significantly increased levels of multiubiquitination (appears as smear) were observed in the wild type GATA3 transfectants compared with control vector, and the levels were significantly increased in the presence of MG132 (Fig. 5*A*, compare *lanes 3* and *4*). The levels of multiubiquitination appeared to be equivalent in the case of the dCT mutant (Fig. 5*A*, *lanes 5* and *6*), slightly decreased in the dCF mutant (*lanes 7* and *8*), and greatly reduced in the dZF mutant (*lanes 9* and *10*). The levels of FLAG-tagged protein in total (nuclear and cytoplasmic) lysates were not reduced in these transfectants (Fig. 5*A*, *right panel*). Thus, multiubiquitination of GATA3 protein can be readily demonstrated, and it appears that the dZF mutant is the least modified among the mutant forms tested. The multiubiquitination on truncated GATA3 mutants was further assessed by anti-FLAG immunoblotting (Fig. 5*B*). The levels of multiubiquitination were slightly decreased in the dCF mutant and greatly reduced in the dZF mutant, indicating again that the C-terminal region of GATA3, including the zinc finger region (residues 261–315), is critical for ubiquitination.

We performed an *in vitro* ubiquitination assay as a further demonstration of the multiubiquitination on GATA3. 293T cells were transfected with FLAG-tagged GATA3, and 3 days later, the cells were treated with MG132 for 2 h. *In vitro* ubiquitination was performed after anti-GATA3 immunoprecipitation, and ubiquitinated GATA3 was detected by immunoblotting with anti-GATA3 (Fig. 5*C*, *left panel*). Concurrently, anti-FLAG immunoprecipitation and anti-FLAG immunoblotting were done (Fig. 5*C*, *right*). Although variable levels of multiubiquitinated GATA3 were detected without *in vitro* ubiquitination (Fig. 5*C*, *lane 4*), significantly increased signals with new bands (indicated by *) were detected after *in vitro* ubiquitination (*lane 8*). Similarly, increased ubiquitination was readily detected after *in vitro* ubiquitination in the anti-FLAG immunoblot (Fig. 5*C*, compare *lanes 12* and *14*).

Next, in order to examine the involvement of activation of the ERK-MAPK cascade in the GATA3 ubiquitination, we assessed the effect of PMA to activate the MAPK cascades and U0126 to inhibit selectively the ERK-MAPK cascade on the ubiquitination of GATA3. 293T cells transfected with FLAG-tagged GATA3 were treated with PMA in the presence or absence of U0126, and then the levels of ubiquitination on GATA3 were assessed (Fig. 5*D*). Treatment with PMA resulted in a reduction in the degree of ubiquitination of GATA3, and this effect could be reversed significantly by the addition of U0126, suggesting that the ubiquitination of GATA3 is regulated by the activation of ERK MAPK. Similarly, in developing Th2 cells, the ubiquitination of GATA3 protein was detected when the cells were treated with MG132 (20 μM) for 2 h, and the levels in ubiquitination were enhanced in the presence of U0126 (data not shown).

Mdm2 Acts as a Ubiquitin E3 Ligase for GATA3—We wanted to identify possible E3 ligase for GATA3 in developing Th2 cells. Since the association with specific substrates is critical for the function of E3 ligases (32, 45), we first examined the physical association of GATA3 with known E3 ligases that are expressed in lymphocytes (Mdm2, Itch, E6-AP, and Cbl-b).

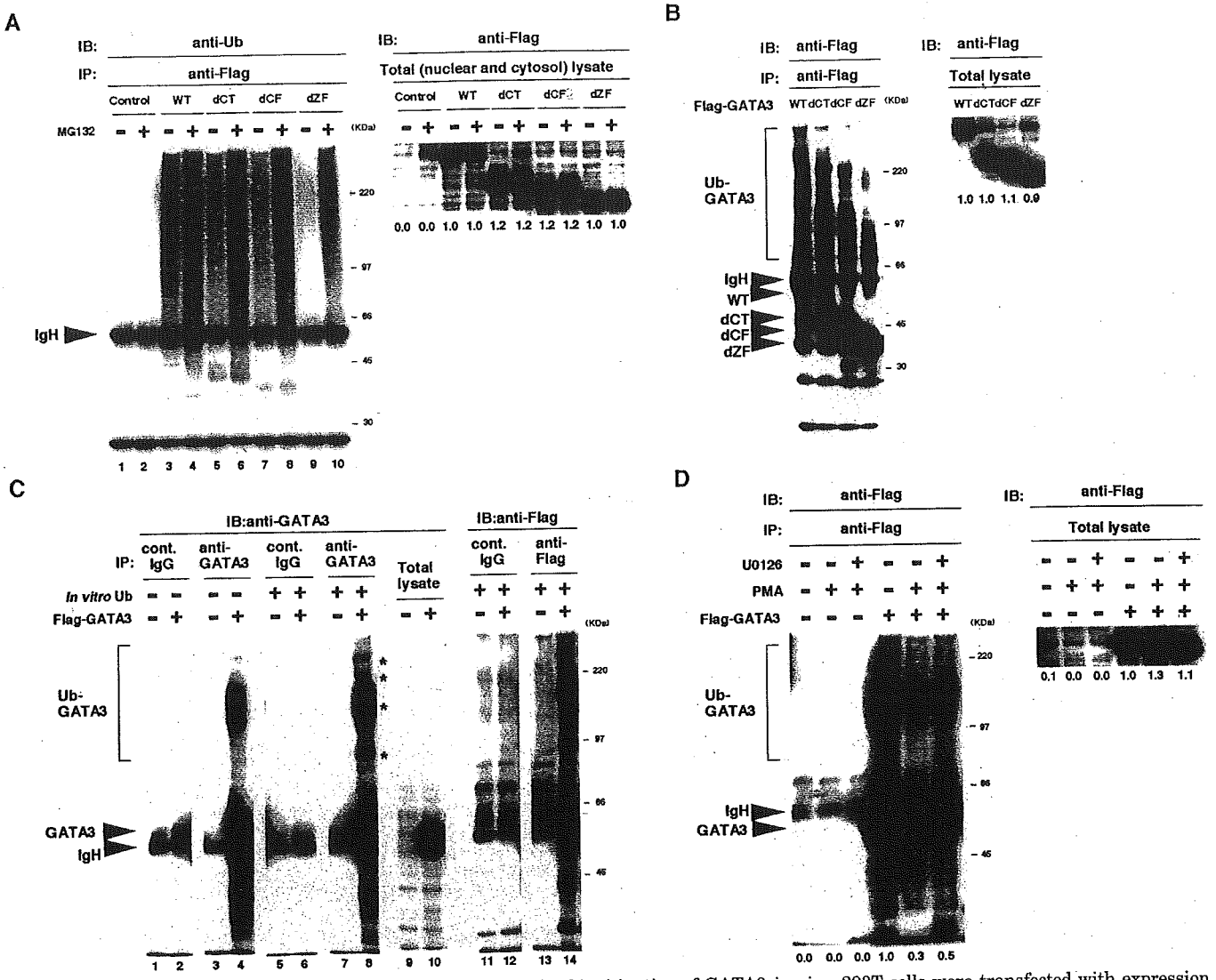

FIG. 5. **GATA3 was ubiquitinated *in vivo* and *in vitro*.** *A*, ubiquitination of GATA3 *in vivo*. 293T cells were transfected with expression plasmids encoding FLAG-tagged GATA3 wild type (*WT*) and mutants (*dCT*, *dCF*, and *dZF*), and 72 h later were treated with MG132 (50 μM) for 2 h. FLAG-tagged GATA3 was immunoprecipitated (*IP*) with anti-FLAG mAb, and the level of ubiquitination was assessed by immunoblotting (*IB*) with anti-Ub mAb (*left panel*). The levels of FLAG-tagged transfected protein in the total (nuclear and cytoplasmic) lysates were also assessed by anti-FLAG immunoblotting (*right panel*). Arbitrary densitometric units are shown below each lane. *B*, FLAG-tagged GATA3 mutants were immunoprecipitated with anti-FLAG mAb, and the levels of ubiquitination were visualized by immunoblotting with anti-FLAG mAb. The positions of migration of ubiquitinated GATA3 (*Ub-GATA3*), nonubiquitinated GATA3 wild type (*WT*), and mutants (*WT*, *dCT*, *dCF*, and *dZF*), and IgH are indicated. Total (nuclear and cytoplasmic) lysates (3 μl) were also run in parallel. Arbitrary densitometric units are shown below each lane. *C*, ubiquitination of GATA3 *in vitro*. 293T cells were transfected with FLAG-tagged GATA3, and 72 h later the cells were treated with MG132 (50 μM) for 2 h. Immunoprecipitates with anti-GATA3 or anti-FLAG mAb were subjected to *in vitro* ubiquitination assay. Ubiquitinated GATA3 was visualized by immunoblotting with anti-GATA3 or anti-FLAG mAb. The positions of migration of ubiquitinated GATA3 (*Ub-GATA3*), nonubiquitinated GATA3 (*GATA3*), and IgH are indicated. The Ub-GATA3 bands that appeared after *in vitro* ubiquitination are indicated by *asterisks*. Total lysates (10 μl) were also run in parallel. *D*, ERK-MAPK cascade controls GATA3 ubiquitination. 293T cells were transfected with FLAG-tagged GATA3. Three days after transfection, the cells were treated with PMA (10 ng/ml) and U0126 (20 μM) for 3 h and then treated with MG132 (50 μM) for 2 h. GATA3 was immunoprecipitated with anti-FLAG mAb and visualized with anti-FLAG immunoblotting. Arbitrary densitometric units of the major Ub-GATA3 band are shown below each lane. Total lysates (3 μl) were also run in parallel.

Freshly isolated splenic CD4 T cells were stimulated with immobilized anti-TCR mAb under Th2− conditions for 3 days in the presence or absence of U0126. Immunoprecipitates with anti-GATA3 mAb were subjected to immunoblotting with anti-Mdm2 mAb and anti-GATA3 mAb (Fig. 6*A*) and with specific antibodies for several E3 ligases (Fig. 6*B*). Large amounts of Mdm2 were detected in the GATA3− precipitates from U0126-treated cells, suggesting association of Mdm2 with GATA3, although the amount of GATA3 is significantly reduced (~1/3) in the U0126-treated cells (Fig. 6*A*). Although there were substantial amounts of E6-AP, Itch, or Cbl-b molecules in developing Th2 cells, no significant quantity of E6-AP, Itch, or Cbl-b was detected in the anti-GATA3 immunoprecipitates under the conditions where substantial amounts of Mdm2 were detected (Fig. 6*B*). Thus, the association of Mdm2 with GATA3 appeared to be more selective than that of other E3 ligases (Itch, E6-AP, and Cbl-b).

To characterize further the Mdm2 association with GATA3, 293T cells were transfected with FLAG-tagged GATA3 and their mutants (dCT, dCF, and dZF) and were treated with MG132 for 2 h. Immunoprecipitates with anti-FLAG mAb were immunoblotted with anti-Mdm2 mAb. As shown in Fig. 6*B*, *upper panel*, association of Mdm2 with GATA3 was readily detected, and the association was apparently decreased in the dCF mutant and almost undetectable in the dZF mutant. The amounts of Mdm2 and FLAG-GATA3 protein in these transfectants were similar (Fig. 6*B*, *middle* and *bottom panels*). Thus Mdm2 appears to be constitutively associated with wild type

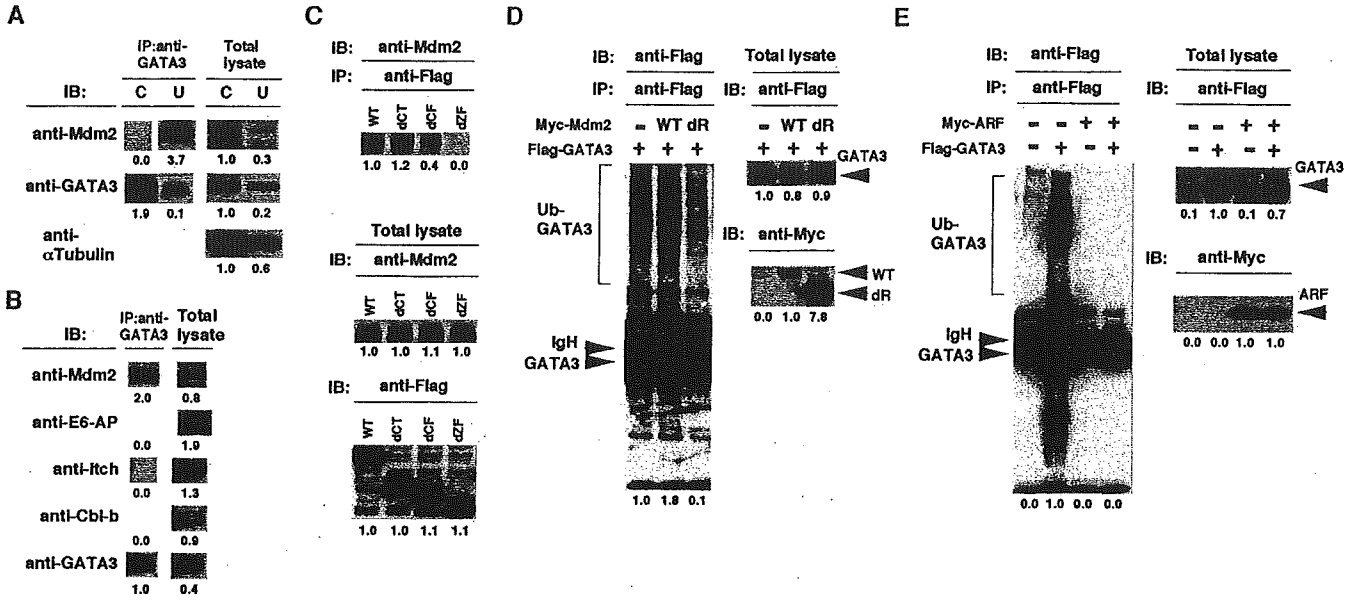

FIG. 6. **Mdm2 acts as an E3 ligase for GATA3.** *A*, Mdm2 is associated with GATA3 in developing Th2 cells. Splenic CD4T cells were cultured under Th2− conditions for 3 days in the presence (*U*) or absence (*C*) of U0126 (20 μM). Immunoprecipitates with anti-GATA3 mAb from the cultured cells were subjected to immunoblotting (*IB*) with anti-Mdm2 and anti-GATA3. Total lysates were also run in parallel. Arbitrary densitometric units are shown below each band. *B*, splenic CD4T cells were cultured under Th2− conditions for 3 days in the presence of U0126 (20 μM). Immunoprecipitates with anti-GATA3 mAb were subjected to immunoblotting with anti-Mdm2, anti-E6-AP, anti-Itch, anti-Cbl-b, and anti-GATA3 antibodies. Total lysates were also run in parallel. Arbitrary densitometric units are shown below each band. *C*, Mdm2 is associated with GATA3 in 293T cells. 293T cells were transfected with FLAG-tagged GATA3 and mutants (*dCT*, *dCF*, and *dZF*). Three days after transfection, cells were treated with MG132 (50 μM) for 2 h. Immunoprecipitates (*IP*) with anti-FLAG mAb were immunoblotted with anti-Mdm2 mAb. Total lysates (10 μl) were run in parallel. Arbitrary densitometric units of the band are shown below each band. *D*, Mdm2 acts as E3 ligase for GATA3. 293T cells were transfected with FLAG-tagged GATA3 and Myc-tagged wild type (*WT*) Mdm2 or Myc-tagged RING finger-deleted Mdm2. Three days after transfection, cells were treated with MG132 (50 μM) for 2 h. Immunoprecipitates with an anti-FLAG mAb were subjected to immunoblotting with anti-FLAG mAb. Mdm2 was detected by immunoblotting with anti-Myc mAb. The positions of migration of ubiquitinated GATA3 (*Ub-GATA3*), nonubiquitinated GATA3 (*GATA3*), IgH, and Myc-tagged wild type Mdm2 (*WT*) and Myc-tagged RING finger-deleted Mdm2 (*dR*) are indicated. Arbitrary densitometric units of the major Ub-GATA3 band are shown below each lane. *E*, overexpression of ARF suppressed multiubiquitination of GATA3. 293T cells were transfected with FLAG-tagged GATA3 and Myc-tagged ARF. FLAG-tagged GATA3 were immunoprecipitated with anti-FLAG mAb, and the levels of ubiquitination were visualized by immunoblotting with anti-FLAG mAb. Transfected Myc-tagged ARF was detected by immunoblotting with anti-Myc mAb. The positions of migration of ubiquitinated GATA3 (*Ub-GATA3*), nonubiquitinated GATA3 (*GATA3*), IgH and Myc-tagged ARF (*ARF*) are indicated. Arbitrary densitometric units of the major Ub-GATA3 band are shown below each lane.

GATA3 in 293T cells, and the C-terminal region including the zinc finger domain is important for association.

To assess whether Mdm2 has E3 ligase activity for GATA, FLAG-tagged GATA3 and Myc-tagged wild type and a RING finger-deleted Mdm2 were expressed in 293T cells. The RING finger domain of Mdm2 is critical for E3 ligase activity for p53 (46). Immunoprecipitates with an anti-FLAG mAb were subjected to immunoblotting with anti-FLAG mAb. Overexpression of wild type Mdm2 led to increased levels of multiubiquitination of GATA3 (Fig. 6*C*, *1st two lanes*). More interestingly, the ubiquitination of GATA3 was greatly reduced when the RING finger-deleted Mdm2 was expressed. The levels were much lower than those in cells without Mdm2 transfection, suggesting a dominant-negative feature of the RING finger-deleted Mdm2 to endogenously expressing Mdm2 in 293T cells. The efficiency of expression of the transfected RING finger-deleted Mdm2 was considerably high (Fig. 6*C*, *right panel*), probably because of the inhibition of ubiquitination itself (47, 48).

It is known that cyclin-dependent kinase inhibitor 2A, a tumor suppressor molecule ($p19^{ARF}$ in the mouse and $p14^{ARF}$ in human cells), binds tightly to Mdm2 and prevents Mdm2-mediated p53 ubiquitination (49). Consequently, we tested the effect of expression of ARF in the GATA3 ubiquitination. As shown in Fig. 6*D*, *left panel*, the introduction of ARF resulted in nearly complete inhibition of the multiubiquitination of GATA3 in 293T cells. Collectively, these results support the notion that Mdm2 has E3 ligase activity for GATA3.

Mdm2 Is Involved in GATA3 Ubiquitination in T Cells—In order to provide additional evidence to support the role of Mdm2 as a major E3 ligase, we attempted the inhibition of GATA3 ubiquitination in T cells by using Mdm2 RNA interference. Mdm2 siRNA was introduced in a T cell line TG40 at a high level. The expression levels of Mdm2 protein were reduced significantly upon the introduction of the Mdm2 siRNA as compared with the control (Fig. 7*A*, *right top panel*). As anticipated, GATA3 ubiquitination was reduced substantially by the Mdm2 siRNA treatment (Fig. 7*A*, *left panel*, *lane 1.0 versus 0.4*). These results help to confirm the involvement of Mdm2 in GATA3 ubiquitination in TG40 T cells.

Finally, we wanted to address the function of Mdm2 in primary developing Th2 cells. The mRNA expression of Mdm2 was similar between developing Th1 and Th2 cells (data not shown). Our attempts to silence Mdm2 by RNA interference were unsuccessful with the primary T cells, probably because of robust proliferation of developing Th2 cells in the *in vitro* cultures. Thus we took an alternative approach to inhibit GATA3 ubiquitination and to facilitate Th2 cell differentiation by introducing a RING finger-deleted Mdm2 (Mdm2-dR) into developing Th2 cells (Fig. 7, *B* and *C*). There was substantial expression of endogenous Mdm2 in primary developing Th2 cells, and furthermore, the level of introduced Mdm2-dR by a retrovirus vector was lower than that of endogenous Mdm2 (Fig. 7*B*, *right top panel*). Nevertheless, GATA3 ubiquitination was significantly reduced (Fig. 7*B*, *left panel*, *lane1.0 versus 0.6*). Moreover, there was significant increase in the generation of IL-4-producing Th2 cells (38.4 *versus* 54.0%) with higher mean fluorescence intensity in IL-4 fluorescence (114.3 *versus* 163.6) when Mdm2-dR was expressed in developing Th2 cells (Fig. 7*C*). Thus, we conclude that Mdm2 is involved in GATA3

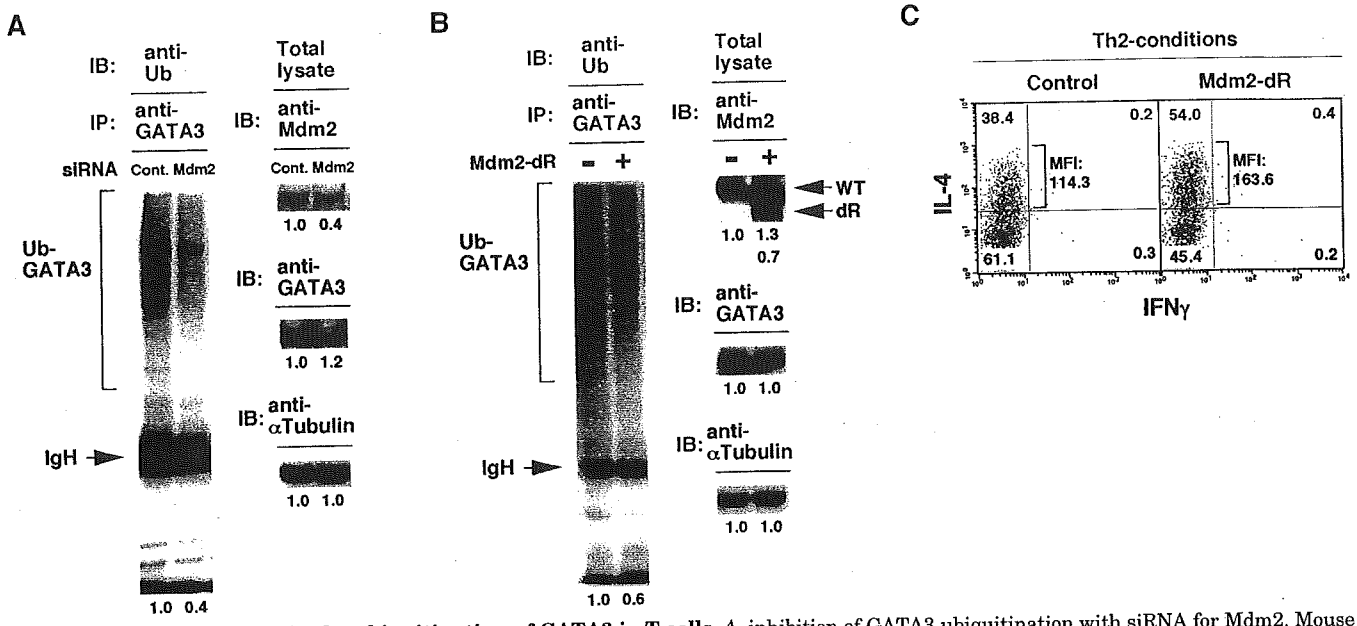

FIG. 7. **Mdm2 is involved in the ubiquitination of GATA3 in T cells.** *A*, inhibition of GATA3 ubiquitination with siRNA for Mdm2. Mouse T cell line TG40 cells were transfected with siRNA specific for Mdm2. Three days after transfection, cells were cultured at 37 °C for 2 h in the presence of MG132 (20 μM). Then the ubiquitination of GATA3 was assessed. Arbitrary densitometric units of the major Ub-GATA3 band are shown below each lane. The expression of Mdm2, GATA3, and α-tubulin is shown on the *right panels*. *IP*, immunoprecipitation; *IB*, immunoblot. *B*, effect of a dominant-negative (Ring-finger deleted) form of Mdm2 (*Mdm2-dR*) on the ubiquitination of GATA3 in developing Th2 cells. Splenic CD4 T cells were stimulated as described in Fig. 1, and the cells were infected with retrovirus encoding Mdm2 mutant bicistronically with human NGFR. Three days after infection, the NGFR-positive population was enriched by MACS and cultured at 37 °C for 2 h in the presence of MG132 (20 μM). Then the ubiquitination of GATA3 was assessed. Arbitrary densitometric units of the major Ub-GATA3 band are shown below each lane. The expression of Mdm2 (WT and dR), GATA3, and α-tubulin is shown on the *right panels*. *C*, expression of dominant-negative Mdm2 enhanced Th2 cell development. Freshly prepared splenic CD4 T cells were stimulated under Th2− skewed conditions and infected with retrovirus encoding Mdm2-dR bicistronically with EGFP on day 2. Three days later, the cells were stimulated with anti-TCR and were subjected to cytoplasmic IFNγ/IL-4 staining. Mean fluorescence intensity (*MFI*) of IL-4-staining is also indicated.

ubiquitination in primary Th2 cells and control Th2 cell differentiation.

DISCUSSION

In this paper, we provide evidence indicating that TCR-mediated activation of the Ras-ERK MAPK cascade controls GATA3 protein stability through the ubiquitin-proteasome pathway. The induction of GATA3 protein in developing Th2 cells is crucial for the differentiation of Th2 cells (18, 19). IL-4-induced STAT6 activation initiates transcription of GATA3 (50). However, among the issues that remain to be clarified is how the expression of GATA3 protein is controlled in developing Th2 cells. Here we demonstrate the following. (i) GATA3 protein is very unstable with a short half-life (~1 h) in transfectants (Fig. 4*A*) and developing Th2 cells (Fig. 4*B*). (ii) The degradation of GATA3 is dependent on the 26 S proteasome pathway (Fig. 4, *A–C*). (iii) GATA3 is ubiquitinated both *in vivo* and *in vitro* (Fig. 5). (iv) The deletion of the possible ubiquitination sites of GATA3 led to stable expression of GATA3 and reduced ubiquitination (Fig. 4*E* and Fig. 5, *A* and *B*). From these results, we conclude that the fate of GATA3 in developing Th2 is highly dependent on degradation through the ubiquitin-proteasome system. Concurrently, we show that activation of the ERK-MAPK cascade facilitated GATA3-mediated chromatin remodeling at the Th2 cytokine gene loci (Fig. 1) and inhibited the degradation (Figs. 2 and 3) and ubiquitination of the GATA3 molecule (Fig. 5*D*). Because the Ras-ERK MAPK cascade in naive CD4 T cells is activated by stimulation of TCR and not of IL-4R (11), the activation of the Ras-ERK MAPK cascade detected in the experiments must be a consequence of TCR-mediated signaling. Therefore, stabilization of GATA3 by the activation of the Ras-ERK MAPK cascade could be the mechanism that accounts for an essential role for TCR-mediated signaling in Th2 cell differentiation.

Our studies identify Mdm2 as a possible E3 ligase for GATA3. Mdm2 was shown to be associated with GATA3 in developing Th2 cells and 293T cells (Fig. 6, *A–C*). The overexpression of wild type Mdm2 induced increased ubiquitination on GATA3, whereas that of RING finger-deleted mutant Mdm2 resulted in the inhibition of GATA3 ubiquitination in 293T cells (Fig. 6*D*). Overexpression of ARF, an inhibitor of Mdm2, resulted in almost complete suppression of multiubiquitination of GATA3 in 293T cells (Fig. 6*E*). Moreover, the introduction of siRNA for Mdm2 into the T cell line TG40 resulted in the reduction in the ubiquitination of GATA3 protein (Fig. 7*A*). The generation of IL-4-producing Th2 cell was enhanced by the expression of RING finger-deleted mutant Mdm2, suggesting a physiological role for Mdm2 in Th2 cell differentiation (Fig. 7*C*).

Mdm2 is known to promote degradation of p53 through a ubiquitin-dependent proteasome pathway (49, 51). Mdm2 acts as an E3 ubiquitin ligase specific for p53 *in vitro* (51). The RING finger domain of Mdm2 is critical for E3 ligase activity for p53 (46). The phosphorylation of p53 at serine 15, threonine 18, and serine 20 led to the reduction of Mdm2 binding and enhancement of p53 stabilization and accumulation (49). Most interestingly, amino acid residues 9–20 (**SVEPPLSQETFS**) of human p53, which are reported to be important for the binding for Mdm2, are highly homologous to amino acid residues 131–142 of human GATA3 (**SVYPPASSSSLS**) and mouse GATA3 (**SVYPPASSSSLA**). In these regions, serine/threonine phosphorylation sites and surrounding proline residues (indicated in boldface above) occur in similar patterns between p53 and GATA3. Moreover, GATA3 has other structural similarities with p53, *e.g.* possible lysine ubiquitination sites at the C-terminal region (364–390 in p53 and 396–422 in GATA3) and a proline-rich regulatory region (69–101 in p53 and 146–178 in

GATA3), which are reported to have important roles in post-translational modification and functions of p53 (52, 53). Thus, it is reasonable to expect that a similar set of molecular events operating in ubiquitination of p53 would occur in the case of GATA3

In our experiments with truncation mutants, truncation of the above-mentioned lysine ubiquitination sites in the C-terminal region 396–422 in GATA3 (dCT mutant) resulted in small effects on degradation (Fig. 4*E*) and multiubiquitination of GATA3 (Fig. 5, *A* and *B*). A small but significant effect was observed in the dCF mutant (Fig. 5, *A* and *B*). A more prominent effect was observed by deletion of residues 261–443 (dZF mutant) (Fig. 4*E* and Fig. 5, *A* and *B*). The 261–315 region contains three lysine residues (293, 303, and 305 in human GATA3) and a nuclear localization signal (KPKRR). It is known that the degradation of p53 is controlled also by the localization of p53 and Mdm2 (54, 55). Thus, similar to p53, the degradation of GATA3 appears to be controlled by both the ubiquitination process and nuclear/cytosol localization of the protein.

The Ras-ERK MAPK cascade regulates stability of various proteins, including Myc, MKP-1, ATF2, and p53 through a mechanism involving serine phosphorylation (56–61). In addition, ERK MAPK-dependent phosphorylation and the subsequent enhancement of the transcriptional activities for GATA2 and GATA4 have been suggested (62, 63). GATA3 was phosphorylated by activated p38 MAPK in cAMP-treated T cells, suggesting a possible regulatory role for the MAPK cascade in GATA3 function (64). In fact, our preliminary results indicate that an active form of ERK2 directly phosphorylates GATA3 protein *in vitro*, and PMA-induced GATA3 phosphorylation was significantly inhibited by U0126 in transfected COS7 cells.[2] GATA3 protein contains numerous Ser/Thr residues (93 residues out of 444 residues) and possesses 35 putative phosphorylation sites, and thus the precise location of critical amino acid residues responsible for the MAPK-dependent phosphorylation remains unclear at this time. Thus, it appears to be reasonable to surmise that the activation of the ERK-MAPK cascade induces GATA3 phosphorylation and prevents its ubiquitin-mediated degradation through the 26 S proteasome.

Our studies with primary T cells indicated that the ERK-MAPK cascade plays a major role in the regulation of GATA3 protein expression. Although we observed the activation of the p38 MAPK cascade after PMA treatment in developing Th2 cells,[2] a specific inhibitor for the p38 MAPK cascade (SB203580) did not affect the GATA3 protein expression. However, it is still possible that the activation of the p38 MAPK cascade may have some effect on the expression of GATA3 protein as well as the function of GATA3 (64).

In summary, TCR-mediated activation of the Ras-ERK MAPK cascade controls the stability of GATA3 protein by a ubiquitin-proteasome-dependent mechanism. IL-4-induced STAT6 activation is required for the induction of GATA3 transcription. Thus, efficient activation of both signaling pathways and resulting stable GATA3 expression, therefore, are crucial for chromatin remodeling at the Th2 cytokine gene loci and successful Th2 cell differentiation.

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Prolonged skin allograft survival by *IL-10* gene-introduced CD4 T cell administration

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Abstract

Both CD4 and CD8 T cells play crucial roles in immune responses in transplantation. Immunosuppressive drugs, such as FK506 and cyclosporin A, block the priming of alloreactive CD4 T_h cells and the subsequent induction of allospecific CD8 cytotoxic effector T cells and inhibit allograft rejection. However, the desire to minimize chronic complications that may arise from the use of immunosuppressive agents drives the search for additional strategies for immunosuppression of allograft rejection. In this study, CD4 or CD8 T cells into which the *IL-10* gene is introduced using an adenovirus vector containing human IL-10 (hIL-10) cDNA (Ad-hIL-10) and into mouse T cells transgenic for the Coxsackie virus and adenovirus receptor form a model system to study the effect of administration of IL-10-secreting T cells on the survival of the allogenic skin grafts. Ad-hIL-10-infected CD4 and CD8 T cells secreted a large amount of hIL-10 for 3–4 days in culture *in vitro*. Ad-hIL-10-infected CD4 T cells administered *in vivo* could be detected in the spleen for 7 days post-transfer. Significantly prolonged survival of grafts was observed in animals that received either Ad-hIL-10-infected activated CD4 T cells or T_h2-skewed CD4 T cells as compared with controls. Furthermore, substantial enhancement of the effect was observed in B6.C-$H2^{bm1}$/ByJ transplants. Thus, a direct manipulation of T cells through the introduction of the immunosuppressive cytokine gene *IL-10* may be a novel strategy for the control of allograft rejection.

Introduction

CD4 and CD8 T cells play critical roles during allograft rejection in transplantation (1, 2). In various experimental allotransplantation systems, rejection appears to be associated with increased production of T_h1 cytokines (IL-2 and IFNγ), and the suppressive roles of T_h2 cytokines such as IL-4 and IL-10 on rejection have been reported (3, 4). Immunosuppressive drugs, such as FK506 and cyclosporin A (CsA), inhibit the priming of alloreactive CD4 T_h cells and the subsequent induction of allospecific CD8 cytotoxic effector T cells very efficiently. Both of these cellular processes are critical in allograft rejection (5, 6). However, the treatment with FK506 and CsA may be accompanied by several side effects, particularly in patients treated for long periods (7, 8). Thus, the establishment of additional strategies for immunosuppression of allograft rejection to minimize complications is desirable.

Several investigators have reported that a strategy for tolerance induction in allograft rejection involves the introduction of immunosuppressive cytokine genes to the host (9–13).

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Several viral vector systems have been used for these kinds of gene therapies (14) and among one of the most popular gene-transfer systems is a retrovirus system (15). Retroviruses are integrated in the genome, and the effect of the transgene can be observed for a long time. However, infection by retroviral vectors requires dividing cells, which limits the application for *in vivo* gene therapy (16, 17). Another system is an adenovirus-mediated gene-transfer system (18). There are several advantages for the adenovirus vector system for the induction of immunosuppression in allograft rejection. (1) Expression is transient and so prospects for side effects due to long-term treatment would be minimal. (2) Preparation of high-titer virus stocks can be easily achieved. (3) Various new adenovirus-related vectors are being investigated, and these may help solve the issue of possible side effects. However, several disadvantages have been reported (19, 20). First, the generation of antibodies against adenoviral antigens may limit the repeated administration of adenovirus vectors or adenovirus-infected cells and may in fact be harmful to the host. Also, CD8 cytotoxic T cells specific for adenovirus may be generated in the host, and infected cells would be eliminated very quickly. Finally, lymphocytes including T cells express a limited amount of Coxsackie virus and adenovirus receptor (CAR) on their cell surface, which may make gene transfer into lymphocytes by adenovirus vectors very difficult from a practical point of view.

IL-10 is produced by T cells, B cells and macrophages and plays potent immunosuppressive roles by inhibiting the production of pro-inflammatory cytokines, the expression of MHC class II antigens and antigen-presenting function (21). In addition, IL-10 can down-regulate IL-12 expression, resulting in a decrease in T_h1 cell differentiation (22). Recently, it has been reported that IL-10 drives the generation of a unique CD4 T cell subset, T regulatory cell type-1, which suppresses antigen-specific immune responses and pathologic inflammation *in vivo* (23, 24).

CAR transgenic (Tg) mice were established in order to overcome the limited expression of CAR on T cells (25). In the present study, we demonstrate the efficient adenovirus vector-mediated *IL-10* gene transfer into CAR Tg T cells and we analyzed the distribution of such adenovirus-infected T cells into the lung, liver and spleen following the administration of these cells into mice. Using an allo-skin graft model system, we observed significant immunosuppressive effects following administration of adenovirus vector containing human *IL-10* gene (Ad-hIL-10)-infected activated CD4 T cells or T_h2 cells. Thus, the introduction of suppressive cytokine genes into CD4 T cells and the subsequent administration of these cells may be a novel cell therapy approach for inducing the long-term survival of allografts.

Methods

Animals

C57BL/6 (H-2^b) male mice were used as recipients, and BALB/c (H-2^d) and B6.C-*H2*^{bm1}/ByJ (Bm1) male mice were used as skin graft donors. C57BL/6 and BALB/c mice were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and Bm1 from the Jackson Laboratories. Tg mice expressing CAR under the control of an *lck* proximal promoter (CAR Tg mice) in the C57BL/6 background have been described previously (25). All mice used in this study were maintained under specific pathogen-free conditions and were used at 7–12 weeks of age (body weight, 20–25 g). Animal care was in accordance with the guidelines of Chiba University.

Flow cytometry analysis

In general, one million cells were stained with antibodies as indicated according to a standard method (26). The reagents used are as follows: anti-CD4–FITC (RM4-1–FITC), anti-CD8–FITC (53.6-72–FITC), anti-CD8–PE (53.6-72–PE) and streptavidin–PE purchased from BD-PharMingen (La Jolla, CA, USA). For detecting human Coxsackie virus and adenovirus receptor (hCAR), biotinylated anti-CAR antibody (RmcB) (25) and streptavidin–PE were used. Two-color flow cytometric analysis was performed using FACSCalibur (Becton Dickinson, Mountain View, CA, USA).

Adenovirus vectors

AdCMVhIL-10 (Ad-hIL-10) and AdCMV*lacZ* (adenovirus vector containing *lacZ* gene, Ad-LacZ) were described previously (11). An empty adenovirus vector AxCAwt (Adex1Cawt) was reported previously (27). The preparation of adenovirus supernatant was performed as described (27). The titer of the viral stocks [infectious units (i.f.u.)] as determined by Adeno-X™ Rapid Titer Kit (BD Bioscience) was 2.8×10^9 i.f.u. ml^{-1}.

Cell culture and adenovirus infection into CAR$^+$ T cells

Splenocytes were stained with anti-CD4–FITC or anti-CD8–FITC, and then the CD4 or CD8 T cells were purified using anti-FITC magnetic beads (Miltenyi Biotec) and an Auto MACS sorter® (Miltenyi Biotec), yielding a purity of >98% as described (28). Naive CD4 and CD8 T cells were stimulated for 6 days under several conditions as described (28, 29): (1) activated CD4 T cells—naive CD4 T cells (2×10^6) were cultured in a 24-well plate (Corning) with immobilized anti-TCR mAb (1μg ml^{-1}) in the presence of IL-2 (25 U ml^{-1}) for 2 days. The cultured T cells were transferred to new wells and cultured for another 4 days in the presence of only IL-2 (25 U ml^{-1}). (2) Type-2 cell differentiation (T_h2 or Tc2)—naive CD4 T cells (2×10^6) or CD8 T cells (1.5×10^6) were stimulated with immobilized anti-TCR mAb for 2 days in the presence of IL-2 (25 U ml^{-1}), IL-4 (100 U ml^{-1}) and anti-IFNγ mAb (R4-6A2, 12.5% culture supernatant). The cultured T cells were transferred to new wells and cultured for another 4 days in the presence of only the cytokines present in the initial culture. Ad-hIL-10 virus vector (2.8×10^8 i.f.u.) was added to the T cell culture on day 6. The amounts of secreted human IL-10 (hIL-10) and mouse IL-10 were determined by ELISA (OptEIA™ Human IL-10 Set, OptEIA™ Set mouse IL-10; BD Bioscience).

Allo-MLR (Mixed lymphocyte reaction)

Responder C57BL/6 CD4 T cells (0.1×10^6 or 0.3×10^6) purified by an Auto MACS sorter® were cultured with irradiated 1×10^6 BALB/c spleen cells (3500 rad). Graded doses of Ad-hIL-10- or Ad-LacZ-infected activated CD4 T cells harvested 12 h after infection were added at the beginning of the culture.

[^{3}H]Thymidine (0.5 μCi) was added in the last 16 h of the 5-day culture.

Skin allograft

Full-thickness abdominal skin grafts were transplanted onto the lateral thorax of the recipients and covered with sterile bactericidal gauze. The entire chest was then wrapped with an elastic bandage. The dressings were removed on day 6, and the grafts were inspected daily until the point of graft rejection, which is defined as >90% necrosis of the graft epithelium as described (30, 31).

On post-operation day (POD) 0 and POD 2, we transferred *IL-10* gene-transduced T cells (5×10^7) into recipients intravenously. A sub-therapeutic dose of FK506 (0.1 mg kg^{-1} per day, Fujisawa Pharmaceutical Co., Osaka, Japan) was administered by the intra-muscular route in some of the experiments.

Statistical analysis

Statistical analyses were performed using the Mann–Whitney U test and log-rank test. $P < 0.05$ was considered as statistically significant.

Results

Adenovirus infection of splenic CD4 T cells from CAR Tg mice

In CAR Tg mice, the majority (>90%) of CD4 and CD8 T cells expressed considerable levels of CAR on the cell surface (25). The function of CAR Tg T cells such as anti-TCR mAb-induced proliferation and the production of cytokines (IL-2, IFNγ and IL-4) is normal (data not shown). Using an adenovirus vector containing *EGFP* gene (Ad-GFP), the efficiency of adenovirus infection in CAR Tg naive and activated CD4 T cells with an immobilized anti-TCR mAb for 16 h was assessed, and highly efficient infection (>90%) was observed in both naive and activated CD4 T cells from CAR Tg mice (Fig. 1A).

IL-10 production from T cells infected with an adenovirus vector encoding the hIL-10 gene

In order to establish the most efficient cell-transfer system, three different CAR Tg T cells were prepared for *IL-10* gene introduction: activated CD4 T cells with immobilized anti-TCR mAb in the presence of IL-2 for 5 days and *in vitro*-differentiated CD4 (T_h2) and CD8 (Tc2) T cells cultured under type-2-skewed conditions (with immobilized anti-TCR, IL-2 and IL-4) for 5 days. The activated CD4 T cells, T_h2 cells and Tc2 cells were infected with 2.8×10^8 i.f.u. of AdCMVhIL-10 virus (Ad-hIL-10) on day 6, and the amount of secreted hIL-10 in the culture supernatants was measured. Very high levels of IL-10 were secreted from all the Ad-hIL-10-infected T cell populations (Fig. 1B). No hIL-10 was produced without Ad-hIL-10 infection. The production of mouse IL-10 from the cultured cells was also measured (Fig. 1C). As we expected, Tc2 cells produced a substantial amount of mouse IL-10, whereas T_h2 cells produced less but still significant levels of IL-10. No significant effect on mouse IL-10 production was observed as the consequence of the infection of Ad-hIL-10. Mouse IL-10 production was not detected in the activated CD4 T cells cultured under non-type-2-skewed conditions.

Inhibition of allo-MLR by the addition of Ad-hIL-10-infected CAR Tg CD4 T cells

In order to confirm the immunosuppressive capacity of Ad-hIL-10-infected CD4 T cells, we used the allo-MLR system in which graded doses of Ad-hIL-10-infected CD4 T cells prepared 2 days after infection were added to the C57BL/6 responder and BALB/c stimulator allo-MLR cultures. Efficient inhibition of the MLR was observed (Fig. 2). Only a few percentage of Ad-hIL-10-infected CD4 T cells caused significant inhibition of the response. No inhibition was observed by the presence of control Ad-LacZ-infected CD4 T cells (Fig. 2) or empty vector-infected CD4 T cells (data not shown). These results suggest that Ad-hIL-10-infected CD4 T cells exert a potent immunosuppressive effect on anti-allo responses *in vitro*.

Distribution of Ad-hIL-10-infected T cells administered intravenously

It was important to analyze the distribution of adenovirus-infected T cells administered intravenously to ensure that the virus infection would not significantly alter the distribution of the transferred cells. Activated CD4 T cells and *in vitro*-differentiated CAR Tg T_h2 and Tc2 cells were infected with Ad-hIL-10, and 2 days later these cells (5×10^7) were administered to C57BL/6 mice. Cell administration was done twice on days 0 and 2. The percentages of CAR-positive CD4 or CD8 T cells were monitored by flow cytometry 1, 3 and 7 days after the first transfer. Figure 3 shows the percentages of CAR-positive cells among CD4 or CD8 T cells in each of the organs analyzed. Without administration of CAR Tg T cells, essentially no CAR-positive cells were detected in these organs (see top control panel). However, large numbers (~15%) of CAR-positive CD4 T cells were detected in the lung and liver on days 3 and 7 in the mice with administration of Ad-hIL-10-infected activated CD4 T cells (Fig. 3A, middle panel). Substantial numbers of CAR-positive transferred CD4 T cells were detected in the spleen, although the frequency of the cells was significantly lower. The kinetics of CAR-positive population were similar between these three different organs. Furthermore, the introduction of sub-therapeutic dose of FK506 had no obvious effect on the distribution of the administered T cells (compare Fig. 3A, middle and bottom panels). These results suggest that combinational treatment with a sub-therapeutic dose of FK506 does not affect the distribution of Ad-hIL-10-infected activated CD4 T cells in the allograft-transplanted hosts.

When we used *in vitro*-differentiated Ad-hIL-10-infected CAR Tg T_h2 cells for transfer, significant numbers (up to 5%) of CAR-positive CD4 T cells were detected in the spleen, liver and lung (Fig. 3B). The levels in the spleen were similar to those of activated CD4 T cells, but those in the lung and liver were significantly lower (compare Fig. 3A and B). The number of CAR-positive cells peaked at the day 3 time point after administration, and the administration of sub-therapeutic doses of FK506 again had no dramatic effects. We also administered non-infected activated CD4 T cells and non-infected T_h2 cells and observed essentially the same distribution

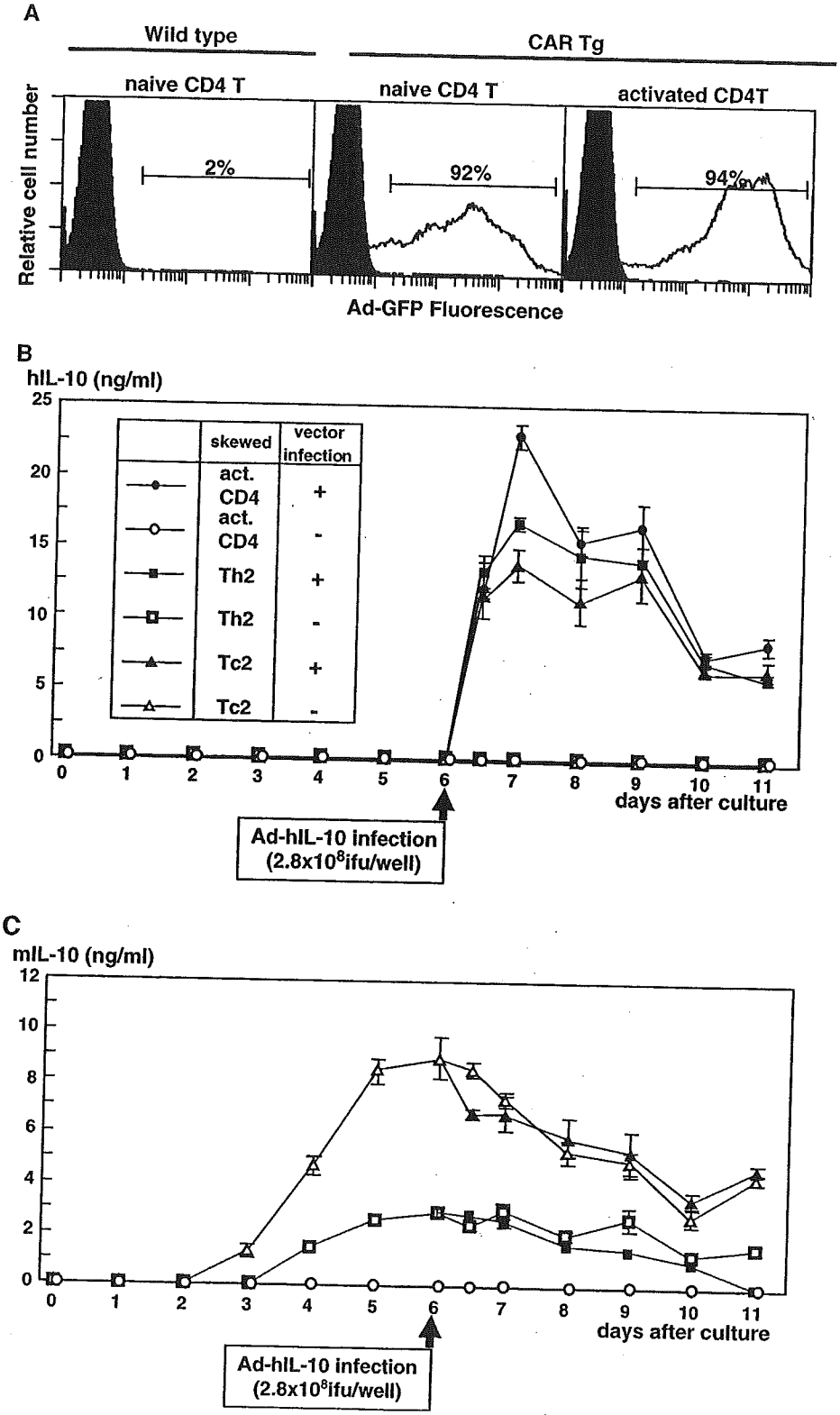


Fig. 1. IL-10 production from T cells infected with Ad-hIL-10. (A) A green fluorescence protein (GFP) gene-encoded adenoviral vector (Ad-GFP) was used for evaluating the efficiency of adenovirus infection in CAR Tg CD4 T cells. Naive CD4 T cells from wild-type and CAR Tg mice and activated CD4 T cells with anti-TCR mAb for 16 h were infected with Ad-GFP. Two days later, GFP expression was assessed by flow cytometry analysis. Percentages of GFP-positive cells are depicted in each panel. (B) The amount of hIL-10 produced by Ad-hIL-10-infected CAR Tg T cells *in vitro*. *In vitro*-differentiated T_h2 and Tc2 cells as well as activated CD4 T cells prepared as described in Methods were infected with 2.8×10^8 i.f.u. of Ad-hIL-10 (arrow), and the amounts of secreted hIL-10 in the culture supernatant were measured by ELISA. (C) The amounts of mouse IL-10 in the culture supernatant of the indicated T cell cultures were measured by ELISA. Each symbol represents the same cell preparations indicated in panel A.

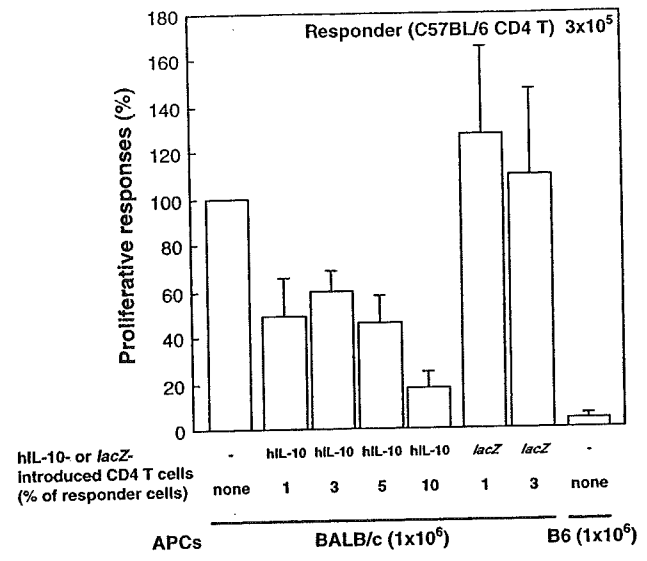


Fig. 2. Inhibition of allo-MLR by Ad-hIL-10-infected CAR Tg CD4 T cells. Graded doses of Ad-hIL-10-infected or Ad-LacZ-infected CD4 T cells prepared 12 h after infection were added to the allo-MLR cultures consisting of C57BL/6 CD4 T cell responder (3×10^5) and BALB/c stimulator cells (1×10^6). Relative values of mean [^{3}H]thymidine uptake (% counts per minute) and standard deviations of triplicate cultures are shown.

in these organs (data not shown). In contrast, we failed to detect CAR-positive CD8 T cells when Ad-hIL-10-infected Tc2 cells were used (Fig. 3C). Significant numbers of CAR-positive CD8 T cells were detected in the spleen when we injected Tc2 cells without Ad-hIL-10 or Ad-LacZ infection (data not shown). Thus, these data suggest that adenovirus infection affects the distribution or survival of this T cell subpopulation.

Effect of administration of Ad-hIL-10-infected activated CD4 T cells and T_h2 cells on BALB/c allo-skin graft survival

The major goal of this study was to determine how the administration of Ad-hIL-10-infected activated CD4 T cells, T_h2 cells or Tc2 cells would affect allo-skin graft survival. We first studied a fully allergenic system. BALB/c allo-skin grafts transplanted on C57BL/6 hosts survive for 7 ± 1days in our assay system (see groups with no administration in Table 1 and Fig. 4). No significant differences in allograft survival was observed among the experimental groups of no administration (7 ± 0.8), non-infected activated CD4 T cells (7 ± 0.9), empty vector-infected activated CD4 T cells (7 ± 0.4) and Ad-LacZ-infected activated CD4 T cells (8 ± 1.1) (Table 1, Exp. 1 and Fig. 4A). In comparison, a significant prolongation was detected in the group that received the administration of Ad-hIL-10-infected activated CD4 T cells (9 ± 1.5). In addition, in the group treated with sub-therapeutic doses of FK506 and Ad-hIL-10-infected activated CD4 T cell administration, significant prolongation was detected compared with the groups with a single treatment (11 ± 2.9 versus 8 ± 1.2, $P < 0.05$ and 11 ± 2.9 versus 9.5 ± 1.5, $P < 0.01$). These results indicate that hIL-10-secreted CD4 T cell administration induced a significant immunosuppressive effect on allo-skin grafts, and in combination with sub-therapeutic doses of FK506 an enhanced effect was obtained.

When Ad-hIL-10-infected T_h2 cells were used, significant prolonged graft survival was detected (Table 1, Exp. 2 and Fig. 4B; 10 ± 1.3 versus 7 ± 1.4, $P < 0.05$). A significant prolongation was also seen in the group with non-infected T_h2 cell administration (9.5 ± 1.9 versus 7 ± 1.4, $P < 0.01$). The addition of the treatment with a sub-therapeutic dose of FK506 appeared to have some effect, but no significant difference was detected. These results suggest that endogenous mouse IL-10 produced by non-infected T_h2 cells can have a significant effect on the survival of allo-skin grafts. No significant effect was observed by the administration of Ad-hIL-10-infected CD8 T cells (data not shown). This may be accounted for by the fact that Ad-hIL-10-infected CD8 T cells were not detected in the spleen, lung or liver (Fig. 3C).

Effect of administration of Ad-hIL-10-infected activated CD4 T cells on Bm1 or allo-skin graft survival

We next examined a system in which the allograft only differed in class I. Bm1 skin grafts were transplanted on C57BL/6 hosts and the effect of the administration of Ad-hIL-10-infected activated CD4 T cells was assessed. As can be seen in Fig. 4(C) and Table 1, Exp. 3, the survival was substantially prolonged when Ad-hIL-10-infected activated CD4 T cells were administrated (11 ± 3.1 versus 18 ± 3.7, $P < 0.0002$). No significant effect was observed with control empty vector-infected activated CD4 T cells (10 ± 2.9) or Ad-LacZ-infected activated CD4 T cells (11 ± 2.8). The addition of the treatment with a sub-therapeutic dose of FK506 appeared to have little effect on survival of Bm1 transplants (11 ± 2.6 versus 11 ± 3.1, non-significant). Even in the presence of a sub-therapeutic dose of FK506, a significant effect of Ad-hIL-10-infected activated CD4 T cells was detected (19.5 ± 1.8 versus 11 ± 2.6, $P < 0.001$). These results indicate that hIL-10-secreted CD4 T cell administration induced significant immunosuppressive effect on Bm1 allo-skin grafts and may suggest that tolerance induction is more effective in transplantation with class I-mismatched allo-skin grafts compared with those with full allo-skin grafts.

Discussion

In this report, we describe efficient *IL-10* gene transfer to CAR Tg T cells using an adenovirus vector and the distribution of the modified cells following transfer *in vivo*. A major goal of this study was to establish an approach to inhibit allograft rejection. To this end we showed that significant immunosuppressive effects could be achieved by the administration of Ad-hIL-10-infected activated CD4 T cells or T_h2 cells in allo-skin graft-rejection models. Introduction of the *IL-10* gene to CD4 T cells and subsequent administration of such cells may offer a novel cell therapy procedure for the induction of allotransplantation tolerance.

IL-10 is a well-known immunosuppressive cytokine. Recombinant cytokines, including IL-10, are, however, short lived and their effects are not easily observed by simple administration *in vivo* (32–34). The serum half-life of recombinant IL-10 after a single injection was reported to be several hours

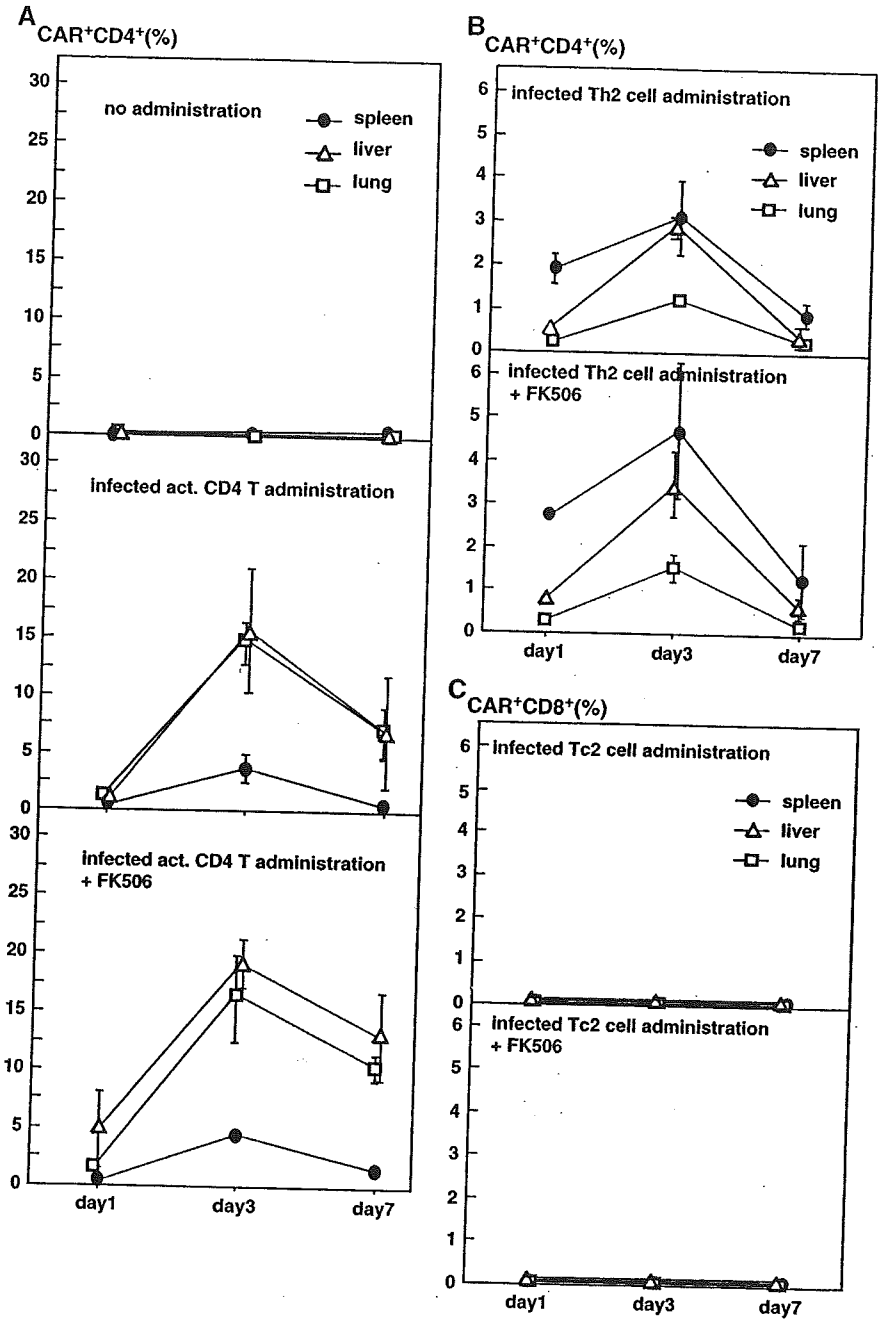


Fig. 3. Distribution of Ad-hIL-10-infected T cells administrated intravenously. Activated CD4 T cells (A), *in vitro*-differentiated T_h2 (B) and Tc2 cells (C) were infected with Ad-hIL-10, and 2 days later the cells (5×10^7) were administered into C57BL/6 mice intravenously on days 0 and 2. The percentages of CAR-positive CD4 or CD8 T cells in the indicated organs were monitored by flow cytometry 1, 3 and 7 days after the initial transfer. Where indicated, host mice were treated with a sub-therapeutic dose of FK506 (0.1 mg kg^{-1} per day). Three mice were used in each group. Mean percentage and standard deviation are shown.

(32, 33). Because of these issues, gene therapy with cytokines such as IL-10 has been considered as an alternative approach. In fact, *IL-10* gene therapy has been performed in several organ transplantation animal models (35), including rat liver (11, 36), mouse heart (9), rat heart (37), sheep cornea (13), rabbit heart (38–41) and rat lung (42, 43). Several different vectors, such as retrovirus, adenovirus, plasmid and liposomal vectors, have been used, but in all reports, the *IL-10* gene was introduced directly into the graft tissues, and potent immunosuppressive effects were generally observed. Coates *et al.* used dendritic cells (DCs) for targets of the *IL-10* gene transduction (44). They used the bovine *IL-10* gene-encoded adenovirus vector, and they observed that DCs transduced with the *IL-10* gene inhibited allostimulation and cytolytic activity.

In contrast to the above-mentioned previous experimental systems, we used CD4 T cells as a target of the *IL-10* gene introduction. It is well known that in most of the inflammatory

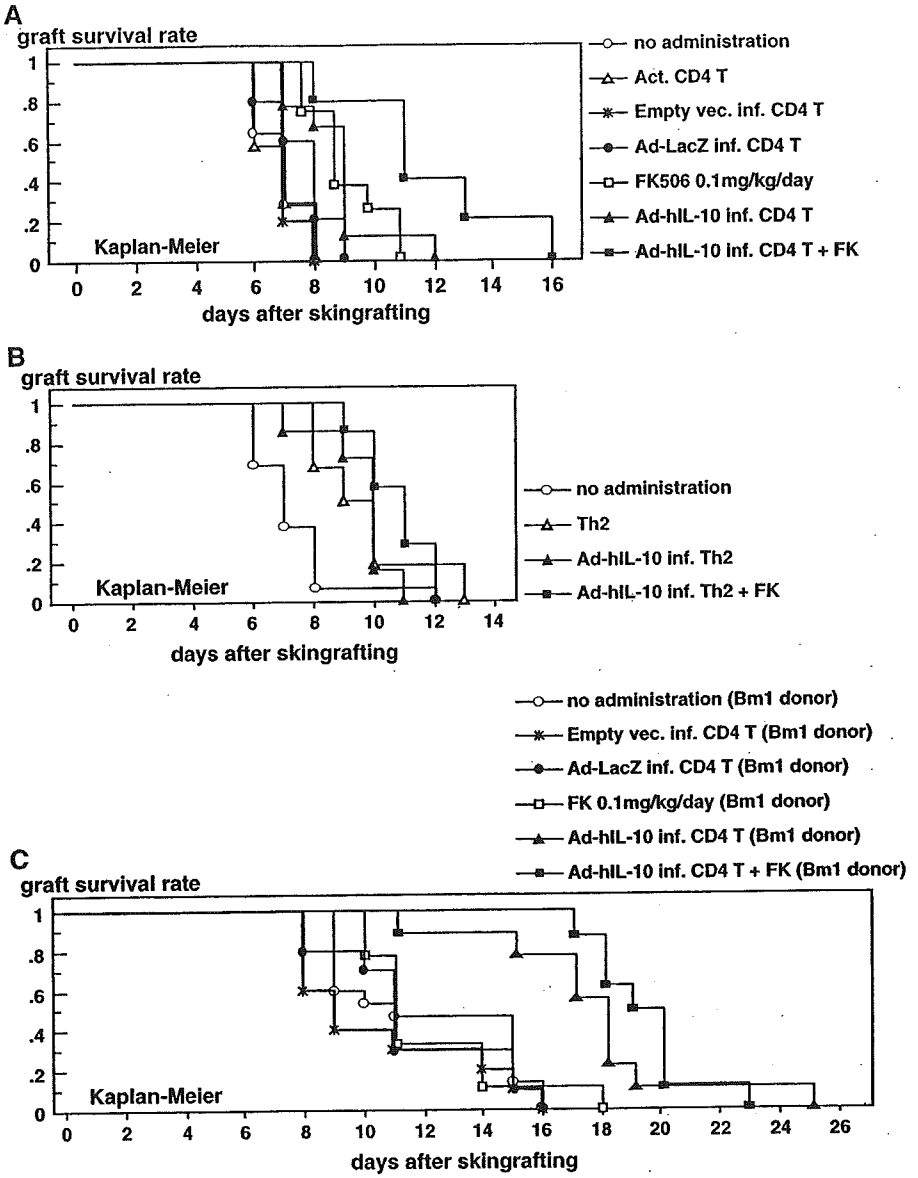


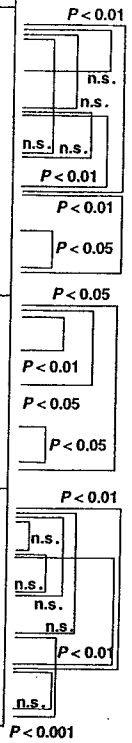
Fig. 4. BALB/c full allo-skin graft and Bm1 allo-skin graft survival after administration of adenovirus-infected activated CD4 T cells or T_h2 cells into C57BL/6 hosts. Graft survival curves with Ad-hIL-10-infected activated CD4 T cells (A) with Ad-hIL-10-infected T_h2 cells (B) in a BALB/c full allo-skin graft system are presented. Bm1 skin graft survival with Ad-hIL-10-infected activated CD4 T cells is shown in (C). The actual numbers are summarized and presented in Table 1. Where indicated, a sub-therapeutic dose of FK506 (0.1 mg kg^{-1} per day) was administered by an intramuscular route.

legions various levels of lymphocyte infiltration including activated CD4 and CD8 T cells are observed, suggesting their regulatory roles in inflammation. Thus, we wished to determine whether the manipulation of immunoregulatory functions of CD4 and CD8 T cells and their administration is an effective approach for the control of inflammation. The results shown in this report indicate that CD4 T cells secreting the immunosuppressive cytokine IL-10 regulate allo-skin graft responses. In addition, one of the advantages of the use of activated lymphocytes for gene introduction is that the effect is transient. The gene-introduced lymphocytes should undergo apoptotic cell death efficiently, and the incidence of tumor generation as well as possible side effects of inducing an immunosuppressive state in hosts would be minimal.

The infection by adenovirus vectors is mediated by CAR (45) whose tissue distribution varies among organs (46). CAR expression is greatest in the liver, while it is very limited on lymphocytes. We overcame this limitation by using CAR Tg mice in which CAR is highly expressed on T cells. For therapeutic considerations in man, to use this approach it may be necessary to establish a new adenovirus-related vector system that can allow human T cells be infected efficiently or alternatively some more efficient means, including lentivirus vector system, to get the *IL-10* gene into

Table 1. Summary of allo-skin graft survival experiments presented in Fig. 4

	Therapy	*n*	Graft survival	MST	SD
Exp. 1	No administration	11	6(x4), 7(x4), 8(x3)	7	0.8
	Act. CD4	7	6(x3), 7, 7, 8, 8	7	0.9
	Empty vec. CD4 T	10	7(x8), 8(x2)	7	0.4
	Ad-LacZ inf. CD4	10	6, 6, 7, 7, 8(x4), 9, 9	8	1.1
	Ad-hIL-10 inf. CD4	9	7, 7, 8, 9(x5), 12	9	1.5
	FK506 0.1 mg kg^{-1} per day	8	7, 7, 8(x3), 9, 10, 10	8	1.2
	Ad-hIL-10 inf. CD4+ FK	5	8, 11, 11, 13, 16	11	2.9
Exp. 2	No administration	19	6(x6), 7(x6), 8(x6), 12	7	1.4
	T_h2	6	8, 8, 9, 10, 10, 13	9.5	1.9
	Ad-hIL-10 inf. T_h2	7	7, 9, 10(x4), 11	10	1.3
	FK506 0.1 mg kg^{-1} per day	8	7, 7, 8(x3), 9, 10, 10	8	1.2
	Ad-hIL-10 inf. T_h2+ FK	7	9, 10, 10, 11, 11, 12, 12	11	1.2
Exp. 3	No administration (Bm1 donor)	15	9(x6), 10, 11, 15(x5), 16, 16	11	3.1
	Empty vec. CD4 T (Bm1 donor)	11	8(x4), 9, 10(x2), 12(x2), 15, 16	10	2.9
	Ad-LacZ inf. CD4 T (Bm1 donor)	10	8, 8, 10, 11(x4), 15, 15, 16	11	2.8
	FK 0.1 mg kg^{-1} per day (Bm1 donor)	9	10, 10, 11(x4), 14, 14, 18	11	2.6
	Ad-hIL-10 inf. CD4 (Bm1 donor)	9	11, 15, 17, 17, 18(x3), 19, 25	18	3.7
	Ad-hIL-10 inf. CD4+ FK (Bm1 donor)	8	17, 18, 18, 19, 20(x3), 23	19.5	1.8



The number of mice examined (*n*), graft survival (days), mean survival time (MST, days) and SD are summarized. Also, the results of statistical analysis with log-rank test are shown. The results of BALB/c full allo-skin grafts with Ad-hIL-10-infected activated CD4 T cells (Exp. 1) and with Ad-hIL-10-infected T_h2 cells (Exp. 2) are shown. Experiment 3 represents the results of Bm1 skin grafts with Ad-hIL-10-infected activated CD4 T cells seen in Fig. 4. Abbreviations: vec., vector; inf., infected; n.s., non-significant.

human T cells. However, once such an efficient gene introduction system is invented, results obtained from experimental model systems such as using CAR Tg T cells would be helpful. Thus, these studies should provide a proof of concept.

As for mechanisms of immunosuppression, one scenario is that the transferred IL-10-producing T cells migrated in the lymphoid organs and inhibited the priming of allospecific CD4 T cells and the subsequent induction of allospecific CD8 CTLs. This is likely because very efficient inhibition of all MLR by Ad-hIL-10-infected CD4 T cells was observed *in vitro* (Fig. 2). Another possible mechanism is that Ad-hIL-10-infected CD4 T cells migrated at the site of skin grafts and directly inhibited the rejection at the effector phase, i.e. inhibiting the function of allospecific cytotoxic CD8 T cells. In fact, we detected significant levels of CAR-positive cells (~0.05% of migrated T cells on day 7) in the skin graft tissue by PCR Southern blotting analysis (our unpublished observation). By immunofluorescent staining assay, no significant numbers of CAR^+ cells were detected in the Bm1 graft of mice with Ad-hIL-10-infected CD4 T cell administration on day 7 or day 14 (our unpublished observation). This could be due to low frequency of CAR^+ administered cells in the skin graft tissue (~0.05%). Moreover, no detectable serum level of IL-10 was observed (1, 4, 8, 24 and 48 h and 4, 7 and 14 days after transfer of Ad-hIL-10-infected CD4 T cells) (our unpublished observation), suggesting a non-systemic action of IL-10. Further investigation is required to clarify the actual mechanism involved. We used allo-skin graft systems for the evaluation of the immunosuppressive effects of the administration of IL-10-secreting CD4 and CD8 T cells; however, it is possible that other transplantation systems may be more appropriate for *IL-10* gene-introduced T cell therapy. In addition, it may be necessary to consider more efficient strategies including the combinational use of other inhibitory cytokines such as transforming growth factor β (47).

We detected a significant enhancing effect by the combined use of administration of low-dose sub-therapeutic FK506 (Fig. 4 and Table 1). This observation would be important because we may be able to reduce the doses of immunosuppressive drugs when transplanted patients are treated in combination with the administration of IL-10-secreting CD4 T cells. Since immune responses during allotransplant rejection consist of various distinct cellular activation processes, the combinational use of inhibitors acting on different processes would be more effective, particularly for minimizing different side effects observed in patients.

In summary, we demonstrate that the administration of adenovirus-delivered IL-10-secreting CD4 T cells inhibits the allograft rejection significantly in a specific graft-rejection model. In addition, the combined use of low-dose sub-therapeutic FK506 led to a substantial enhancement of the therapy. Thus, the direct manipulation of T cells by the

introduction of immunosuppressive genes may offer a novel approach for the control of allograft rejection.

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Abbreviations

Ad-GFP	adenovirus vector containing *EGFP* gene
Ad-hIL-10	adenovirus vector containing human *IL-10* gene
Ad-LacZ	adenovirus vector containing *lacZ* gene
Bm1	B6.C-$H2^{bm1}$/ByJ
CAR	Coxsackie virus and adenovirus receptor
CsA	cyclosporin A
DC	dendritic cell
hIL-10	human IL-10
i.f.u.	infectious units
POD	post-operation day
Tg	transgenic

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Gene Silencing of Virus Replication by RNA Interference

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Abstract Small interfering RNAs (siRNAs) are as effective as long double-stranded RNAs (dsRNAs) at targeting and silencing genes by RNA interference (RNAi). siRNAs are widely used for assessing gene function in cultured mammalian cells or early developing vertebrate embryos. They are also promising reagents for developing gene-specific therapeutics. The specific inhibition of viral replication is particularly well suited to RNAi, as several stages of the viral life cycle and many viral and cellular genes can be targeted. The future success of this approach will depend on the recent advances in siRNA-based clinical trials.

Keywords RNA interference · Gene silencing · Virus · Toll-like receptors · Virus escape

1 General Mechanism of RNAi

Eukaryotes have evolved a cellular defense system that responds to double-stranded (ds)RNAs and protects their genomes against these invading foreign elements. dsRNA delivery into cells has been used to elucidate the role of cellular genes that are homologous in sequence to the introduced dsRNAs by means of sequence-specific gene silencing (Fire et al. 1998). RNA interference (RNAi)-based reverse genetic analysis now provides a rapid link between se-

quence data and biological function. RNAi is particularly useful for the analysis of gene function in *Caenorhabditis elegans* (for reviews, see Hope 2001; Kim 2001). Effective gene silencing typically requires long dsRNAs (Parrish et al. 2000; Elbashir et al. 2001c). However, its application in vertebrates, including mammals, has proved to be difficult because of the presence of additional dsRNA-triggered pathways that mediate the non-specific suppression of gene expression (Caplen et al. 2000; Nakano et al. 2000; Oates et al. 2000; Zhao et al. 2001). These non-specific responses to long dsRNAs are not, however, triggered by small interfering RNAs (siRNAs) (Bitko and Barik 2001; Caplen et al. 2001; Elbashir et al. 2001a; Zhou et al. 2002). siRNAs can target genes as effectively as long dsRNAs (Elbashir et al. 2001c) and are widely used for assessing gene function in cultured mammalian cells or early developing vertebrate embryos (Harborth et al. 2001; Elbashir et al. 2002; Zhou et al. 2002). siRNAs are also promising reagents for developing gene-specific therapeutics (Tuschl and Borkhardt 2002). However, another major problem for using RNAi as a tool to inhibit viral replication is predicting the effectiveness of a specific siRNA. The difficulty lies in making siRNAs trigger silencing in a gene-specific manner without causing non-target-related biological effects or the emergence of escape variants by foreign siRNAs. Work over the past 2 years has allowed investigators to meet this challenge, and the siRNA approach has now been adopted as a standard methodology for sequence-specific silencing in mammalian cells. This review focuses on RNAi as it relates to mammalian systems and the application of siRNAs for targeting genes that are expressed in virus-infected cell lines.

Studies in plants and *Drosophila* have provided fundamental insights into the mechanism of RNAi, following the demonstration that RNAi was activated by dsRNAs and the suggestion that it might involve a derivative of dsRNAs (Fire et al. 1998; Fig. 1).

Biochemical characterization has shown that siRNAs are 21- to 23-nt dsRNA duplexes with symmetric 2- to 3-nt 3′ overhangs, and 5′-phosphate and 3′-hydroxyl groups (Elbashir et al. 2001b; Fig. 1). This structure is characteristic of an RNase III-like enzymatic cleavage pattern, which led to the identification of the highly conserved Dicer family of RNase III enzymes as the mediators of dsRNA cleavage (Bernstein et al. 2001; Billy et al. 2001; Ketting et al. 2001).

Extensive biochemical and genetic evidence has allowed a better understanding of how long dsRNAs trigger degradation of the target messenger RNAs (mRNAs) (Fig. 1; for recent reviews, see Sharp 2001; Hannon 2002; McManus and Sharp 2002; Zamore 2002). Several studies have shown that this process is restricted to the cytoplasm (Hutvagner and Zamore 2002; Zeng and Cullen 2002; Kawasaki and Taira 2003). In the first step, Dicer cleaves long dsRNAs to produce siRNAs, which are incorporated into a multiprotein RNA-inducing silencing complex (RISC). There is a strict requirement for the siRNAs to be 5′ phosphorylated in order to enter into the RISC (Nykanen et al. 2001; Schwarz et al. 2002). siRNAs that lack a 5′ phosphate are rapidly phosphorylated by

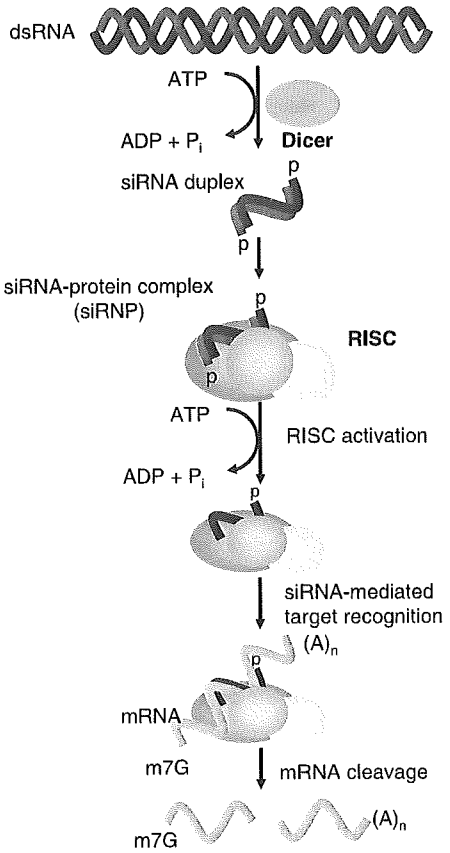

Fig. 1 Model for RNA-mediated interference and silencing. The cellular RNase III enzyme Dicer processes double-stranded RNA (*dsRNA*) to 21- to 23-nt short-interfering RNA (*siRNA*) duplexes in an ATP-dependent manner. The siRNAs are incorporated into a siRNA–ribonucleoprotein complex (*siRNP*), which uses ATP to rearrange itself into the RNA-induced silencing complex (*RISC*) by unwinding the siRNA duplex. Once unwound, the single-stranded antisense siRNA guides the RISC to mRNA with a complementary sequence, causing endonucleolytic cleavage of the target mRNA. The mRNA-cleavage products are then released and the RISC can be reactivated for another round of catalytic target RNA cleavage

an endogenous kinase (Schwarz et al. 2002). The duplex siRNA is unwound, leaving the antisense strand to guide the RISC to its homologous target mRNA for endonucleolytic cleavage. The target mRNA is cleaved at a single site in the center of the duplex region between the guide siRNA and the target mRNA, 10 nt from the 5′ end of the siRNA (Elbashir et al. 2001a,b).

Interestingly, endogenously expressed siRNAs have not been found in mammals. However, related microRNAs (miRNAs) have been cloned from various organisms and cell types (Pasquinelli 2002; Fig. 2). These short (22 nt) RNA