International Immunology, Vol. 17, No. 6, pp. 759–768
doi:10.1093/intimm/dxh256

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

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Keywords: Bm1, CAR Tg, gene therapy, skin graft

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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Transmitting editor: A. Singer

Received 3 February 2005, *accepted* 10 March 2005

Advance Access publication 17 May 2005

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

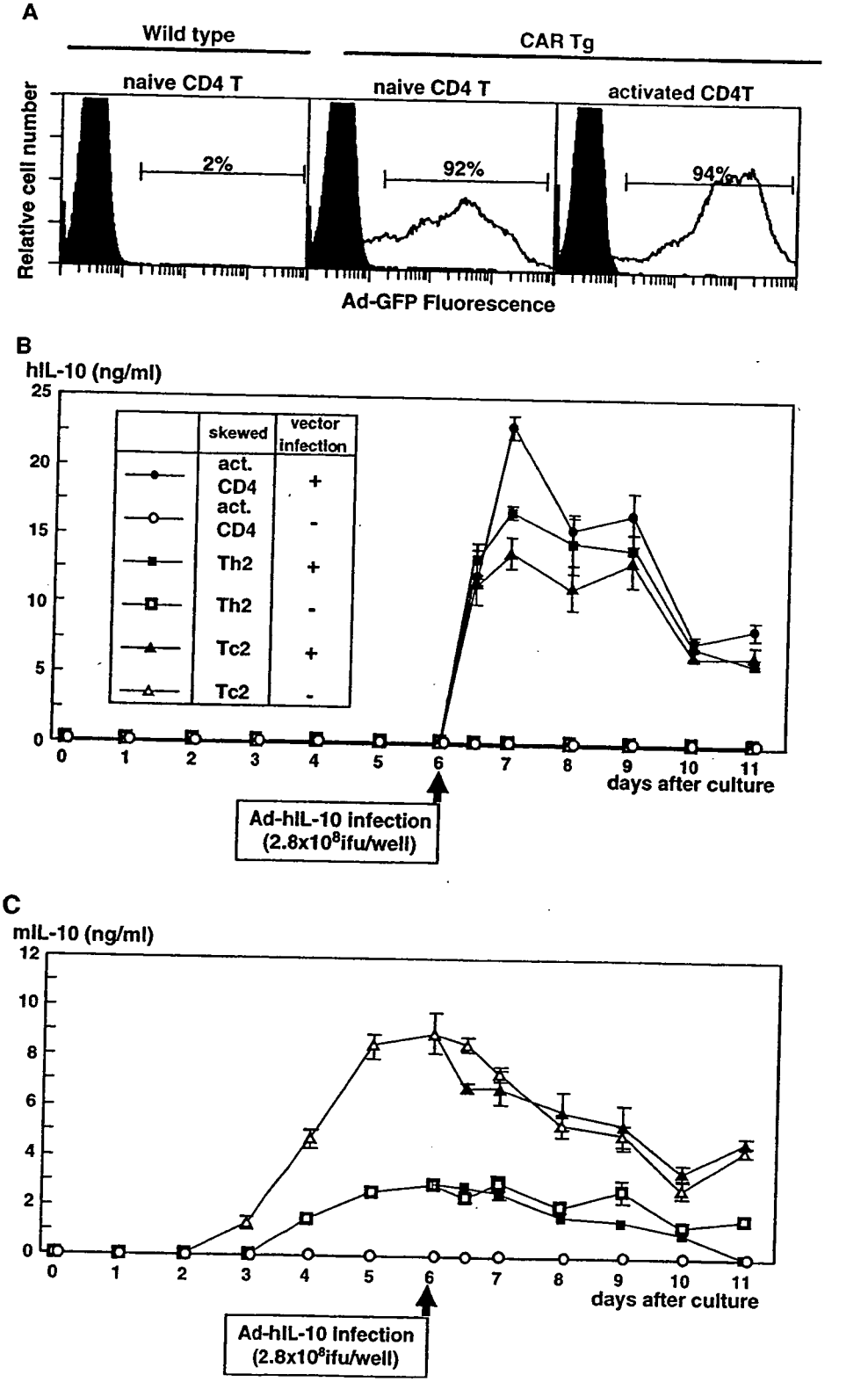


Fig. 1. IL-10 production from T cells infected with Ad-hIL-10. (A) A green fluorescence protein (GFP) gene-encoded adenoviral vector (Ad-GFP) was used for evaluating the efficiency of adenovirus infection in CAR Tg CD4 T cells. Naive CD4 T cells from wild-type and CAR Tg mice and activated CD4 T cells 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.

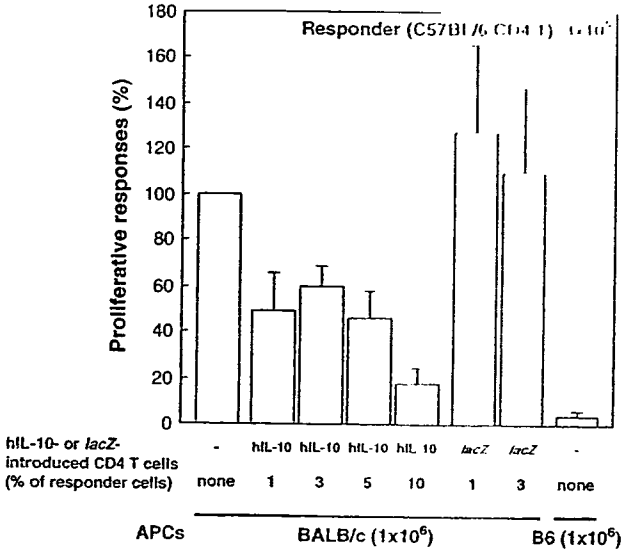


Fig. 2. Inhibition of allo-MLR by Ad-hIL-10-infected CAR Tg CD4 T cells. Graded doses of Ad-hIL-10-infected or Ad-LacZ-infected CD4 T cells prepared 12 h after infection were added to the allo-MLR cultures consisting of C57BL/6 CD4 T cell responder (3×10^5) and BALB/c stimulator cells (1×10^6). Relative 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

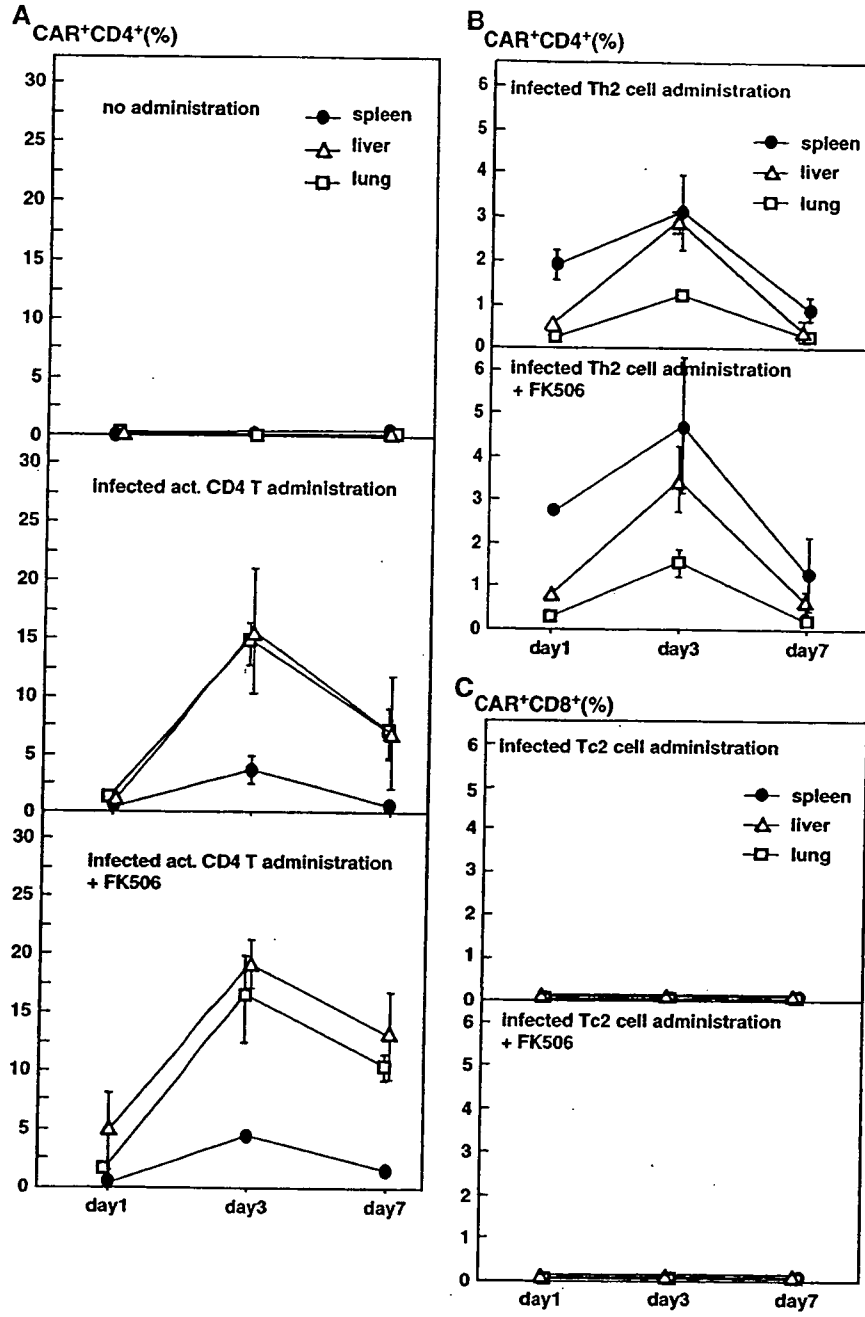


Fig. 3. Distribution of Ad-hIL-10-infected T cells administrated intravenously. Activated CD4 T cells (A), *in vitro*-differentiated T_h2 (B) and Tc2 cells (C) were infected with Ad-hIL-10, and 2 days later the cells (5×10^7) were administered into C57BL/6 mice intravenously on days 0 and 2. The percentages 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

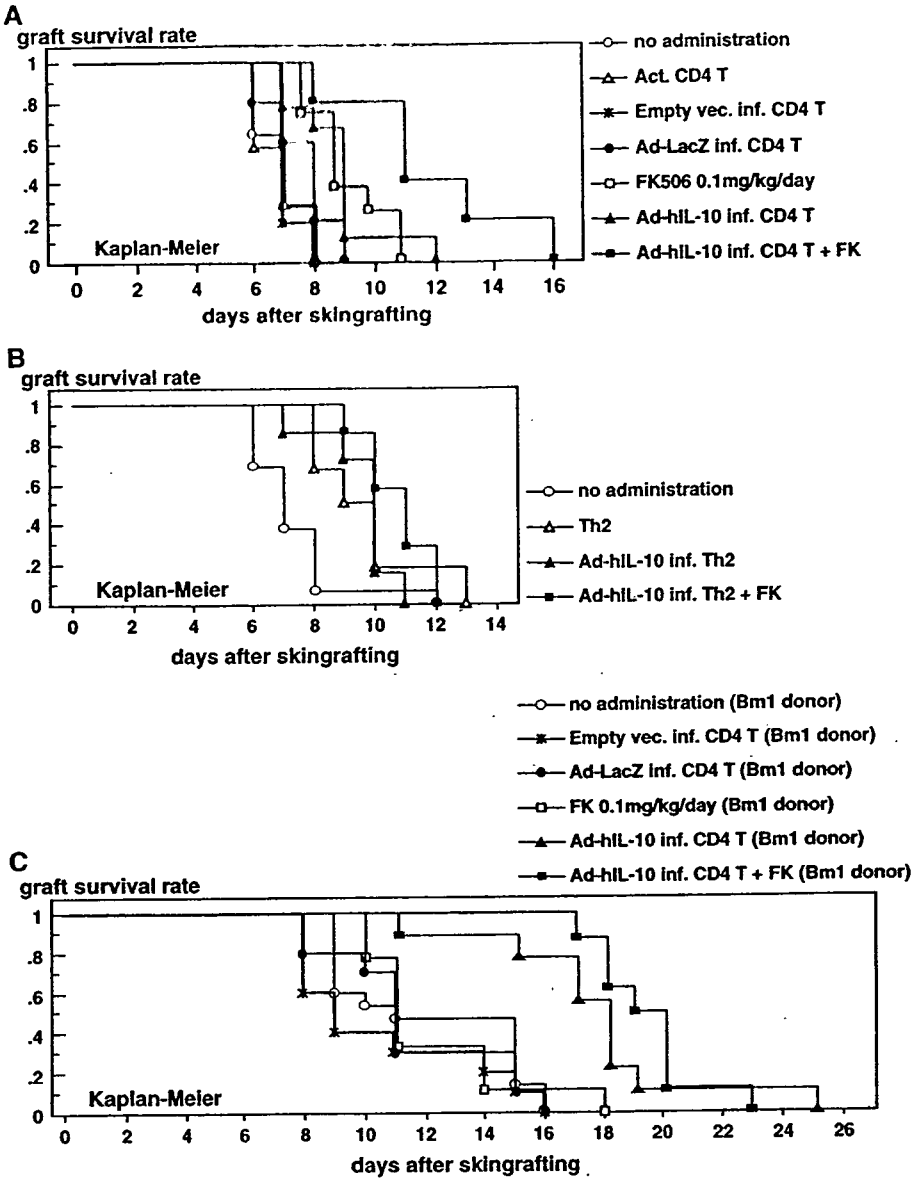


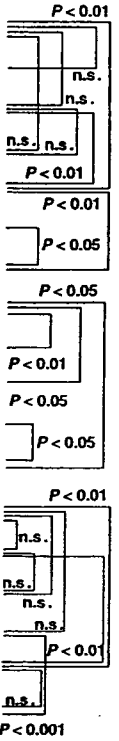
Fig. 4. BALB/c full allo-skin graft and Bm1 allo-skin graft survival after administration of adenovirus-infected activated CD4 T cells or T_h2 cells into C57BL/6 hosts. Graft survival curves with Ad-hIL-10-infected activated CD4 T cells (A) with Ad-hIL-10-infected T_h2 cells (B) in a BALB/c full allo-skin 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

Acknowledgements

The authors are grateful to Ralph T. Kubo for helpful comments and constructive criticisms in the preparation of the manuscript. The authors also thank Ms Kaoru Sugaya for excellent technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan) (Grants-in-Aid for: Scientific Research, Priority Areas Research #13218016 and #16043211; Scientific Research B #14370107; Scientific Research C #16616003 and #15790248 and Special Coordination Funds for Promoting Science and Technology), the Ministry of Health, Labor and Welfare (Japan), the Program for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (Japan) and the Japan Health Science Foundation, Uehara Memorial Foundation, Kanae Foundation and Mochida Foundation.

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

References

1 Zelenika, D., Adams, E., Humm, S., Lin, C. Y., Waldmann, H. and Cobbold, S. P. 2001. The role of $CD4^+$ T-cell subsets in determining transplantation rejection or tolerance. *Immunol. Rev.* 182:164.
2 Rocha, P. N., Plumb, T. J., Crowley, S. D. and Coffman, T. M. 2003. Effector mechanisms in transplant rejection. *Immunol. Rev.* 196:51.
3 Nickerson, P., Steiger, J., Zheng, X. X. *et al.* 1997. Manipulation of cytokine networks in transplantation: false hope or realistic opportunity for tolerance? *Transplantation* 63:489.
4 Piccotti, J. R., Chan, S. Y., VanBuskirk, A. M., Eichwald, E. J. and Bishop, D. K. 1997. Are Th2 helper T lymphocytes beneficial, deleterious, or irrelevant in promoting allograft survival? *Transplantation* 63:619.
5 Ruhlmann, A. and Nordheim, A. 1997. Effects of the immunosuppressive drugs CsA and FK506 on intracellular signalling and gene regulation. *Immunobiology* 198:192.
6 Schreiber, S. L. and Crabtree, G. R. 1992. The mechanism of action of cyclosporin A and FK506. *Immunol. Today* 13:136.
7 Kahan, B. D. 1993. Cyclosporine: the base for immunosuppressive therapy—present and future. *Transplant. Proc.* 25:508.
8 Starzl, T. E. 1993. FK 506 versus cyclosporine. *Transplant. Proc.* 25:511.
9 Qin, L., Chavin, K. D., Ding, Y. *et al.* 1996. Retrovirus-mediated transfer of viral IL-10 gene prolongs murine cardiac allograft survival. *J. Immunol.* 156:2316.
10 Brauner, R., Nonoyama, M., Laks, H. *et al.* 1997. Intracoronary adenovirus-mediated transfer of immunosuppressive cytokine genes prolongs allograft survival. *J. Thorac. Cardiovasc. Surg.* 114:923.
11 Shinozaki, K., Yahata, H., Tanji, H., Sakaguchi, T., Ito, H. and Dohi, K. 1999. Allograft transduction of IL-10 prolongs survival following orthotopic liver transplantation. *Gene Ther.* 6:816.
12 David, A., Chetritt, J., Guillot, C. *et al.* 2000. Interleukin-10 produced by recombinant adenovirus prolongs survival of cardiac allografts in rats. *Gene Ther.* 7:505.
13 Klebe, S., Sykes, P., Coster, D., Krishnan, R. and Williams, K. 2001. Prolongation of sheep corneal allograft survival by *ex vivo* transfer of the gene encoding interleukin-10. *Transplantation* 71:1214.
14 Lundstrom, K. 2003. Latest development in viral vectors for gene therapy. *Trends Biotechnol.* 21:117.
15 Blaese, R. M., Culver, K. W., Miller, A. D. *et al.* 1995. T lymphocyte-directed gene therapy for ADA^- SCID: initial trial results after 4 years. *Science* 270:475.
16 Verma, I. M. and Somia, N. 1997. Gene therapy—promises, problems and prospects. *Nature* 389:239.
17 Anderson, W. F. 1998. Human gene therapy. *Nature* 392:25.
18 Benihoud, K., Yeh, P. and Perricaudet, M. 1999. Adenovirus vectors for gene delivery. *Curr. Opin. Biotechnol.* 10:440.
19 Kafri, T., Morgan, D., Krahl, T., Sarvetnick, N., Sherman, L. and Verma, I. 1998. Cellular immune response to adenoviral vector infected cells does not require *de novo* viral gene expression: implications for gene therapy. *Proc. Natl Acad. Sci. USA* 95:11377.
20 Molinier-Frenkel, V., Gahery-Segard, H., Mehtali, M. *et al.* 2000. Immune response to recombinant adenovirus in humans: capsid components from viral input are targets for vector-specific cytotoxic T lymphocytes. *J. Virol.* 74:7678.
21 Moore, K. W., de Waal Malefyt, R., Coffman, R. L. and O'Garra, A. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 19:683.
22 Kennedy, M. K., Picha, K. S., Shanebeck, K. D., Anderson, D. M. and Grabstein, K. H. 1994. Interleukin-12 regulates the proliferation of Th1, but not Th2 or Th0, clones. *Eur. J. Immunol.* 24:2271.
23 Groux, H., O'Garra, A., Bigler, M. *et al.* 1997. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737.
24 Buer, J., Lanoue, A., Franzke, A., Garcia, C., von Boehmer, H. and Sarukhan, A. 1998. Interleukin 10 secretion and impaired effector function of major histocompatibility complex class II-restricted T cells anergized *in vivo*. *J. Exp. Med.* 187:177.
25 Wan, Y. Y., Leon, R. P., Marks, R. *et al.* 2000. Transgenic expression of the coxsackie/adenovirus receptor enables adenoviral-mediated gene delivery in naive T cells. *Proc. Natl Acad. Sci. USA* 97:13784.
26 Nakayama, T., June, C. H., Munitz, T. I. *et al.* 1990. Inhibition of T cell receptor expression and function in immature $CD4^+CD8^+$ cells by CD4. *Science* 249:1558.
27 Kanegae, Y., Lee, G., Sato, Y. *et al.* 1995. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucleic Acids Res.* 23:3816.
28 Omori, M., Yamashita, M., Inami, M. *et al.* 2003. CD8 T cell-specific downregulation of histone hyperacetylation and gene activation of the IL-4 gene locus by ROG, repressor of GATA. *Immunity* 19:281.
29 Yamashita, M., Kimura, M., Kubo, M. *et al.* 1999. T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. *Proc. Natl Acad. Sci. USA* 96:1024.
30 Inamura, N., Nakahara, K., Kino, T. *et al.* 1988. Prolongation of skin allograft survival in rats by a novel immunosuppressive agent, FK506. *Transplantation* 45:206.
31 Wekerle, T., Sayegh, M. H., Hill, J. *et al.* 1998. Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. *J. Exp. Med.* 187:2037.
32 Wissing, K. M., Morelon, E., Legendre, C. *et al.* 1997. A pilot trial of recombinant human interleukin-10 in kidney transplant recipients receiving OKT3 induction therapy. *Transplantation* 64:999.
33 Chernoff, A. E., Granowitz, E. V., Shapiro, L. *et al.* 1995. A randomized, controlled trial of IL-10 in humans. Inhibition of inflammatory cytokine production and immune responses. *J. Immunol.* 154:5492.
34 Ettinghausen, S. E. and Rosenberg, S. A. 1986. Immunotherapy of murine sarcomas using lymphokine activated killer cells: optimization of the schedule and route of administration of recombinant interleukin-2. *Cancer Res.* 46:2784.
35 Chen, D., Sung, R. and Bromberg, J. S. 2002. Gene therapy in transplantation. *Transplant Immunol.* 9:301.

36 Drazan, K. E., Wu, L., Olthoff, K. M., Jurim, O., Busuttil, R. W. and Shaked, A. 1995. Transduction of hepatic allografts achieves local levels of viral IL-10 which suppress alloreactivity *in vitro*. *J. Surg. Res.* 59:219.
37 Wang, C. K., Zuo, X. J., Carpenter, D. *et al.* 1999. Prolongation of cardiac allograft survival with intracoronary viral interleukin-10 gene transfer. *Transplant. Proc.* 31:951.
38. Tung, T. C., Oshima, K., Cui, G., Laks, H. and Sen, L. 2003. Dual upregulation of Fas and Bax promotes alloreactive T cell apoptosis in IL-10 gene targeting of cardiac allografts. *Am. J. Physiol. Heart Circ. Physiol.* 285:H964.
39 Sen, L., Hong, Y. S., Luo, H., Cui, G. and Laks, H. 2001. Efficiency, efficacy, and adverse effects of adenovirus vs. liposome-mediated gene therapy in cardiac allografts. *Am. J. Physiol. Heart Circ. Physiol.* 281:H1433.
40 Oshima, K., Sen, L., Cui, G. *et al.* 2002. Localized interleukin-10 gene transfer induces apoptosis of alloreactive T cells via FAS/FASL pathway, improves function, and prolongs survival of cardiac allograft. *Transplantation* 73:1019.
41 Hong, Y. S., Laks, H., Cui, G., Chong, T. and Sen, L. 2002. Localized immunosuppression in the cardiac allograft induced by a new liposome-mediated IL-10 gene therapy. *J. Heart Lung Transplant.* 21:1188.
42. Itano, H., Mora, B. N., Zhang, W. *et al.* 2001. Lipid-mediated *ex vivo* gene transfer of viral interleukin 10 in rat lung allotransplantation. *J. Thorac. Cardiovasc. Surg.* 122:29.
43 de Perrot, M., Fischer, S., Liu, M. *et al.* 2003. Impact of human interleukin-10 on vector-induced inflammation and early graft function in rat lung transplantation. *Am. J. Respir. Cell Mol. Biol.* 28:616.
44 Coates, T., Krishnan, R., Chew, G. *et al.* 2001. Dendritic cell TH2 cytokine gene therapy in sheep. *Transplant. Proc.* 33:180.
45 McDonald, D., Stockwin, L., Matzow, T., Blair Zajdel, M. E. and Blair, G. E. 1999. Coxsackie and adenovirus receptor (CAR)-dependent and major histocompatibility complex (MHC) class I-independent uptake of recombinant adenoviruses into human tumour cells. *Gene Ther.* 6:1512.
46 Fechner, H., Haack, A., Wang, H. *et al.* 1999. Expression of coxsackie adenovirus receptor and alpha$_v$-integrin does not correlate with adenovector targeting *in vivo* indicating anatomical vector barriers. *Gene Ther.* 6:1520.
47 Letterio, J. J. and Roberts, A. B. 1998. Regulation of immune responses by TGF-b. *Annu. Rev. Immunol.* 16:137.

HYBRIDOMA
Volume 24, Number 5, 2005

Production and Characterization of a Monoclonal Antibody Specific to Nef-Associated Factor 1 (Naf1)/A20-Binding Inhibitor of NF-κB Activation (ABIN-1)

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ABSTRACT

Cellular protein Naf1 (Nef-associated factor 1) or ABIN-1 (A20-binding inhibitor of NF-κB activation) is an important cellular protein, expressed in various human tissues and T-cell lines. Naf1 protein has two isoforms (Naf1α and Naf1β) with different C-termini, produced by alternative splicing. Naf1α and Naf1β have approximately 2800 and 2600 nucleotides, with an open reading frame of 1941 and 1781 nucleotides, encoding the 72-kDa Naf1α and 68-kDa Naf1β proteins, respectively. In the present study, we generated a monoclonal antibody (MAb) against human Naf1, which recognizes full-length, endogenous Naf1 of both isotypes. For this purpose, recombinant 6×His and myc-tagged N-terminal Naf1$^{38-135}$, Naf1(N) protein was produced by using the baculovirus expression system. Recombinant Naf1(N) protein was used to immunize Balb/c mice, and a hybridoma cell line producing stable and highly specific MAb with strong affinity to Naf1 was established. We further characterized this antibody by immunofluorescent assay and Western blot analysis to confirm effectiveness in detecting recombinant and endogenous Naf1. By Western blot analysis of recombinant Naf1-N fusion proteins with overlapping N-terminal sequences, the epitope targeted by anti-Naf1 MAb was determined as the 81–88–amino acid region of human Naf1.

INTRODUCTION

NAF1 (Nef-associated factor-1) or ABIN-1 (A20-binding inhibitor of NF-κB activation) is a cellular protein with four putative leucine zippers and four predicted regions of coiled coil structures. The gene encoding Naf1, which consists of 18 exons, is located on human chromosome 5q 32-33.1.[1] Naf1 mRNA is ubiquitously expressed in several human tissues, with strong expression in peripheral blood lymphocytes, spleen, and skeleton muscles. Naf1 is also detected in various human hematopoietic cell lines, such as Jurkat, Molt-4, H-9, and HL60.[1,2] According to the previous reports, Naf1 associates with human immunodeficiency virus type-1 (HIV-1) viral proteins Nef and matrix in the yeast two-hybrid system and pull-down assay using transfected human cell lysates.[1,2]

HIV-1 Nef not only enhances the viral infectivity, but also plays an important role in viral replication and pathogenesis.[3–10] Nef-defective HIV-1 virions are isolated from some cases of long-term non-progressors.[11] Nef induces down-regulation of cell surface expression levels of CD4 and major histocompatibility complex (MHC) class I molecules in HIV infection.[12–14] Thereby, Nef helps the virus to evade host defense and to increase viral infectivity.[15–17] CD4 is the primary receptor for HIV-1 and interferes with the infectivity of HIV-1 particles released from T cells.[18] Down-regulation of CD4 reduces the formation of complexes between CD4 and newly synthesized HIV-1 envelope protein on the infected cell surface, facilitating the release of HIV-1 virions. Nef-induced degradation of CD4 is also reported to result in the release of the normally CD4-bound tyrosine kinase, lck, and this could have a marked effect on signaling pathway in cellular activation. Because MHC class I is required to present viral peptide epitopes to cytotoxic T lymphocytes (CTL), down-regulation of cell surface MHC class I could inhibit the CTL-mediated lysis of HIV-

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1–infected cells. Naf1 overexpression increases cell surface CD4 levels, but overexpression of Nef, in turn, inhibits Naf1-induced CD4 augmentation.[1]

Naf1 (ABIN-1) also interacts with A20 zinc finger protein in yeast two-hybrid screening. Zinc finger protein A20 has been characterized as a dual inhibitor of nuclear factor–kappa B (NF-κB) activation and tumor necrosis factor (TNF)–induced apoptosis.[19–24] NF-κB plays a pivotal role in immune and inflammatory responses through the regulation of the expression of several proteins, including pro-inflammatory cytokines, chemokines, and adhesion molecules. Uncontrolled activation of the NF-κB pathway is involved in the pathogenesis of several chronic inflammatory diseases and autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, and asthma. A20 binding protein Naf1 (ABIN-1) has been reported to inhibit NF-κB–dependent gene expression induced by TNF-α, and IL-1. Overexpression of Naf1 blocks NF-κB activation by TNF-α, and it is also thought that the expression of Naf1 is NF-κB dependent. It has been suggested that Naf1 takes a role in negative feedback regulation of NF-κB expression by competing with IKK-γ and also acts as an endogenous brake for the expression of some TNF-α–driven genes.[24–27] Moreover, Naf1 appeared to attenuate the EGF/ERK2 nuclear signaling, which is important for cell growth, differentiation, and cell death.[28] Nevertheless, intriguing questions regarding the mechanism of functions and regulation of Naf1 as well as the importance of physical associations between Naf1 and Naf1 interacting proteins, to carry out their functions in the molecular signaling pathways, remain to be answered.

For further understanding of the molecular mechanisms of the functions of Naf1, the most important tasks are to investigate intracellular localization, to investigate the relationship between the nucleocytoplasmic shuttling of Naf1 and its functions, and to discover Naf1-interacting proteins along with their functions. To perform these tasks, anti-Naf1 MAb is an essential tool, and in this report, we describe the production, characterization, and epitope mapping of an MAb specific to human cellular protein Naf1/ABIN-1. We also report the expression and subcellular localization of endogenous Naf1 in primary and various cell lines such as human PBL, Jurkat, MT-4, Molt-4, 293, and U-937 cell lines by using this MAb.

MATERIALS AND METHODS

Cell cultures

Spodoptera frugiperda (Sf9) cells were grown and maintained in complete Grace's insect medium (Gibco) supplemented with 10% fetal bovine serum (FBS), and *Trichoplusia ni* (High Five) cells in serum-free Sf-900II medium (Gibco). Both cell lines were cultured in monolayers at 27°C. SP2/0 murine myeloma cells, human PBL from a healthy adult donor, Jurkat, MT-4, Molt-4, 293, and U-937 cells were maintained in RPMI-1640 medium (Sigma) supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin.

Construction of Naf1(N)-fusion protein cDNAs

cDNA encoding 6×His and myc-tagged Naf1(N) (amino acid residues 38–135) was constructed by polymerase chain re-

TABLE 1. OLIGONUCLEOTIDE PRIMERS USED FOR POLYMERASE CHAIN REACTION

Gene	*Primer*	*Sequences (5′ → 3′)*
Naf1(N)	His-Naf1(N)/F	CGCGGATCCGCGCATCATCATCATCATCATCAAGGGAT AAAGATGTTAGGGGAGC
	myc-Naf1(N)/R	CCGCTCGAGCGGCTAATTCAAGTCCTCTTCAGAAATG AGCTTTTGCTCCATTGAATTCTGCTCCTCAGGAGTGA
Naf1(N)-1	His-Naf1(N)-1/F	CGCGGATCCGCGCATCATCATCATCATCATATGCAAGG GATAAAGATGTTAGGG
	myc-Naf1(N)-1/R	CCCAGCTTGCTCTAATTCAAGTCCTCTTCAGAAATGA GCTTTTGCTCCAT CTCAGCCAGGGGGTCG
Naf1(N)-2	His-Naf1(N)-2/F	CGCGGATCCGCGCATCATCATCATCATCATGAGGAGCT AGTGAAGGACAACGA
	myc-Naf1(N)-2/R	CCCAAGCTTGCTCTAATTCAAGTCCTCTTCAGAAATGA GCTTTTGCTCCATGACTGGTGCTGGCTTGTCAC
Naf1(N)-3	His-Naf1(N)-3/F	CGCGGATCCGCGCATCATCATCATCATCATCTCACAGG AAAGGACTCAAATGTC
	His-Naf1(N)-3/R	CCCAAGCTTGCTCTAATTCAAGTCCTCTTCAGAAATGA GCTTTTGCTCCATTGAATTCTGCTCCTCAGGAGTG
Naf1(N)-A	His-Naf1(N)-A/F	CGCGGATCCGCGCATCATCATCATCATCAT GAGGAGCTAGTGAAGG ACAACGAGCTGCTCC
	myc-Naf1(N)-A/R	CCCAAGCTTCCATGGGCTCTAATTCAAGTCCTCTTCAG AAATGAGCTTTTGCTCCATAGGTGGTGGGAGCAGCT
Naf1(N)-B	His-Naf1(N)-B/F	CGCGGATCCGCGCATCATCATCATCATCATAACGAGCT GCTCCCACCACCTTCTCCCT
	myc-Naf1(N)-B/R	CCCAAGCTTCCATGGGCTCTAATTCAAGTCCTCTTCAG AAATGAGCTTTTGCTCCATGCCCAAGGAGGGAGAAG
Naf1(N)-C	His-Naf1(N)-C/F	CGCGGATCCGCGCATCATCATCATCATCAT
	myc-Naf1(N)-C/R	CCCAAGCTTCCATGGGCTCTAATTCAAGTCCTCTTCAG AAATGAGCTTTTGCTCCATCTCAGCCAGGGGGTCGA

action (PCR) technology, and the plasmid containing cDNA of full-length Naf1 was used as the template. The primers used in PCR reaction consisted of sense and antisense oligonucleotides containing *Bam*HI and *Xho*I sites respectively. The amplified cDNA was restricted by *Bam*HI/*Xho*I digestion, and subcloned into *Bam*HI/*Xho*I–digested pMelBacA baculovirus transfer vector (Invitrogen). cDNAs encoding 6 × His and myc-tagged Naf1(N) fusion protein fragments 1, 2, and 3, and A, B, and C were prepared by PCR amplification using specific primers with *Bam*HI and *Hind*III restriction sites, and ligated into pMAL-C2 vector containing MBP (maltose binding protein) sequence (Table 1). All constructs were confirmed by sequencing.

Generation of recombinant baculovirus in Sf9 cells

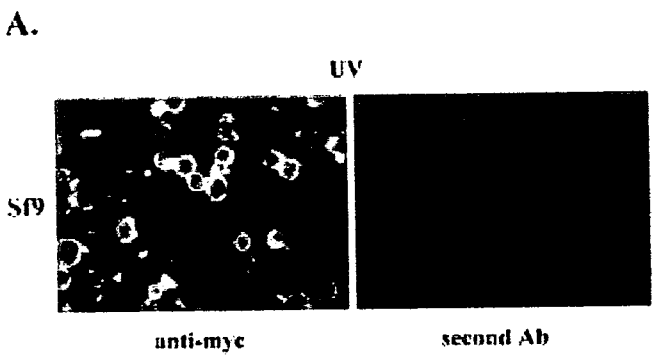
Recombinant baculovirus expressing Naf1(N) was produced by homologous recombination using Bac-N-Blue transfection kit (Invitrogen) according to procedures described previously.[29] Single viral clones were isolated by plaque assay and amplified by infecting Sf9 cells at a multiplicity of infection (MOI) of less than one. Infectivity was determined by titration and immunofluorescence staining of infected cells with mouse anti-myc MAb, followed by fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse second antibody. Virus stock was stored in aliquots at −80°C.

Production of recombinant Naf1(N) protein in High Five cells

Exponentially growing High Five cells in 75-cm^2 tissue culture flasks were infected with the recombinant baculovirus at an MOI of 10–20 plaque-forming units (pfu)/cell and incubated at 27°C for 3–5 days. High Five cells were then sedimented by centrifugation, and supernatant was collected for purification using Ni-NTA columns (polyhistidine tag at the amino terminus of the recombinant Naf1(N) protein binds to Ni-NTA resin).[30–31] Purification of recombinant Naf1(N) protein was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining.

Mice and immunization

Immunization, hybridoma preparation, and purification of MAb were done according to the procedures previously described.[32] In summary, three 6-week-old adult female Balb/c mice were immunized with three subcutaneous (s.c) injections of purified Naf1(N) protein at 2-week intervals. 100 μL of purified



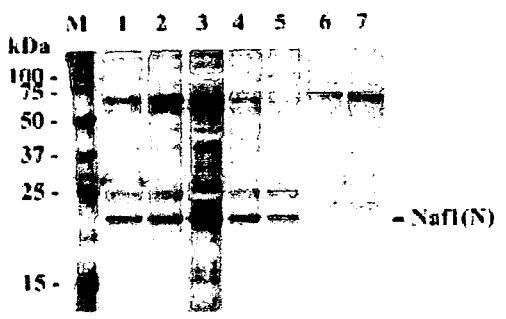


FIG. 1. (A) Immunofluorescence staining of Sf9 cells with anti-myc monoclonal antibody (MAb). Sf9 cells infected with baculovirus expressing 6×His and myc-tagged Naf1(N) were fixed in methanol and immunostained with anti-myc MAb, followed by fluorescein isothiocyanate (FITC)–conjugated second antibody (left panel) or with second antibody (Ab) only (right panel). (B) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Silver staining of purified recombinant Naf1(N) protein. M, unstained precision plus protein marker; lane 1–5, elusion fraction E1 to E5; lane 6, wash; lane 7, flow-through. The arrowhead at the right indicates the purified target of Naf1(N) protein.

Naf1(N) protein was emulsified in the same amount of Freund's complete adjuvant (Wako) for the first injection and in Freund's incomplete adjuvant for the following two booster injections. After the third injection, immune response was assessed by screening blood samples from the immunized mice with Western blot analysis of purified Naf1(N) protein. The mouse showing highest immune response was given a final intraperitoneal booster injection with 100 μL of antigen, 2-weeks after the third injection and 3 days before fusion with myeloma cells.

Hybridoma preparation

Three days after the last immunization, the mice were killed and the spleens removed aseptically. Spleen cells were mixed with SP 2/0 myeloma cells at a ratio of 5:1 and fused in the presence of polyethylene glycol (PEG 1500; Roche) at 37°C. The cells were then pelleted and resuspended in RPMI 1640 medium (Sigma) supplemented with 20% fetal bovine serum (FBS; Cansera International Inc), 100 U/mL penicillin, 100 g/mL streptomycin, and 2% HAT (100 μM hypoxanthine, 400 nM aminopterin, and 16 μM thymidine) for hybrid selection. Aliquots of the cell suspension were plated in 96-well plates and cultured at 37°C in 5% CO_2. After 24 h, 100 μL of HAT-selective medium was added, and every 2 or 3 days half of the medium from each well was replaced with fresh HAT medium. Between the 12th and 15th days, cell growth appeared in the majority of wells, and the supernatants were screened by immunofluorescence staining for the specific antibody-secreting clones. Cells from the positive hybridomas were transferred to a 24-well plate and cultured in 1 mL of 1% HT medium. As hybridoma cells grew well, aminopterin was omitted from the medium. At days 2 and 4, 0.5 mL of HT medium was added to each well, and supernatants were screened again by immunofluorescence staining. Among five positive hybridomas, one strongly positive hybridoma (Hybridoma-4) was selected and subcloned by limiting dilution in HT medium (GIBCO), followed by screening and selection of the strongest clone (C-3). Hybridoma clone (C-3) was cultured in large scale for inoculation into mice.

Purification and isotyping of MAb

Hybridoma cells (2×10^6) were inoculated into the peritoneal cavity of three BALB/c mice treated 1 week before with pristine (Sigma) to generate ascitic fluids containing anti-Naf1 MAb. After 10 days, ascitic fluid was collected and purified using HiTrap-Protein G Sepharose columns (Pharmacia Biotech). Immuno-globulin isotyping was performed using mouse MAb isotyping kit (Amersham) according to the manufacturer's instructions.

Immunofluorescence staining

Immunofluorescence staining of Sf9 cells infected with recombinant baculovirus was done as described in the previous report.[33] In summary, cells were fixed in methanol for 5 min and incubated for 1 h at 37°C with anti-myc or anti-Naf1 MAb, followed by washing three times with PBS. Cells were then incubated with FITC-conjugated goat anti-mouse second antibody (American Qualex) for 1 h. As for control, the second antibody only was used. After thorough washings, cells were evaluated under fluorescence microscope (BX50F, Olympus Optical Co., Ltd.).

Silver staining and Western blot analysis

The extent of the purification of recombinant Naf1(N) protein was analyzed by silver staining and Western blot analysis. Aliquots of elusion, flow-through, and wash fractions were subjected to 12% SDS-PAGE, followed by silver staining.[34]

Cell lysates of 293 cells transfected with full-length Naf1 and Naf1(N) cDNA were subjected to Western blot analysis. Cell lysates were separated on a 12% SDS gel and blotted on an Immobilon-P (Millipore) transfer membrane. After incubation with anti-Naf1 MAb (1:1000 dilutions) and second anti-mouse antibody conjugated with horseradish peroxidase, antibody binding was detected by chemiluminescence reagent.

Epitope determination of anti-Naf1 MAb

For the epitope mapping of the anti-Naf1 MAb, truncated forms of Naf1(N) fusion proteins were expressed in *Escherichia coli*, as described previously.[35] cDNAs encoding 6×His and myc-tagged Naf1(N)-1,2,3 and Naf1(N)-A,B,C fusion protein fragments, ligated in pMAL-C2 vector after digestion with *Bam*H1 and *Hind*III were transformed into *E. coli* (XL 2-Blue). Single colonies of transformed *E. coli* were grown in Luria-Bertani (LB) medium with kanamycin (25 μg/mL). Protein expression was induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) and incubated at 37°C for 3 h. The cells were then pelleted by centrifugation and resuspended in 100 μL of lysis buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA; 100 mM NaCl) followed by sonication. Cell lysates were applied to SDS-PAGE, followed by Coomassie Brilliant Blue staining or immunoblotting with anti-Naf1 MAb.

Immunofluorescence staining and confocal microscopy

Unstimulated peripheral blood lymphocytes (PBL), Jurkat, MT-4, and Molt-4 cells were washed with PBS and fixed in 2% paraformaldehyde for 20 min on ice. Cells were washed again with PBS containing 0.1% BSA, treated with 2% normal goat serum for 30 min to block non-specific binding, and then incubated with anti-Naf1 MAb (1:500 dilution) or control mouse IgG for 1 h at room temperature. After washing, cells were incubated with FITC-conjugated goat anti-mouse IgG antibody for 1 h at room temperature. Cells were then washed and mounted with Fluorescent Mounting Medium (Dako). Fluorescent images of endogenous Naf1 were evaluated by confocal microscopy using a Zeiss (LSM-510, V-2.5) Axioplan-2, laser-imaging confocal microscope.

RESULTS

Expression and purification of recombinant Naf1(N) protein

To generate MAb against Naf1 cellular protein, we first constructed the recombinant baculovirus encoding Naf1(N) cDNA by homologous recombination of pMelBac A-Naf1(N) cDNA with the replication-deficient baculovirus DNA in Sf9 cells using Bac-N-Blue transfection kit. Recombinant virus encoding Naf1(N) was then used to infect High Five cells for large-scale

protein production. Expression of the recombinant Naf1(N) protein was examined by immunofluorescence staining of baculovirus-infected Sf9 cells with anti-myc MAb (Fig. 1A). After protein purification, the purity of recombinant Naf1(N) protein was analyzed by SDS-PAGE and Silver staining, showing recombinant Naf1(N) protein as a major band as shown in Figure 1B. Silver staining of purified Naf1(N) protein denoted the presence of minor contaminants; however, none of these bands reacted with purified MAb in Western blot analysis (Fig. 2B), indicating that the contaminants are not the components of recombinant Naf1(N) protein.

Production and characterization of anti-Naf1 MAb

Female Balb/c mice, subcutaneously immunized with purified recombinant Naf1(N) protein, revealed antibody responses in Western blot analysis using mice sera, and we selected a mouse with highest antibody response for hybridoma preparation. We obtained five positive hybridomas, and from them, one strongly positive hybridoma (Hybridoma number-4) was selected by immunofluorescent assay. We then subcloned the hybridoma by limiting dilution, and the strongest clone (C-3) was selected and cultured in large scale to be inoculated into the peritoneal cavity of BALB/c mice. Ascitic fluid, containing anti-Naf1 MAb, was collected and purified by using Protein G Sepharose columns. Immunoglobulin isotyping showed the isotype of the purified anti-Naf1 MAb as IgG1 with a lambda (λ) light chain. The specificity of MAb was examined by immunofluorescence staining of Sf9 cells infected with recombinant baculovirus (Fig. 2A) and Western blot analysis using purified anti-Naf1 MAb (Fig. 2B, C). Anti-Naf1 MAb recognized recombinant Naf1(N) protein, but not control BSA (Fig. 2B), as well as full-length Naf1 and Naf1(N) in transfected 293 cell lysates (Fig. 2C). These results clearly show that anti-Naf1 MAb is specific to Naf1 protein, not to histidine or myc, tagged to the recombinant Naf1(N) antigen. According to previous reports, it has already been shown that hexahistidine tagging takes advantage of a high-affinity to Ni-NTA resin in the purification procedure without interfering with the protein function.

Epitope analysis of MAb

To complete the characterization of MAb, we investigated the Naf1 epitope recognized by this MAb. Polyhistidine and myc-tagged Naf1(N) fusion protein fragments (1, 2, and 3) with

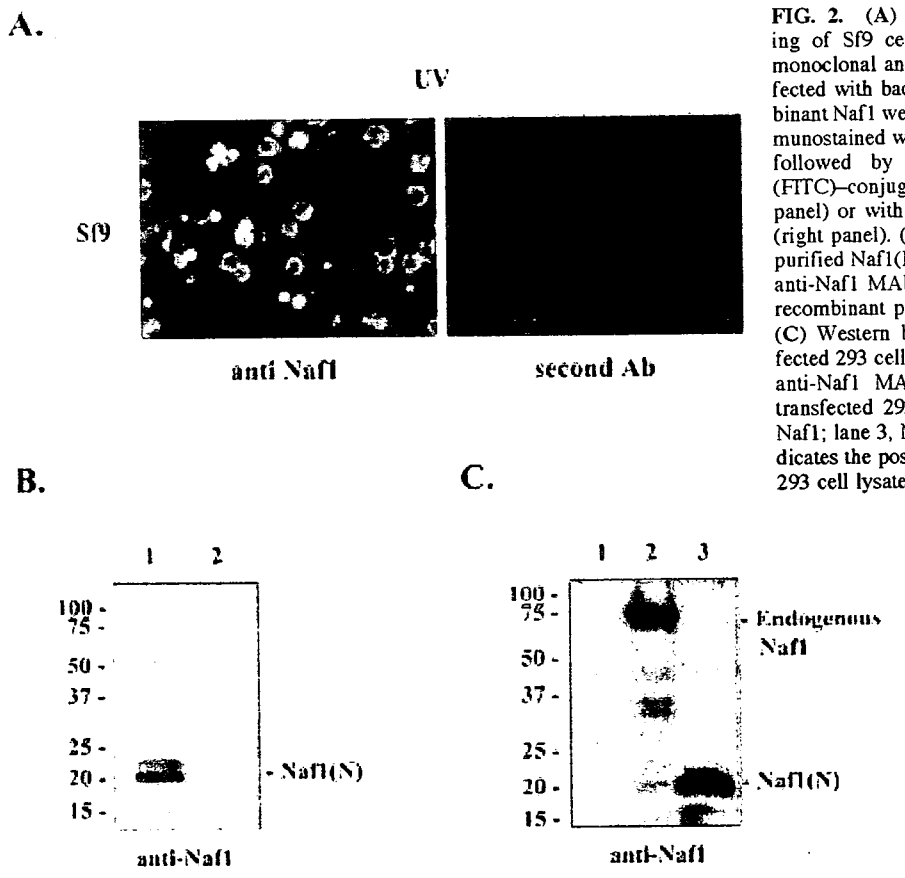


FIG. 2. (A) Immunofluorescence staining of Sf9 cells with purified anti-Naf1 monoclonal antibody (MAb). Sf9 cells infected with baculovirus expressing recombinant Naf1 were fixed in methanol and immunostained with purified anti-Naf1 MAb, followed by fluorescein isothiocyanate (FITC)–conjugated second antibody (left panel) or with second antibody (Ab) only (right panel). (B) Western blot analysis of purified Naf1(N), showing the reactivity of anti-Naf1 MAb. Lane 1, purified Naf1(N) recombinant protein; lane 2, control BSA. (C) Western blot analysis of Naf1-transfected 293 cell lysates for the specificity of anti-Naf1 MAb. Lane 1, control vector transfected 293 lysate; lane 2, full-length Naf1; lane 3, Naf1 (N). The arrowhead indicates the position of endogenous Naf1 in 293 cell lysate.

overlapping regions between amino acid residues 38 and 135, were generated (Fig. 3A). Protein expressions in induced *E. coli* were analyzed by SDS-PAGE and the Coomassie blue staining (Fig. 3B). Western blot analysis of Naf1(N) protein fragments using anti-myc antibody and the purified MAb shows that protein fragments were thoroughly produced in *E. coli*, and the epitope detected by the MAb was located at the overlapping region of protein fragments 1 and 2, between amino acid residues 64 and 88 (Fig. 3C).

We again constructed Naf1 fusion protein fragments (Fig. 3A–C) with overlapping regions between amino acid residues 64 and 88 (Fig. 3D). Expression of Naf1(N) protein fragments was ascertained by SDS-PAGE, followed by Coomassie blue staining (Fig. 3E) and Western blot analysis using anti-myc antibody (Fig. 3F). According to the result of Western blot analysis using anti-Naf1 MAb, as shown in Figure 3F, the MAb was found to have a specific binding ability to Naf1(N) fragment C. These results indicate that the epitope recognized by anti-Naf1 MAb was located at amino acid residues 81–88 region of Naf1.

Endogenous Naf1 expression and subcellular localization

The ability of the anti-Naf1 MAb to detect endogenous Naf1 in various cells was also investigated by Western blot analysis and the immunofluorescent staining of cells followed by confocal microscopy (Fig. 4A,B). Cell lysates of unstimulated human PBL, Jurkat, MT-4, Molt 4, 293, and U-937 cells were applied to 12% gel SDS-PAGE, and immunoblotting with Naf1 MAb showed endogenous Naf1 protein with the molecular weight of about 72 kDa (Fig. 4A). Immunofluorescent staining of human PBL, Jurkat, MT-4, and Molt-4 cells with anti-Naf1 MAb, followed by the assess-

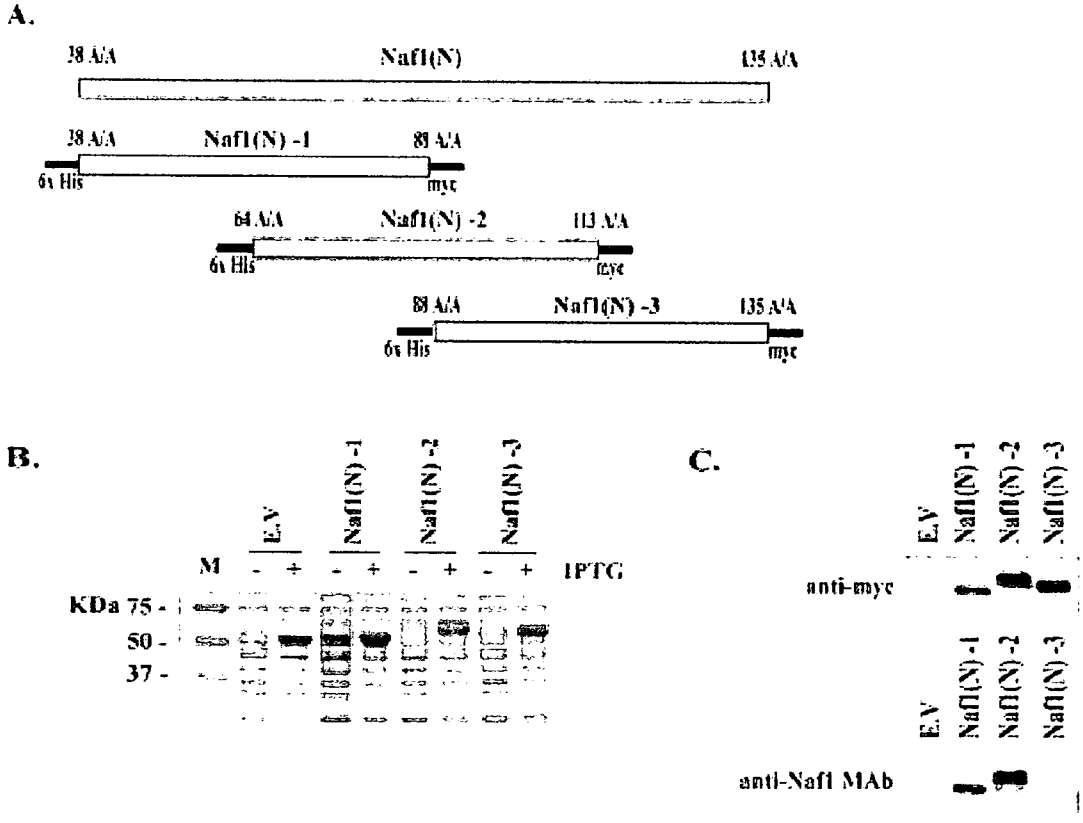


FIG. 3. Mapping of the Naf1 epitope recognized by anti-Naf1 monoclonal antibody (MAb). (**A, and D**) Schematic representation of N-terminally truncated Naf1 fusion proteins. The fusion proteins contain 6×His tag at N-terminus and myc tag at C-terminus. The numbers refer to the position of amino acids in Naf1 protein. (**B,E**) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining for the expression of Naf1 fusion proteins from un-induced (−) or isopropyl-D-thiogalactopyranoside (IPTG) induced (+) *E. coli*, transformed with control empty vector (EV) or with pMAL-6×His-Naf1-myc cDNAs. (**C,F**) Western blot analysis of induced bacterial lysates expressing Naf1 fusion proteins. Lysates of induced *E. coli* were electrophoresed in 12% acrylamide gels and immunoblotted with anti-Naf1 MAb for the specific epitope (upper panals) or anti-myc MAb for the protein input control (lower panals).

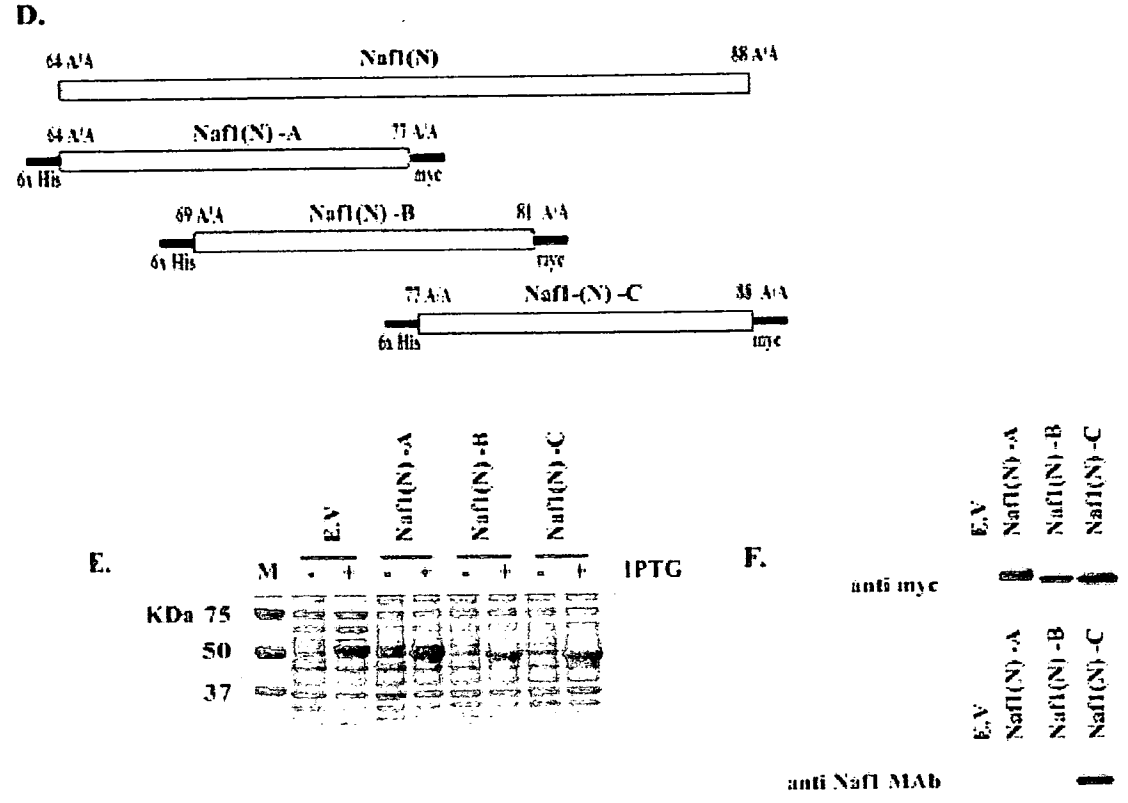


FIG. 3. *Continued.*

ment under confocal microscope, showed predominantly cytoplasmic localization of endogenous Naf1 (Fig. 4B).

DISCUSSION

In this report, we present the production and characterization of an MAb specific for the cellular protein, Naf1/ABIN-1. We used the baculovirus expression system to synthesize recombinant Naf1(N) protein. High Five cells were described to achieve higher protein production when compared with Sf9 cells.[30] Previous reports showed that hexahistidine tagging takes advantage of a high affinity to Ni-NTA resin in the purification procedure and does not interfere with the protein function.[29]

The ability of anti-Naf1 MAb to recognize endogenous Naf1 protein in various cell lines was examined by Western blotting and immunostaining. We also investigated the expression and localization of endogenous Naf1 in human PBL and other cell lines showing its cytoplasmic localization. According to previous reports, Naf1 associates with HIV-1 viral proteins, Nef, and matrix in the yeast two-hybrid system and pull-down assays using Naf1-overexpressed cell lysates.[1,2] HIV-1 Nef contributes substantially to AIDS pathogenesis by augmenting virus replication and markedly perturbing the T cell functions. Host cell activation by viral protein Nef has been supposed to be a result of interaction between Nef with cellular protein Naf1, which is involved in the signal transduction of host cells. Naf1 has also been known to interact with A20 zinc finger protein, previously characterized as an inhibitor of NF-κB activation and apoptosis. Naf1 (ABIN-1) has been reported to inhibit NF-κB–dependent gene expression induced by TNF and IL-1. Naf1 blocks NF-κB activation by negative feedback regulation of NF-κB, and it has also been suggested that Naf1 has the potential to inhibit TNF-induced activation of NF-κB upon overexpression.[24,26] However, most of these earlier experiments were performed with Naf1-overexpressed cells because of the lack of specific MAb to the endogenous Naf1. To investigate the Naf1-interacting proteins and their molecular functions under physiological condition, anti-Naf1 MAb is required to access endogenous Naf1 in some specific examinations, such as immunoprecipitation and immunostaining assays. The improvement in understanding of the activation and regulation of NF-κB has opened the way for the development of new treatments in inflammatory diseases as well

as in HIV infection. From a therapeutic point of view, genetic engineering of specific human cells to express an NF-κB inhibitor such as Naf1 will offer a novel therapeutic tool in gene therapy.

In conclusion, anti-Naf1 MAb will be an indispensable tool in various immunoassay and functional investigations concerning the endogenous Naf1. We hope this MAb may lead to new findings and understanding of Naf1 in many aspects relating to HIV infection and various inflammatory diseases in human beings.

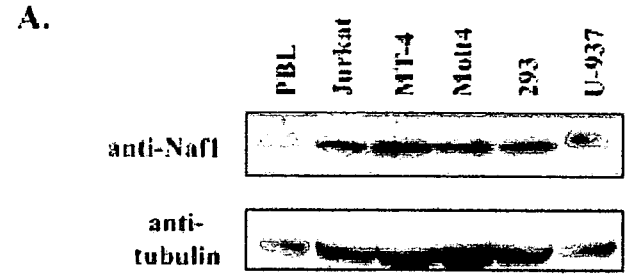
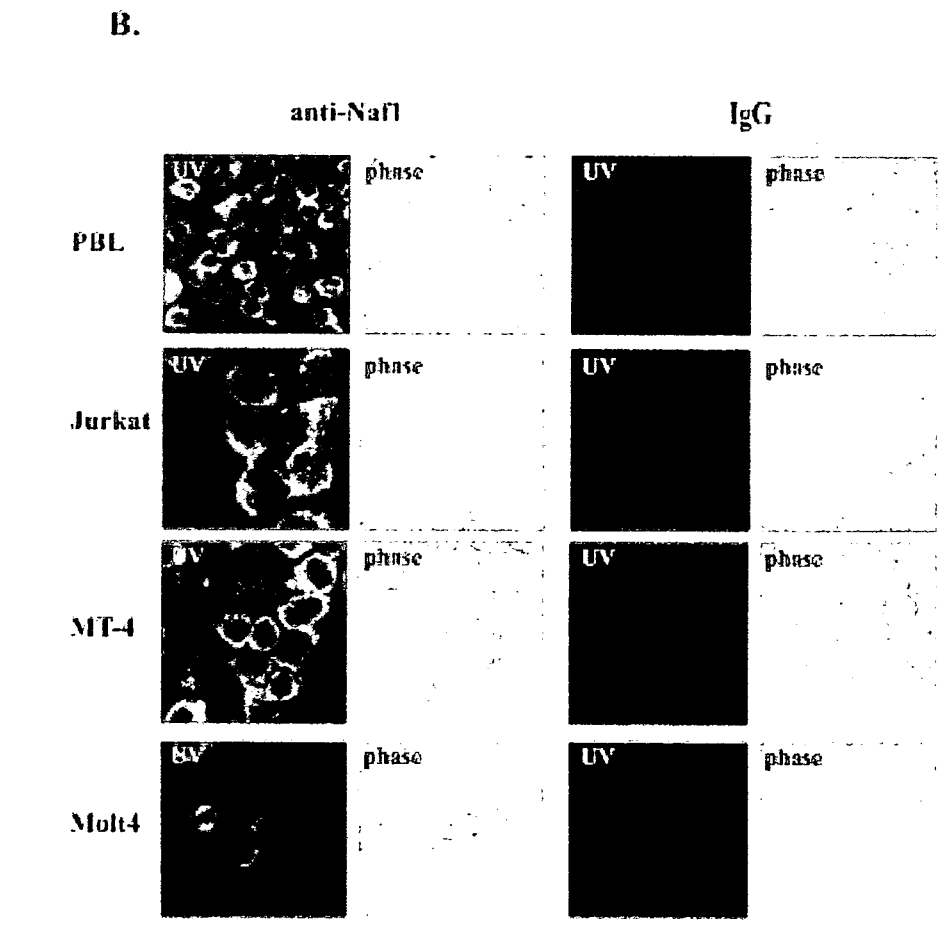


FIG. 4. Naf1 expression in human PBL, Jurkat, MT-4, Molt-4, 293, and U-937 cell lines. (A) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of cell lysates for the expression of endogenous Naf1 using anti-Naf1 monoclonal antibody (MAb). (B) Subcellular localization of Naf1 in human PBL, Jurkat, MT-4, and Molt-4 cells by immunofluorescence staining with anti-Naf1 MAb or control IgG and assessment under confocal microscopy.



ACKNOWLEDGMENTS

We thank Dr. Kazuo Terashima and Masami Koushi for their excellent technical assistance in immunostaining and confocal microscopy. This work was supported by grants from the Ministry of Education, Science and Culture, and the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

1. Fukushi M, Dixon J, Kimura T, Tsurutani N, Michael Dixon M, and Yamamoto N: Identification and cloning of a novel cellular protein Naf1, Nef-associated factor 1, that increases cell surface CD4 expression. FEBS Lett 1999;442:83–88.
2. Gupta K, Ott D, Hope TJ, Siliciano RF, and Boeke JD: A Human nuclear shuttling protein that interacts with human immunodeficiency virus type 1 matrix is packed into virions. J Virol 2000;74: 11811–11824.
3. Kestler HW 3rd, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, and Desrosiers RC: Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell 1991;65:651–662.
4. Miller MD, Warmerdam MT, Gaston I, Greene WC, and Feinberg MB: The human immunodeficiency virus–1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J Exp Med 1994;179:101–113.
5. Spina CA, Kwoh TJ, Chowers MY, Guatelli JC, and Richman DD: The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. J Exp Med 1994;179:115–123.
6. Chowers MY, Spina CA, Kwoh TJ, Fitch NJ, Richman DD, and Guatelli JC: Optimal infectivity *in vitro* of human immunodeficiency virus type 1 requires an intact nef gene. J Virol 1994;68:2906–2914.
7. Harris M: From negative factor to a critical role in virus pathogenesis: the changing fortunes of Nef J Gen Virol 1996;77:2379–2392.
8. Kawano Y, Tanaka Y, Misawa N, Tanaka R, Kira JI, Kimura T, Fukushi M, Sano K, Goto T, Nakai M, Kobayashi T, Yamamoto N, and Koyanagi Y: Mutational analysis of human immunodeficiency virus type 1 (HIV-1) accessory genes: requirement of a site in the nef gene for HIV-1 replication in activated $CD4^+$ T cells *in vitro* and *in vivo*. J Virol 1997;71:8456–8466.
9. Cullen BR: HIV-1 auxiliary proteins: making connections in a dying cell. Cell 1998;93:685–692.
10. Joseph AM, Kumar M, and Mitra D: Nef: "necessary and enforcing factor" in HIV infection. Curr HIV Res 2005;3:87–94.
11. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, and Desrosiers RC: Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N Engl J Med 1995; 332:228–232.
12. Piguet V, Chen YL, Mangasarian A, Foti M, Carpentier JL, and Trono D: Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the μ chain of adaptor complexes. EMBO J 1998; 17:2472–2481.
13. Kim YH, Chang SH, Kwon JH, and Rhee SS: HIV-1 Nef plays an essential role in two independent processes in CD4 down-regulation: dissociation of the CD4–$p56^{lck}$complex and targeting of CD4 to lysosomes. Virology 1999;257:208–219.
14. Swann SA, Williams M, Story CM, Bobbitt KR, Fleis R, and Collins KL: HIV-1 Nef blocks transport of MHC class I molecules to the cell surface via a PI 3-kinase-dependent pathway. Virology 2001;282:267–277.
15. Glushakova S, Munch J, Carl S, Greenough TC, Sullivan JL, Margolis L, and Kirchhoff F: CD4 down-modulation by human immunodeficiency virus type 1 Nef correlates with the efficiency of viral replication and with $CD4^+$ T-cell depletion in human lymphoid tissue *ex vivo*. J Virol 2001;75:10113–10117.
16. Lundquist CA, Tobiume M, Zhou J, Unutmaz D, and Aiken C: Nef-mediated downregulation of CD4 enhances human immunodeficiency virus type 1 replication in primary T lymphocytes. J Virol 2002;76:4625–4633.
17. Casartelli N, Di Matteo G, Potesta M, Rossi P, and Doria M: CD4 and major histocompatibility complex class I downregulation by the human immunodeficiency virus type 1 nef protein in pediatric AIDS progression. J Virol 2003;77:11536–11545.
18. Cortes MJ, Wong-Staal F, and Lama J: Cell surface CD4 interferes with the infectivity of HIV-1 particles released from T cells. J Biol Chem 2002;277:1770–1779.
19. Christiane F, Stroka DM, Badrichani AZ, Cooper JT, Wrighton CJ, Soares M, Grey ST, and Bach FH: A20 inhibits NF-κB activation in endothelial cells without sensitizing to tumor necrosis factor–mediated apoptosis. Blood 1998;91:2249–2258.
20. Heyninck K, De Valck D, Vanden Berghe W, Van Criekinge W, Contreras R, Fiers W, Haegeman G, and Beyaert R: The zinc finger protein A20 inhibits TNF-induced NF-κB–dependent gene expression by interfering with an RIP- or TRAF2-mediated transactivation signal and directly binds to a novel NF-κB inhibiting protein ABIN. J Cell Biol 1999;145:1471–1482.
21. Lee EG, Boone DL, Chai S, Libby SL, Chien M, Lodolce JP, and Ma A: Failure to regulate TNF-induced NF-κB and cell death responses in A20-deficient mice. Science 2000;289:2350–2354.
22. Klinkenberg M, Van Huffel S, Heyninck K, and Beyaert R: Functional redundancy of the zinc fingers of A20 for inhibition of NF-κB activation and protein-protein interactions. FEBS Lett 2001; 498:93–97.
23. Yamamoto Y, and Gaynor RB: IκB kinases: key regulators of the NF-κB pathway. Trends Biochem Sci 2004;29:72–79.
24. Beyaert R, Heyninck K, and Van Huffel S: A20 and A20-binding proteins as cellular inhibitors of nuclear factor-κB-dependent gene expression and apoptosis. Biochem Pharmacol 2000;60: 1143–1151.
25. Heyninck K, Kreike MM, and Beyaert R: Structure-function analysis of the A20-binding inhibitor of NF-κB activation, ABIN-1. FEBS Lett 2003;536:135–140.
26. Gallagher J, Howlin J, McCarthy C, Murphy EP, Bresnihan B, FitzGerald O, Godson C, Brady HR, and Martin F: Identification of Naf1/ABIN-1 among TNF-α–induced expressed genes in human synoviocytes using oligonucleotide microarrays. FEBS Lett 2003;551:8–12.
27. El Bakkouri K, Wullaert A, Haegman M, Heyninck K, and Beyaert R: Adenoviral gene transfer of the NF-κB inhibitory protein ABIN-1 decreases allergic airway inflammation in a murine asthma model. J Biol Chem 2005;280:17938–17944.
28. Zhang S, Fukushi M, Hashimoto S, Gao C, Huang L, Fukuyo Y, Nakajima T, Amagasa T, Enomoto S, Koike K, Miura O, Yamamoto N, and Tsuchida N: A new ERK2 binding protein, Naf1, attenuates the EGF/ERK2 nuclear signaling. Biochem Biophys Res Commun 2002;297:17–23.
29. Massotte D, Baroche L, Simonin F, Yu L, Kieffer B, and Pattus F: Characterization of δ, κ, and μ human opioid receptors overexpressed in baculovirus-infected insect cells. J Biol Chem 1997;272: 19987–19992.
30. Kozasa T, and Gilman AG: Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits. Characterization of α_{12} and inhibition of adenylyl cyclase by α_z. J Biol Chem 1995;270:1734–1741.
31. Grennan Jones F, Wolstenholme A, Fowler S, Smith S, Ziemnicka K, Bradbury J, Furmaniak J, and Rees Smith B: High-level expression of recombinant immunoreactive thyroid peroxidase in the High Five insect cell line. J Mol Endocrinol 1996;17:165–174.

32. Nelson PN, Reynolds GM, Waldron EE, Ward E, Giannopoulos K, and Murray PG: Monoclonal antibodies. Mol Pathol 2000;53: 111–117.
33. Horiuchi S, Ampofo W, Koyanagi Y, Yamashita A, Waki M, Matsumoto A, Yamamoto M, and Yamamoto N: High-level production of alternatively spliced soluble interleukin-6 receptor in serum of patients with adult T-cell leukaemia/HTLV-I–associated myelopathy. Immunology 1998;95:360–369.
34. Rabilloud T, Vuillard L, Gilly C, and Lawrence JJ: Silver-staining of proteins in polyacrylamide gels: a general overview. Cell Mol Biol 1994;40:57–75.
35. Na BK, Chung GT, and Song CY: Production, characterization, and epitope mapping of a monoclonal antibody against aspartic proteinase of *Candida albicans*. Clin Diagn Lab Immunol 1999;6: 429–433.

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Received for publication June 17, 2005. Accepted for publication July 14, 2005.