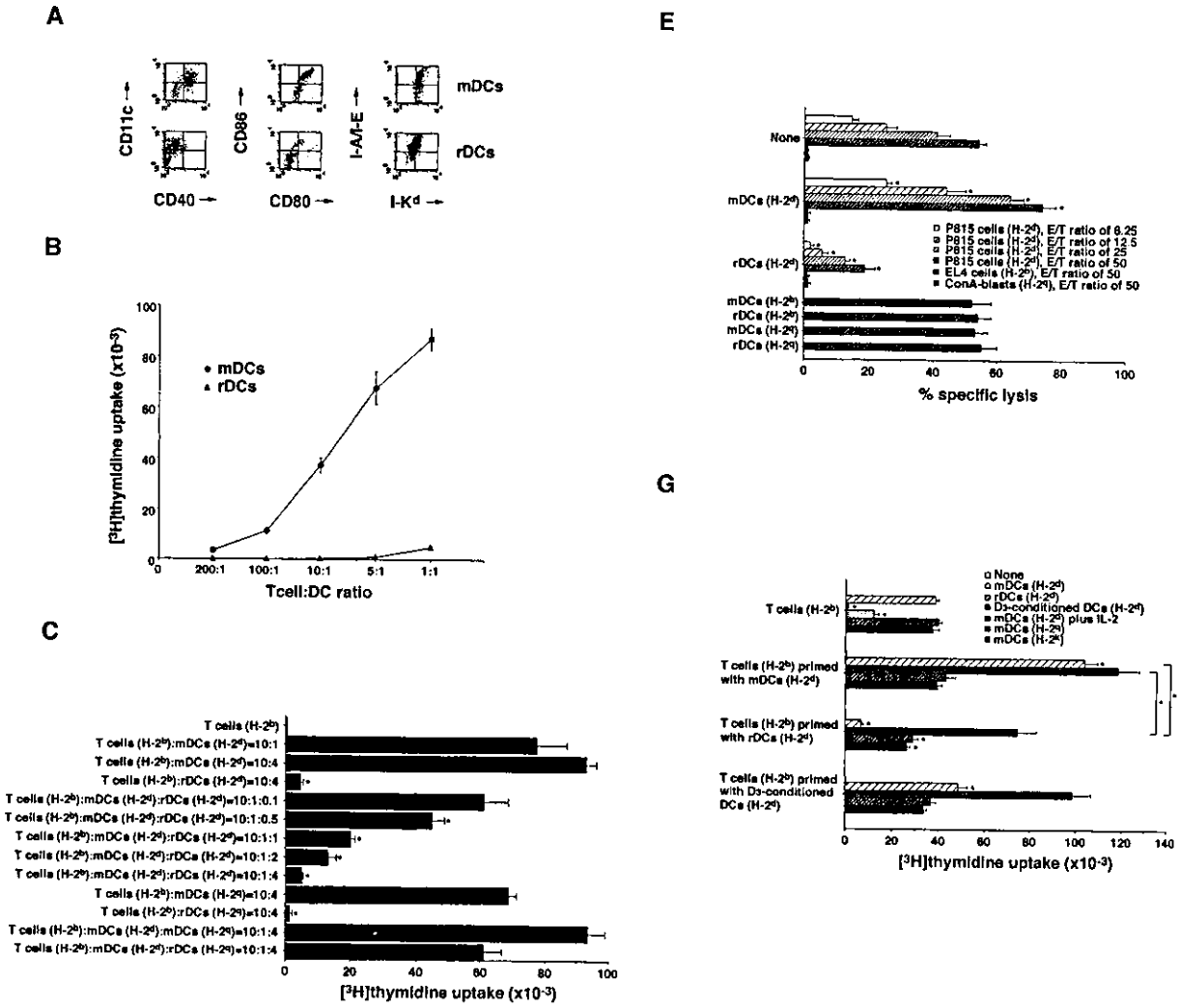


Figure 1. rDCs Regulate the Function of T Cells

(A) Cells were stained with the stated mAbs, and cell surface expression was analyzed by flow cytometry. Data are represented by a dot plot.
(B) $CD4^+$ T cells ($H\text{-}2^b$) were cultured with mDCs or rDCs ($H\text{-}2^d$) at various T cell:DC ratios, and the proliferative response was measured.
(C and D) $I\text{-}K^{b+}CD4^+$ T cells or $I\text{-}K^{d+}CD4^+$ T cells obtained from the transplanted recipients ($H\text{-}2^d$) (C) or ($H\text{-}2^q$) (D) were cultured with mDCs ($H\text{-}2^d$) (C) or ($H\text{-}2^q$) (D) in the presence or absence of mDCs or rDCs obtained from the indicated strains at various T cell:DC ratios, and the proliferative response was measured. Primed $CD4^+$ T cells ($H\text{-}2^b$) plus mDCs ($H\text{-}2^d$) (C) or primed $CD4^+$ T cells ($H\text{-}2^d$) plus mDCs ($H\text{-}2^q$) (D) versus any other group, $P < 0.01$.
(E and F) $I\text{-}K^{b+}CD8^+$ T cells or $I\text{-}K^{d+}CD8^+$ T cells obtained from the transplanted recipients ($H\text{-}2^d$) (E) or ($H\text{-}2^b$) (F) were cultured with medium alone (none) or the indicated types of DCs at a T cell/DC ratio of 10:1 and were subjected to CTL assay against the targeted cells obtained from various strains. $CD8^+$ T cells alone versus any other group, $P < 0.01$.
(G and H) $CD4^+$ T cells ($H\text{-}2^b$) (G) or ($H\text{-}2^d$) (H) were cultured with or without the indicated types of DCs ($H\text{-}2^d$) (G) or ($H\text{-}2^q$) (H) at a T cell/DC ratio of 10:1 in a first coculture. In another experiment, $CD4^+$ T cells obtained from a first coculture were then cultured with medium alone (none) or mDCs obtained from various strains at a T cell/DC ratio of 10:1 in the presence or absence of IL-2 in a second coculture, and the proliferative response was measured. Primed $CD4^+$ T cells ($H\text{-}2^b$) plus mDCs (G) or primed $CD4^+$ T cells ($H\text{-}2^d$) plus mDCs (H) versus any other group, $P < 0.01$. Five replicate experiments with similar results were pooled.

weight loss, became apparent within 6 days. On the other hand, a single injection of mDCs (1.5×10^6/mouse, $H\text{-}2^d$) 2 days following transplantation enhanced the lethality caused by acute GVHD ($P < 0.01$, Figure 2A). In contrast, a single injection with the host-matched rDCs (1.5×10^6/mouse, $H\text{-}2^d$), but not host-mismatched rDCs ($H\text{-}2^b$ and $H\text{-}2^q$), 2 days following transplantation completely protected the recipients from this lethality, and they survived over 60 days with little or no signs of acute GVHD ($P < 0.01$, Figures 2A and 2B). In addition, the therapeutic effect of rDCs on acute GVHD was stronger than that of D_3-conditioned DCs ($P < 0.01$, Figure 2B). We also observed similar specific efficacy of rDCs in other strain combinations (Figure 2C).

We evaluated the therapeutic efficacy of rDCs ($H\text{-}2^d$) in the recipients ($H\text{-}2^q$) of BMS ($H\text{-}2^b$) (Figure 2D). The therapeutic effect of rDCs on acute GVHD was observed in a dose-dependent fashion (1.5×10^4–1.5×10^6/mouse) when the recipients received a single injection with rDCs 2 days after transplantation, and the repetitive

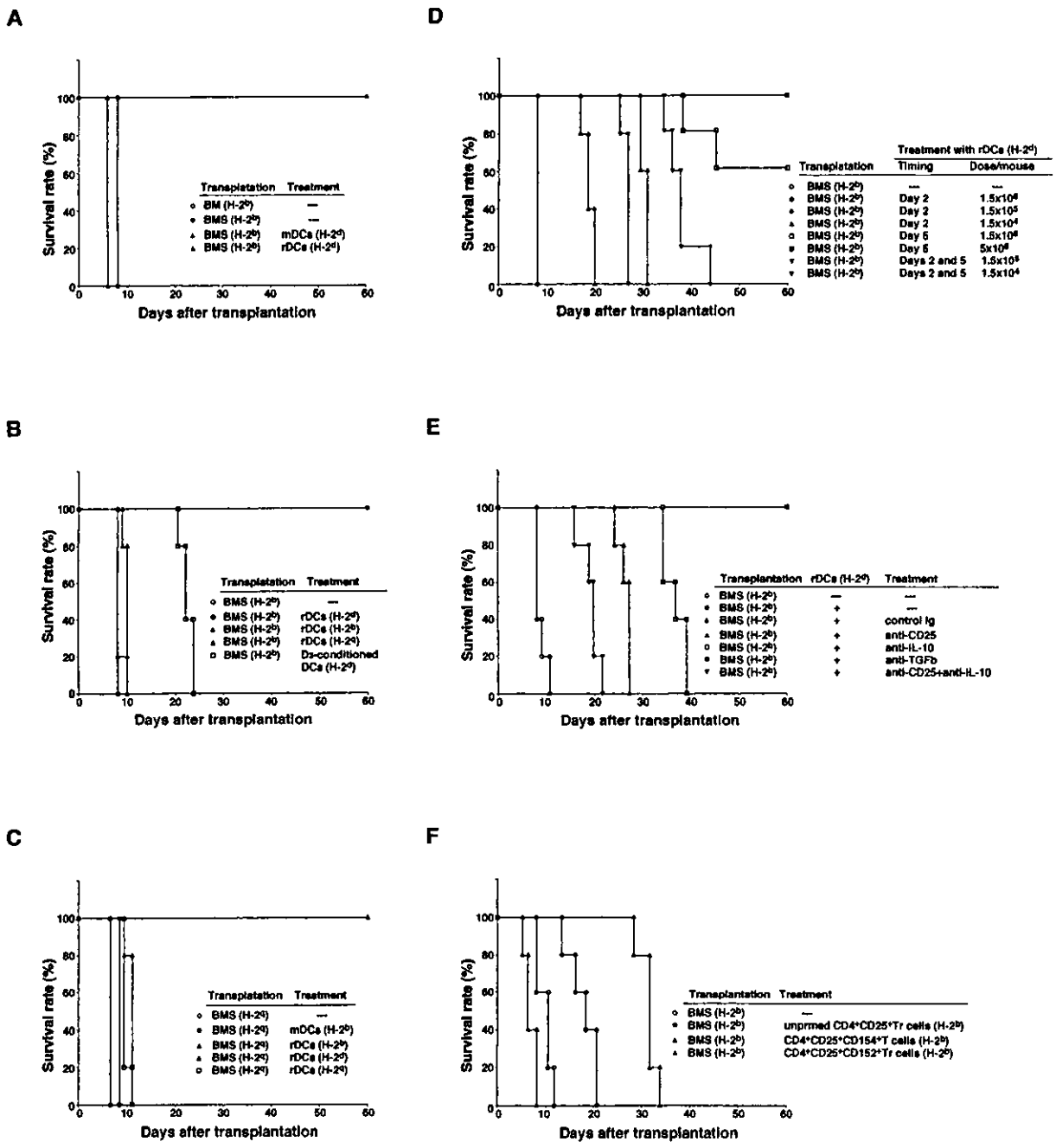


Figure 2. rDCs Protected the Recipients of Allogeneic BMS from the Lethality Caused by Acute GVHD

(A and B) Recipients (H-2^d) of BM cells or BMS (H-2^b) were injected with or without the indicated types of DCs 2 days after transplantation. (C) Recipients (H-2^b) of BMS (H-2^d) were injected with or without the indicated types of DCs 2 days after transplantation. (D) Recipients (H-2^d) of BMS (H-2^b) were injected with or without various doses of rDCs (H-2^d) on the indicated days after transplantation. (E and F) Recipients (H-2^d) of BMS (H-2^b) injected with rDCs (H-2^d) were treated with the indicated Abs (E) or injected with the indicated types of CD4$^+$CD25$^+$ T cells (H-2^b) (F) after transplantation as described in Experimental Procedures. Untreated recipients of allogeneic BMS versus any other group, $P < 0.01$. Two replicate experiments with similar results were pooled.

injections of rDCs (1.5×10^4 and 1.5×10^5/mouse) 2 and 5 days after transplantation enhanced the survival rate in the recipients ($P < 0.01$). On the other hand, a single injection with rDCs (1.5×10^6/mouse) 5 days after transplantation reduced the therapeutic effect whereas a high-dose injection (5×10^6/mouse) with rDCs completely protected the recipients from the lethality.

Analysis of the Regulatory Effect of rDCs in the Recipients of Allogeneic BMS

We examined the I-K^{b+} donor-derived T cells in spleen mononuclear cells in the recipients (H-2^d) of BMS (H-2^b) 5 days after transplantation (Figure 3A). I-K^{b+}CD3$^+$, I-K^{b+}CD4$^+$, and I-K^{b+}CD8$^+$ subpopulations were greater in the recipients injected with mDCs following trans-

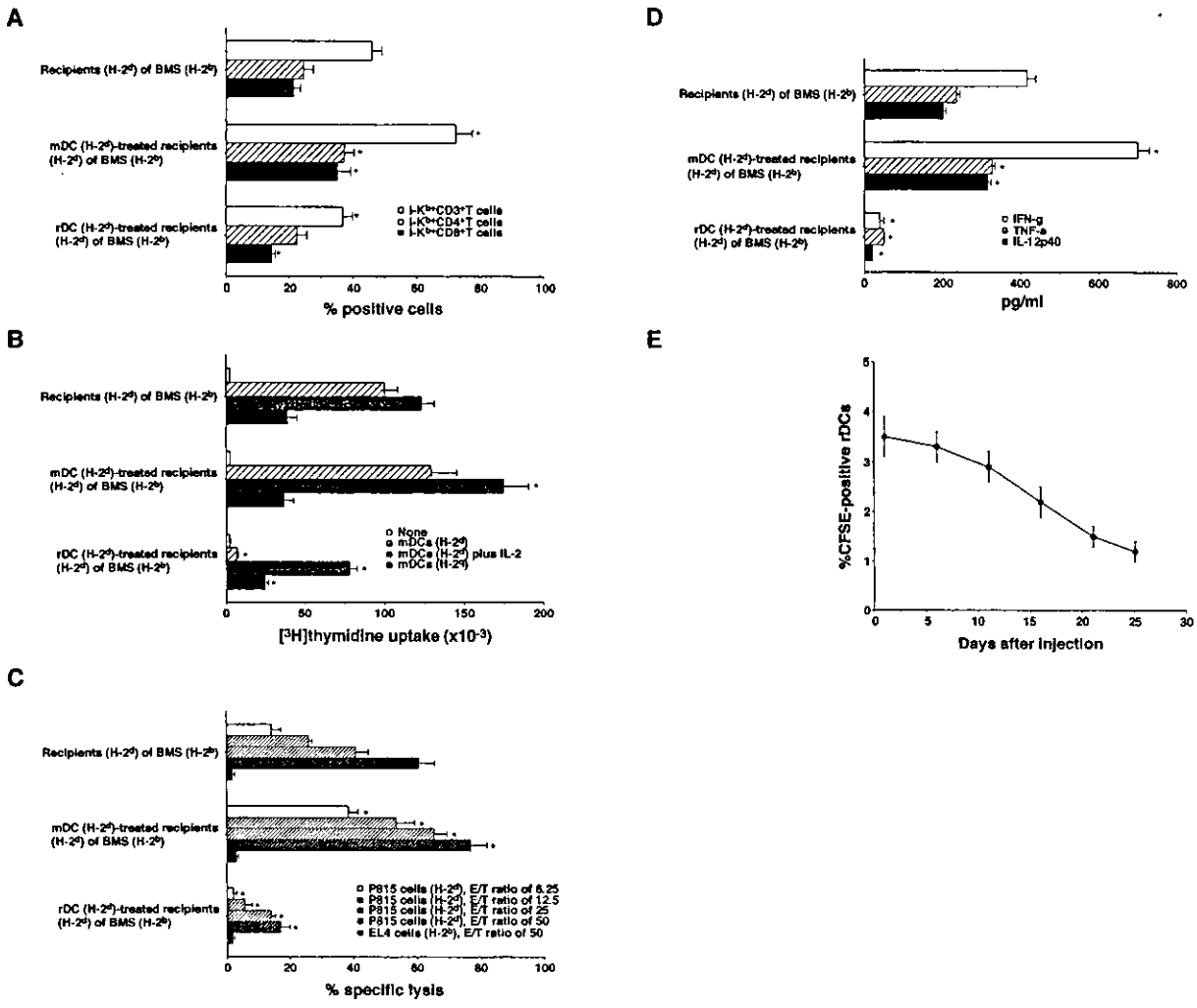


Figure 3. rDCs Impair Allogeneic Ag-Specific Responses of CD4+ T Cells and CD8+ T Cells in Transplanted Mice

Recipients (H-2d) of BMS (H-2b) were injected with or without mDCs or rDCs (H-2d), and spleen mononuclear cells and sera were obtained from each group 5 days after transplantation. (A) Cells were stained with the stated mAbs, and cell surface expression was analyzed by flow cytometry. Data are expressed as percent positive cells. (B) I-Kb+CD4+ T cells obtained from spleen mononuclear cells were cultured with medium alone (none) or mDCs (H-2d or H-2q) in the presence or absence of IL-2 at a T cell/DC ratio of 10:1, and the proliferative response was measured. (C) I-Kb+CD8+ T cells obtained from spleen mononuclear cells were subjected to CTL assay against P815 cells or EL4 cells. (D) Concentrations of IFN-γ, TNF-α, and IL-12p40 in sera were evaluated by ELISA. Untreated recipients of allogeneic BMS versus any other group, $P < 0.01$. (E) Recipients (H-2d) of BMS (H-2b) were injected with CFSE-labeled rDCs (H-2d) 2 days after transplantation, and CFSE-labeled rDCs in spleen mononuclear cells were determined at the indicated days after injection by flow cytometry. Five replicate experiments with similar results were pooled.

plantation (mDC-treated recipients) than untreated recipients of allogeneic BMS (untreated recipients). On the other hand, I-Kb+CD3+ and I-Kb+CD8+ subpopulations were decreased in the recipients injected with rDCs following transplantation (rDC-treated recipients) as compared with untreated recipients, although there were no significant differences among I-Kb+CD4+ subpopulations.

We also examined the allogeneic response of I-Kb+CD4+ T cells to mDCs (Figure 3B). I-Kb+CD4+ T cells obtained from untreated recipients and mDC-treated recipients responded vigorously to mDCs (H-2d). In contrast, I-Kb+CD4+ T cells obtained from rDC-treated recipients were hyporesponsive to mDCs (H-2d). Furthermore, the addition of IL-2 to cultures partly restored this response, and the response of these I-Kb+CD4+ T cells to mDCs (H-2q) was slightly lower than those of other groups of recipient mice.

To examine CTL activity of I-Kb+CD8+ T cells to recipient tissues (H-2d), we examined the lytic activity of I-Kb+CD8+ T cells obtained from the recipients against P815 cells and EL4 cells (Figure 3C). I-Kb+CD8+ T cells obtained from mDC-treated recipients showed a higher lytic activity against P815 cells than those obtained from untreated recipients. In contrast, I-Kb+CD8+ T cells obtained from rDC-treated recipients exhibited a reduced lytic activity against P815 cells. We also observed little or no cytotoxic activity of these I-Kb+CD8+ T cells against EL4 cells, indicating that their cytotoxicity was H-2d specific.

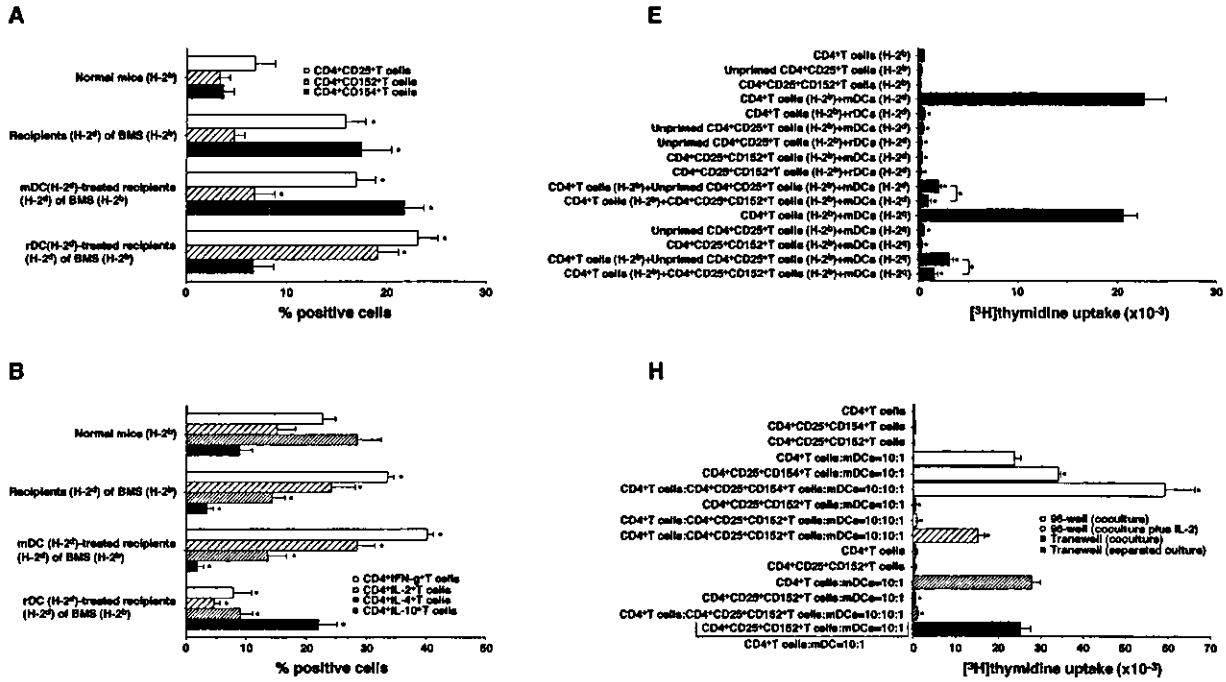


Figure 4. Characterization of Donor-Derived CD4$^+$CD25$^+$ T Cells in Transplanted Mice

Allogeneic transplantation was performed as described in Figure 3, and I-K^b$^+$CD4$^+$ T cells were obtained from spleen mononuclear cells in each group.

(A and B) I-K^b$^+$CD4$^+$ T cells were assayed for phenotype (A) and cytokine profile (B) by flow cytometry. Data are expressed as percent positive cells. CD4$^+$ T cells obtained from normal mice versus any other group, $P < 0.01$.

(C) CD4$^+$CD25$^+$ T cells isolated from normal mice (H-2^b) or I-K^b$^+$CD4$^+$CD25$^+$ T cells isolated from I-K^b$^+$CD4$^+$ T cells in each group of recipients were assayed for phenotype by flow cytometry. Data are represented by a dot plot.

(D) Unprimed CD4$^+$CD25$^+$ T cells (H-2^b) or I-K^b$^+$CD4$^+$ CD25$^+$ T cells isolated from rDC-treated recipients on the indicated days after transplantation were assayed for CD152 expression by flow cytometry. Data are expressed as percent positive cells. Unprimed CD4$^+$CD25$^+$ T cells versus any other group, $P < 0.01$.

(E) CD4$^+$ T cells (H-2^b), unprimed CD4$^+$CD25$^+$ T cells (H-2^b), and/or I-K^b$^+$CD4$^+$CD25$^+$CD152$^+$ T cells were cultured with or without mDCs or rDCs (H-2^d or H-2^q) at a T cell/DC ratio of 10:1, and the proliferative response was measured.

(F) CD4$^+$ T cells (H-2^b) were cultured with mDCs (H-2^d) at a T cell/DC ratio of 10:1 in the presence or absence of the different numbers of unprimed CD4$^+$CD25$^+$ T cells or I-K^b$^+$CD4$^+$CD25$^+$CD152$^+$ T cells isolated from rDC-treated recipients on day 5 after transplantation, and the proliferative response was measured.

(G) CD4$^+$ T cells (H-2^b) were cultured with mDCs (H-2^d) in the presence or absence of unprimed CD4$^+$CD25$^+$ T cells or I-K^b$^+$CD4$^+$CD25$^+$ T cells isolated from rDC-treated recipients on the indicated days after transplantation at a T cell/CD25$^+$T cell/DC ratio of 10:1:1, and the proliferative response was measured.

(H) CD4$^+$ T cells (H-2^b) were cultured with or without mDCs (H-2^d) in the presence or absence of I-K^b$^+$CD4$^+$CD25$^+$ CD154$^+$ T cells, I-K^b$^+$CD4$^+$CD25$^+$CD152$^+$ T cells, or IL-2 at a T cell/CD25$^+$T cell/DC ratio of 10:10:1. For Transwell experiments, I-K^b$^+$CD4$^+$CD25$^+$CD152$^+$ T cells plus mDCs (H-2^d) were either added directly to the coculture of CD4$^+$ T cells (H-2^b) with mDCs (H-2^d) or were separated in 24-well plates. Following depletion of mDCs, T cells were transferred to 96-well plates to measure the proliferative response. CD4$^+$ T cells plus mDCs (E, G, H) or CD4$^+$ T cells, unprimed CD4$^+$CD25$^+$ T cells plus mDCs (G) versus any other group, $P < 0.01$. Five replicate experiments with similar results were pooled.

We further evaluated the levels of proinflammatory cytokines in serum in the recipients of allogeneic BMS 5 days after transplantation (Figure 3D). The levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-12p40 in serum obtained from mDC-treated recipients were higher than those in untreated recipients, whereas the production of these proinflammatory cytokines was significantly reduced in rDC-treated recipients (Figure 3D).

To better understand the half-life of the injected rDCs, the recipients (H-2^d) of BMS (H-2^b) were injected with carboxyfluorescein diacetate-succinimidyl ester (CFSE)-labeled rDCs (H-2^d), and their trafficking in spleen was examined by flow cytometry (Figure 3E). We detected approximately 4% of CFSE-labeled rDCs in spleen mononuclear cells the next day after transplantation, and the half-life of the injected rDCs was around 18 days after transplantation.

To examine the stability of rDCs under inflammatory conditions after in vivo infusion, we examined the phenotype and allogeneic T cell stimulatory capacity of rDCs obtained from rDC (H-2^d)-treated recipients (H-2^b) of BMS (H-2^q) 5 days after transplantation (Table 1). We observed little or no change in the property of rDCs following in vivo infusion. These results indicate that rDCs retain their property even under inflammatory conditions in vivo.

Analysis of CD4$^+$CD25$^+$ T Cells in the Protected Mice

CD4$^+$CD25$^+$ regulatory T (Tr) cells, which suppress the function of CD4$^+$ T cells, are supposed to maintain im-

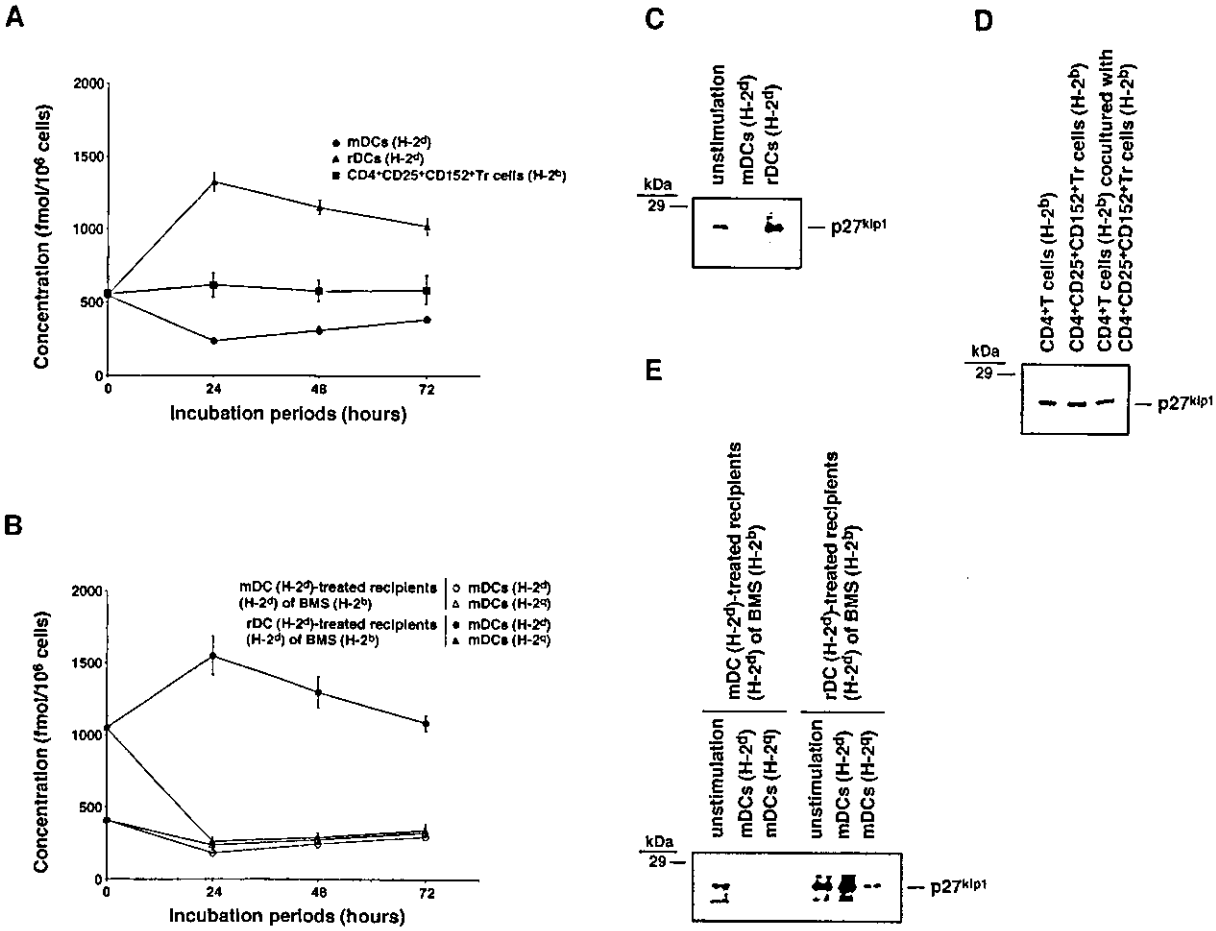


Figure 5. Involvement of cAMP and $p27^{kip1}$ in an Induction of Allogeneic Ag-Specific Tolerant $CD4^+$ T Cells by rDCs

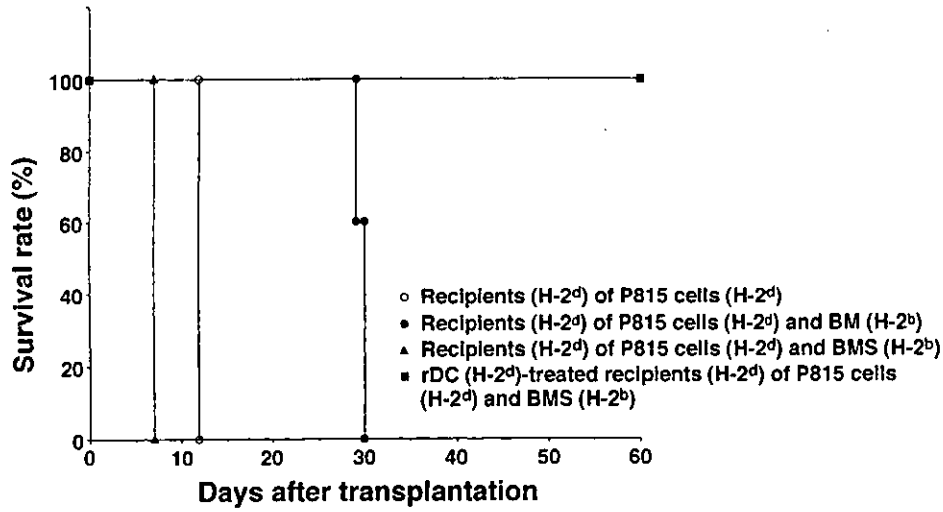
(A, C, and D) $CD4^+$ T cells (H-2^b) were obtained from the coculture of $CD4^+$ T cells (H-2^b) with the indicated types of DCs (H-2^d) or I-$K^{b+}CD4^+$ $CD25^+CD152^+$ Tr cells as described in Figure 4 at a T cell/DC ratio of 10:1 or at a T cell/$CD25^+$ T cell ratio of 1:1. (B and E) Allogeneic transplantation was performed as described in Figure 3, and I-$K^{b+}CD4^+$ T cells obtained from spleen mononuclear cells in each group were then cultured with mDCs (H-2^d or H-2^q) at a T cell/DC ratio of 10:1. Following these procedures, $CD4^+$ T cells were isolated from the coculture. (A and B) The concentration of intracellular cAMP was measured. (C, D, and E) The expression of $p27^{kip1}$ was analyzed by immunoblot. Five replicate experiments with similar results were pooled.

munologic self-tolerance or control immunopathogenic diseases (Thornton and Shevach, 1998; Sakaguchi, 2000; Maloy and Powrie, 2001). We therefore examined the subpopulations of transplanted I-$K^{b+}CD4^+$ T cells and their ability to produce cytokines in mDC- or rDC (H-2^d)-treated recipients (H-2^d) of BMS (H-2^b). $CD25^+$ and $CD154^+$ subpopulations were increased in I-$K^{b+}CD4^+$ T cells obtained from untreated recipients and mDC-treated recipients whereas $CD25^+$ and $CD152^+$ subpopulations were increased in I-$K^{b+}CD4^+$ T cells obtained from rDC-treated recipients as compared with $CD4^+$ T cells obtained from normal mice (unprimed $CD4^+$ T cells, H-2^b) (Figure 4A). On the other hand, IFN-γ- and IL-2-producing cells were increased in I-$K^{b+}CD4^+$ T cells obtained from untreated recipients and mDC-treated recipients, whereas IL-10-producing cells were increased in I-$K^{b+}CD4^+$ T cells obtained from rDC-treated recipients as compared with unprimed $CD4^+$ T cells (H-2^b) (Figure 4B).

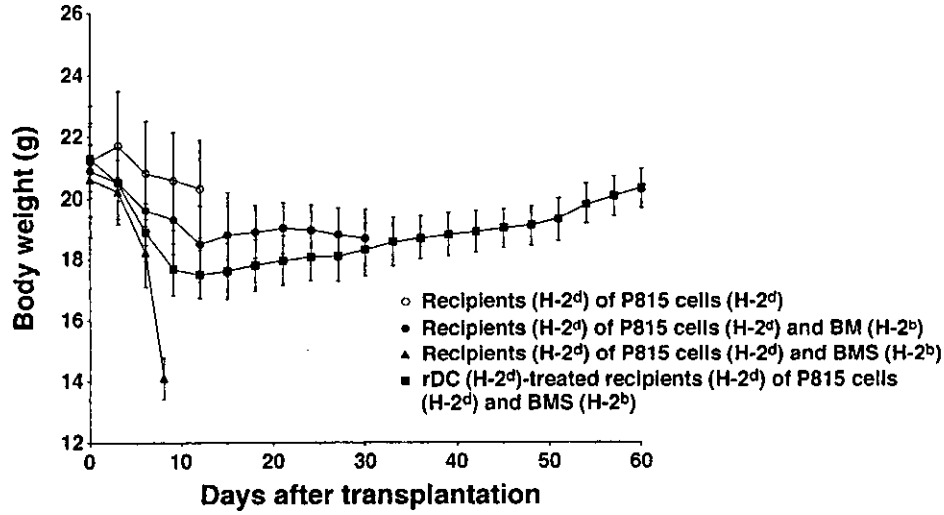
We examined the phenotype of I-$K^{b+}CD4^+CD25^+$ T cells obtained from mDC- or rDC-treated recipients. Similar to the previous report (Takahashi et al., 2000), a part of $CD4^+CD25^+$ T cells obtained from normal mice (unprimed $CD4^+CD25^+$ T cells, H-2^b) constitutively expressed CD152, whereas they did not express CD154 (Figure 4C; see supplemental data at http://www.immunity.com/cgi/content/full/18/3/367/DC1). On the other hand, $CD4^+CD25^+$ T cells obtained from mDC-treated recipients predominantly expressed CD154, whereas a large proportion of $CD4^+CD25^+$ T cells obtained from rDC-treated recipients expressed CD152 (Figure 4C; see supplemental data at http://www.immunity.com/cgi/content/full/18/3/367/DC1). In addition, the expression level of CD152 was increased in $CD4^+CD25^+$ T cells after the injection of rDCs in the recipients of allogeneic BMS, and they retained their expression level until 60 days after transplantation (Figure 4D; see supplemental data at http://www.immunity.com/cgi/content/full/18/3/367/DC1).

We also examined the functional difference of I-$K^{b+}CD4^+CD25^+$ T cells obtained from mDC- or rDC-treated recipients (referred to hereafter as $CD4^+CD25^+$

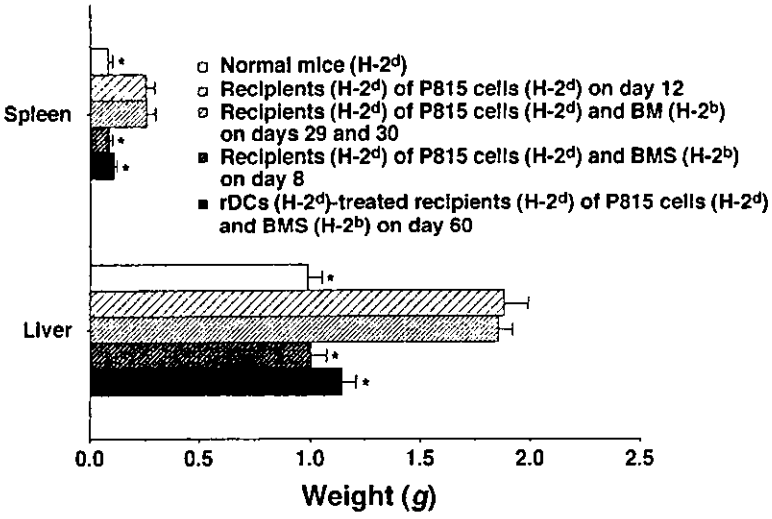
A


Survival rate (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Days after transplantation
○ Recipients (H-2d) of P815 cells (H-2d)
● Recipients (H-2d) of P815 cells (H-2d) and BM (H-2b)
▲ Recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b)
■ rDC (H-2d)-treated recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b)

B


Body weight (g)
26
24
22
20
18
16
14
12
0
10
20
30
40
50
60
Days after transplantation
○ Recipients (H-2d) of P815 cells (H-2d)
● Recipients (H-2d) of P815 cells (H-2d) and BM (H-2b)
▲ Recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b)
■ rDC (H-2d)-treated recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b)

C


Spleen
Liver
0.0
0.5
1.0
1.5
2.0
2.5
Weight (g)
Normal mice (H-2d)
Recipients (H-2d) of P815 cells (H-2d) on day 12
Recipients (H-2d) of P815 cells (H-2d) and BM (H-2b) on days 29 and 30
Recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b) on day 8
rDCs (H-2d)-treated recipients (H-2d) of P815 cells (H-2d) and BMS (H-2b) on day 60

$CD154^+$ T cells or $CD4^+CD25^+CD152^+$ T cells, respectively). Unprimed $CD4^+CD25^+$ T cells ($H\text{-}2^b$) or $CD4^+CD25^+CD152^+$ T cells not only showed poor response to mDCs and rDCs ($H\text{-}2^d$ or $H\text{-}2^q$) but also inhibited the proliferation of $CD4^+$ T cells ($H\text{-}2^b$) in response to mDCs ($H\text{-}2^d$ or $H\text{-}2^q$) (Figures 4E), indicating that their suppression is an Ag nonspecific. Furthermore, $CD4^+CD25^+CD152^+$ T cells showed a more potent suppression than unprimed $CD4^+CD25^+$ T cells (Figures 4E and 4F). In addition, the suppressive activity was upregulated in $CD4^+CD25^+$ T cells after the injection of rDCs in the recipients of allogeneic BMS, and they retained their activity until 60 days after transplantation (Figure 4G). On the other hand, the separation of $CD4^+CD25^+CD152^+$ T cells virtually abolished this inhibitory effect, and the addition of IL-2 restored the response of activated $CD4^+$ T cells at least partially (Figure 4H). We also observed that $CD4^+CD25^+$ $CD154^+$ T cells showed a vigorous response to mDCs ($H\text{-}2^d$), and the addition of $CD4^+CD25^+CD154^+$ T cells did not affect the response of $CD4^+$ T cells ($H\text{-}2^b$) to mDCs ($H\text{-}2^d$) (Figure 4H). These results indicate that $CD4^+CD25^+CD152^+$ T cells as well as unprimed $CD4^+CD25^+$ T cells act as Tr cells.

We further examined the role of IL-10-producing $CD4^+$ T cells and $CD4^+CD25^+$ $CD152^+$ Tr cells in the therapeutic effect of rDCs on acute GVHD. In vivo blockade experiments showed that treatment with anti-CD25 monoclonal antibody (mAb) or anti-IL-10 Ab, but not anti-TGF-β mAb and control IgG (control Ig), significantly abrogated the therapeutic effect of rDCs on acute GVHD ($P < 0.01$), and the treatments with both anti-CD25 mAb and anti-IL-10 Ab showed a more potent suppression (Figure 2E).

We also tested the effect of a single injection of unprimed $CD4^+CD25^+$ Tr cells, $CD4^+CD25^+CD154^+$ T cells, or $CD4^+CD25^+CD152^+$ Tr cells (each $H\text{-}2^b$) following transplantation on acute GVHD-induced lethality. $CD4^+CD25^+CD152^+$ Tr cells showed a more potent protective effect than unprimed $CD4^+CD25^+$ Tr cells whereas $CD4^+CD25^+CD154^+$ T cells enhanced the lethality ($P < 0.01$, Figure 2F).

Involvement of cAMP and $p27^{kip1}$ in an Induction of Allogeneic Ag-Specific Tolerant $CD4^+$ T Cells by rDCs

The accumulation of cyclic AMP (cAMP) leads to the upregulation of $p27^{kip1}$ cyclin-dependent kinase (CDK) inhibitor, and these molecular events play a crucial role in in vitro induction of tolerant $CD4^+$ T cells (Boussiotis et al., 2000). We therefore examined the role of cAMP and $p27^{kip1}$ in in vitro induction of Ag-specific tolerant $CD4^+$ T cells by allogeneic rDCs (Figures 5A and 5C). $CD4^+$ T cells ($H\text{-}2^b$) stimulated with rDCs ($H\text{-}2^q$) had increased amounts of cAMP and expression levels of $p27^{kip1}$ whereas $CD4^+$ T cells ($H\text{-}2^b$) stimulated with mDCs ($H\text{-}2^q$) had decreased amounts of cAMP and expression levels of $p27^{kip1}$ compared to unstimulated $CD4^+$ T cells ($H\text{-}2^b$) in vitro. We observed similar amounts of cAMP and expression levels of $p27^{kip1}$ between unstimulated $CD4^+$ T cells and $CD4^+CD25^+CD152^+$ Tr cells, which were obtained from rDC ($H\text{-}2^q$)-treated recipients ($H\text{-}2^d$) of BMS ($H\text{-}2^b$) 5 days after transplantation, and these Tr cells did not affect them in $CD4^+$ T cells following coculture (Figures 5A and 5D).

We also tested the involvement of cAMP and $p27^{kip1}$ in the induction of Ag-specific tolerant $CD4^+$ T cells in rDC ($H\text{-}2^q$)-treated recipients ($H\text{-}2^d$) of BMS ($H\text{-}2^b$) (Figures 5B and 5E). $I\text{-}K^{b+}CD4^+$ T cells obtained from mDC ($H\text{-}2^d$)-treated recipients 5 days after transplantation showed decreased amounts of cAMP and expression levels of $p27^{kip1}$ following in vitro stimulation with mDCs ($H\text{-}2^d$ and $H\text{-}2^q$). In contrast, $I\text{-}K^{b+}CD4^+$ T cells obtained from rDC-treated recipients 5 days after transplantation showed constitutive high amounts of cAMP and expression levels of $p27^{kip1}$. Furthermore, in vitro stimulation of these $CD4^+$ T cells with mDCs ($H\text{-}2^d$) enhanced the amount of cAMP and the expression level of $p27^{kip1}$ whereas the amount of cAMP and the expression level of $p27^{kip1}$ were significantly decreased following in vitro stimulation with mDCs ($H\text{-}2^q$).

GVL Effect in rDC-Treated Leukemia-Bearing Recipients of Allogeneic BMS

The current preclinical approach failed to control the balance between anti-GVHD effect and GVL effect. We therefore examined the effect of rDCs ($H\text{-}2^q$) on GVL effect in leukemia ($H\text{-}2^d$)-bearing recipients ($H\text{-}2^q$) of BMS ($H\text{-}2^b$). All leukemia-bearing mice that received total body irradiation (TBI) died 12 days after transplantation (Figures 6A and 6B) with marked hepatosplenomegaly (Figure 6C), indicating that these recipients exhibited a poor GVL effect. On the other hand, leukemia-bearing recipients of allogeneic BM cells (approximately 4% of $CD8^+$ T cells in the BM graft) survived until 30 days after transplantation ($P < 0.01$, Figures 6A and 6B), and they died probably due to the leukemia, because they manifested massive hepatosplenomegaly (Figure 6C). In contrast, rDC-treated leukemia-bearing recipients of allogeneic BMS survived over 60 days ($P < 0.01$, Figures 6A and 6B), and they did not show apparent acute GVHD and hepatosplenomegaly ($P < 0.01$, Figure 6C), whereas leukemia-bearing recipients of allogeneic BMS died 8 days after transplantation due to acute GVHD (Figures 6A and 6B). These results indicate that rDC-treated recipients of allogeneic BMS showed an undetectable severity of acute GVHD but retained a strong GVL effect.

Discussion

In this study, we established a novel immunotherapeutic approach involving rDCs to prevent acute GVHD and leukemia relapse in allogeneic BMT in mice bearing leukemia mediated through the control of the ability of the

Figure 6. GVL Effect in rDC-Treated Leukemia-Bearing Recipients of Allogeneic BMS

Leukemia ($H\text{-}2^d$)-bearing recipients ($H\text{-}2^q$) received BM ($H\text{-}2^b$), BMS ($H\text{-}2^b$), and/or rDCs ($H\text{-}2^q$) as described in Experimental Procedures. (A and B) Recipients were monitored for survival (A) and body weight (B). (C) Liver and spleen were obtained from representative recipients at the time of death or 60 days after transplantation to determine their weight. Recipients that received TBI plus the inoculation with P815 cells versus any other group, $P < 0.01$. Two replicate experiments with similar results were pooled.

transplanted T cells to induce acute GVHD and GVL effect.

In order to exploit Ag-specific immnunotherapy for acute GVHD and leukemia relapse, we successfully generated rDCs from the culture of BM cells with granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-10, transforming growth factor (TGF)-β1, and lipopolysaccharide (LPS) as therapeutic agents in mice. Furthermore, rDCs retained their T cell regulatory property in vitro and in vivo even under inflammatory conditions. However, the use of LPS is unlikely to be suitable for preparation of these cells in a clinical setting. On the other hand, TNF-α as well as LPS could generate rDCs from the culture of BM cells with GM-CSF, IL-10, and TGF-β1 (data not shown). In addition, we have recently reported that human modified DCs obtained from the culture of monocytes with GM-CSF, IL-4, IL-10, TGF-β1, and TNF-α also act as rDCs to cause a potent immunoregulation in vitro (Sato et al., in press). Therefore, the strategy involving the use of rDCs is potentially useful for the establishment of therapeutic approach in a clinical setting.

Analysis of phenotype and function of mDCs suggests that they could efficiently deliver signal to T cell receptor for Ag (TCR) ligands (signal 1) plus signal to costimulatory molecules (signal 2) (Viola and Lanzavecchia, 1996), resulting in the potent activation of T cells. Furthermore, repetitive stimulations with mDCs may cause clonal expansion of alloreactive T cells resulting in the appearance of their further allogeneic response. Conversely, rDCs exhibited high levels of MHC molecules plus poor expressions of costimulatory molecules, whereas D_3-conditioned DCs showed moderate expression levels of these molecules. In addition, rDCs showed lower activation of allogeneic T cells in the priming and more potent induction of their tolerance upon restimulation with mDCs than D_3-conditioned DCs. These phenomena imply that the condition responsible for the induction of T cell tolerance by tolerant DCs is that the degree of T cell activation in the priming is much lower than that on restimulation.

Previous study has shown that fibroblasts transfected with HLA-DR7 alone (t-DR7) induced anergy in $CD4^+$ T cell clones specific for HLA-DR7, whereas fibroblasts transfected with HLA-DR7 and B7-1 (t-DR7/B7-1) induced productive stimulation (Boussiotis et al., 2000). Therefore, the characteristic expression profile of MHC and costimulatory molecules in rDCs is associated with their potent tolerance-inducing ability, in which they may deliver potent signal 1 plus poor signal 2 to alloreactive $CD4^+$ T cells.

A single injection of mDCs enhanced the lethality caused by acute GVHD in the recipients of allogeneic BMS. In addition, these recipients exhibited the increased proportions and allogeneic responses of the transplanted T cells as well as the enhanced productions of proinflammatory cytokines. The results suggest that the injected mDCs activate the transplanted alloreactive T cells via delivery of potent signal 1 plus signal 2 resulting in the enhancement of the lethality caused by acute GVHD.

Although in vitro analysis of tolerant DCs such as iDCs (Jonuleit et al., 2000), IL-10-treated DCs (Steinbrink et al., 1997; Sato et al., 2002), and D_3-conditioned DCs (Penna and Adorini, 2000) has been extensively performed in humans, the information about in vivo efficacy of tolerant DCs as therapeutic agents for immunopathogenic diseases is limited in humans and animals. This paper reports that tolerant DCs showed a prominent therapeutic effect on immunopathogenic disease in which the treatment began even after disease progression. A dose response and timing analysis suggests that the increasing doses of rDCs and their sequential infusions enhance their therapeutic efficacy on murine acute GVHD. On the other hand, the results obtained from various strain combinations in allogeneic BMT suggest that in vivo efficacy of rDC is closely associated with the compatibility of MHC haplotypes between host and rDCs. Interestingly, host APCs reportedly play a crucial role in the initiation and the progression of acute GVHD, and their inactivation leads to the prevention of acute GVHD (Shlomchik et al., 1999). We showed that the infused rDCs were still found in spleen in syngeneic recipients for >18 days after allogeneic transplantation. Thus, a long-term existence of host MHC-matched rDCs might be crucial for their therapeutic efficacy.

We showed that rDCs directly suppressed not only the effecor functions of in vivo-primed allogeneic $CD4^+$ and $CD8^+$ T cells but also their responsiveness to the restimulation in vitro. Analysis of rDC-treated recipients revealed that they showed the impaired allogeneic Ag-specific responses of the transplanted T cells as well as the reduced productions of proinflammatory cytokines. Thus, the mechanism responsible for the therapeutic effect on acute GVHD involves the induction of tolerant T cells as well as direct suppression on effector T cells in vivo.

Donor-type $CD4^+CD25^+$ Tr cells reportedly suppressed acute GVHD-induced lethality in mice, and this suppressive effect was partly mediated through IL-10 (Hoffmann et al., 2002; Taylor et al., 2002). On the other hand, the suppressor function of $CD4^+CD25^+$ Tr cells reportedly could occur independently of TGF-β1 (Piccirillo et al., 2002). We showed that IL-10-producing $CD4^+$ T cells and $CD4^+CD25^+CD152^+$ Tr cells were increased in rDC-treated recipients. The deletion of $CD25^+$ cells as well as the neutralization of IL-10, but not TGF-β1, significantly impaired the therapeutic effect of rDCs on acute GVHD, and their combination enhanced the suppressive effect. Furthermore, $CD4^+CD25^+CD152^+$ Tr cells showed a more potent protective effect on acute GVHD than unprimed $CD4^+CD25^+$ Tr cells. Thus, our results suggest that IL-10-producing $CD4^+$ T cells and $CD4^+CD25^+CD152^+$ Tr cells play a redundant role in protection, and they are crucial for the therapeutic effect of rDCs on acute GVHD mediated through the inhibition on the transplanted alloreactive T cells.

A single injection of mDCs or rDCs increased $CD4^+CD25^+CD154^+$ T cells or $CD4^+CD25^+CD152^+$ Tr cells in spleen by approximately 5-fold, respectively. Thus, antigenic stimulation of unprimed $CD4^+$ T cells with allogeneic DCs may be necessary for the enhancement of these $CD4^+CD25^+$ T cells. Allogeneic mDCs reportedly induced $CD4^+CD25^+CD154^+$ T cells from naive $CD4^+$ T cells via the delivery of potent signal 1 plus signal 2 (Banchereau et al., 2000; Jonuleit et al., 2000). These phenomena imply that signal 1 plus additional signal(s) may be needed for the enhancement of

$CD4^+CD25^+CD152^+$ Tr cells by allogeneic rDCs. Although the precise mechanism remains unknown, several possibilities may account for this regulation. We observed that 7%–10% of $CD4^+$ T cells in BMS inoculum were $CD4^+CD25^+$ T cells (data not shown). It is possible that these cells may preferentially survive in rDC-treated recipients of allogeneic BMS. Thus, rDCs may provide an environment promoting the naturally existing $CD4^+CD25^+$ Tr cell-mediated infectious tolerance (Maloy and Powrie, 2001; Waldmann and Cobbold, 2001). Further study will be needed to test this possibility. On the other hand, the mechanism underlying the generation of $CD4^+CD25^+$ Tr cells from their precursor cells, including $CD4^+CD25^-$ T cells, has been debated (Maloy and Powrie, 2001; Shevach, 2001). It is possible that allogeneic rDCs can directly convert alloreactive $CD4^+CD25^-$ T cells into $CD4^+CD25^+CD152^+$ Tr cells mediated through the interaction between unknown surface-bound molecule(s) on rDCs and receptor(s) on $CD4^+CD25^-$ T cells because $CD4^+CD25^+CD152^+$ Tr cells as well as unprimed $CD4^+CD25^+$ Tr cells showed a poor proliferative response to mDCs and rDCs. To test this possibility, we are conducting experiments to detect the molecules specifically expressed on rDCs.

It has been reported that stimulation with t-DR7 induced increased intracellular cAMP followed by the upregulation of $p27^{kip1}$ in DR7-specific $CD4^+$ T cell clones, and these molecular events arrested at the early G1 phase of cell cycle, resulting in the inhibition of their clonal expansion and the induction of their anergic state (Boussiotis et al., 2000). We showed that in vivo and in vitro stimulation with rDCs increased the amount of cAMP and the expression level of $p27^{kip1}$ in allogeneic $CD4^+$ T cells in an Ag-specific manner whereas $CD4^+CD25^+CD152^+$ Tr cells failed to induce these events. Therefore, rDCs may specifically activate signaling events involving cAMP and $p27^{kip1}$ in alloreactive $CD4^+$ T cells mediated through the delivery of potent signal 1 plus poor signal 2, and that result in the induction and maintenance of their tolerance as well as suppression of their clonal expansion via the blockade of cell cycle progression.

Transplanted $CD8^+$ T cells reportedly participated in a GVL effect possibly mediated through the perforin pathway (Tsukada et al., 1999). Our results suggest that allogeneic rDCs induced a more potent tolerance in $CD4^+$ T cells than in $CD8^+$ T cells. On the other hand, leukemia-bearing recipients of allogeneic BM cells exhibited slight GVL effect although they survived without symptoms of acute GVHD until 30 days after transplantation. We hypothesized that allogeneic rDC-regulated cytotoxic activity in transplanted $CD8^+$ T cells, which fail to cause acute GVHD, may be sufficient to cause an efficient GVL effect.

Collectively, the immunotherapeutic strategy using rDCs provides benefits including prominent anti-GVHD effect and GVL effect as well as undetectable side effects for the treatment of acute GVHD and leukemia relapse in murine allogeneic BMT. To establish a clinically useful strategy with rDCs for other immunopathologic diseases including autoimmune diseases and allogeneic/xenogeneic graft rejection, preclinical studies with rDCs in animal immunopathogenic models are being conducted in our laboratories.

Experimental Procedures

Cell Preparation

mDCs were prepared by culturing BM cells obtained from female BALB/c mice ($H\text{-}2^d$), C57/BL6 mice ($H\text{-}2^b$), DBA/1 mice ($H\text{-}2^q$), or CBA/J mice ($H\text{-}2^k$) (all from Charles River Laboratories, Raleigh, NC) with murine GM-CSF (20 ng/ml; Pepro Tech., London, England) for 6 days followed by LPS (1 μg/ml, Sigma, St. Louis, MO) for 2 days. rDCs were also generated from BM cells ($H\text{-}2^d$, $H\text{-}2^b$, or $H\text{-}2^q$) cultured with murine GM-CSF (20 ng/ml), murine IL-10 (20 ng/ml, Pepro Tech.), and human TGF-β1 (20 ng/ml, Pepro Tech.) for 6 days followed by LPS (1 μg/ml) for 2 days. D_3-conditioned DCs were prepared from the culture of BM cells with GM-CSF (20 ng/ml) plus D_3 (10 nM, Sigma) for 6 days followed by LPS (1 μg/ml) for 2 days as described previously (Griffin et al., 2001). T cells were negatively selected from spleen mononuclear cells obtained from normal mice ($H\text{-}2^d$, $H\text{-}2^b$, or $H\text{-}2^k$) with mAbs to Ly-76, B220, Ly-6G, and I-A/I-E (all from BD PharMingen, San Diego, CA) plus sheep anti-rat IgG mAb-conjugated immunomagnetic beads (Dynal, Oslo, Norway). Subsequently, $CD4^+$ T cells or $CD8^+$ T cells were negatively selected from T cells with anti-CD8 mAb or anti-CD4 mAb (both from BD PharMingen) in combination with sheep anti-rat IgG mAb-conjugated immunomagnetic beads. These T cell preparations were typically >97% pure as indicated by FACS analysis (Becton Dickinson, Mountain View, CA).

In Vitro Priming of T Cell Subsets

T cell subsets (10^7) were cultured in medium alone or primed with allogeneic mDCs, rDCs, or D_3-conditioned DCs (10^6) at a T cell/DC ratio of 10:1 for 3 days. After incubation, in vitro primed T cell subsets were negatively selected with DC-matched anti-I-K mAb (BD PharMingen) plus goat anti-mouse IgG mAb-conjugated immunomagnetic beads for their depletion. We collected viable T cell subsets ($1.5–2 \times 10^7$, $7–8 \times 10^6$, or $8–9 \times 10^6$) from the coculture with allogeneic mDCs, rDCs, or D_3-conditioned DCs, respectively. These preparations typically contained <0.1% of allogeneic DCs ($CD11c^+$ and allogeneic $I\text{-}K^+$ cells) as indicated by FACS analysis. In vitro primed T cell subsets were rested in medium containing murine IL-2 (10 U/ml, Pepro Tech.) for 3 days and used for subsequent experiments.

Models for Acute GVHD and Leukemia Relapse

Recipients ($H\text{-}2^d$ or $H\text{-}2^b$, five animals in each group) received lethal TBI (10 Gy from a ^{60}Co source, MBR-1505R2, Hitachi Medical, Tokyo, Japan). Alternatively, recipients ($H\text{-}2^d$, five animals in each group) were inoculated intravenously (i.v.) with P815 cells (2×10^5/0.2 ml, $H\text{-}2^d$; RIKEN Cell Bank, Tsukuba, Japan) 2 days before TBI to evaluate the GVL effect (Tsukada et al., 1999). Allogeneic transplantation was performed by a single injection of 0.2 ml of PBS containing host-mismatched nucleated BM cells (1.5×10^7/mouse) alone or 0.4 ml of PBS containing host-mismatched BM cells (1.5×10^7/mouse) plus spleen mononuclear cells (1.5×10^7/mouse) into recipients through the tail vein. The day of transplantation was designated as day 0. Subsequently, recipients received a single or repetitive i.v. injection(s) of host-matched or mismatched mDCs, rDCs, or D_3-conditioned DCs (1.5×10^4–5×10^6/0.2 ml/mouse) 2 and/or 5 days after transplantation. Recipients were monitored once every day from the day of transplantation until they succumbed naturally to GVHD and/or tumor burden or 60 days after transplantation to determine survival time and body weight. In another experiment, recipients were killed on the indicated days after transplantation to obtain serum and spleen. Alternatively, liver and spleen were obtained from representative mice bearing leukemia at the time of death or 60 days after transplantation to determine their weight.

In Vivo Blockade Experiments

Recipients ($H\text{-}2^d$) of BMS (3×10^7/mouse, $H\text{-}2^b$) were injected with rDCs (1.5×10^6/mouse, $H\text{-}2^d$) 2 days after transplantation. The i.v. injections of anti-CD25 mAb (clone PC61, BD PharMingen), neutralizing anti-IL-10 polyclonal Ab (model AB-417-NA, R&D System, Minneapolis, MN), neutralizing anti-TGF-β mAb (clone 1D11, R&D System), or preimmune rat IgG used as control Ig (each 500 μg/mouse) were performed on days 3, 5, 7, 9, 10, 13, and 15 after

transplantation. Treatment with anti-CD25 mAb resulted in the depletion of >98% of spleen $CD4^+CD25^+$ T cells in rDC-treated recipients 16 days after transplantation. In another experiment, recipients ($H\text{-}2^d$) of BMS ($H\text{-}2^b$) were injected 2 days after transplantation with unprimed $CD4^+CD25^+$ T cells (10^6/mouse, $H\text{-}2^b$), $CD4^+CD25^+$ T cells (10^6/mouse, $H\text{-}2^b$) obtained from mDC-treated recipients 5 days after transplantation, or $CD4^+CD25^+$ T cells (10^6/mouse, $H\text{-}2^b$) obtained from rDC-treated recipients 60 days after transplantation. Recipients were monitored once every day from the day of transplantation as described above.

In Vivo Priming of T Cell Subsets

Transplantation with allogeneic BMS (3×10^7/mouse) was performed in the following strain combinations: recipients ($H\text{-}2^d$) versus BMS ($H\text{-}2^b$), recipients ($H\text{-}2^b$) versus BMS ($H\text{-}2^d$), and recipients ($H\text{-}2^d$) versus BMS ($H\text{-}2^d$). Spleen mononuclear cells were >95% donor-matched $I\text{-}K^+$ cells 5 days after transplantation, and they were obtained by the depletion of recipient cells with recipient-matched anti-I-K mAb plus goat anti-mouse IgG mAb-conjugated immunomagnetic beads at $<2 \times 10^7$/mouse, and donor-derived $CD4^+$ T cells and $CD8^+$ T cells were then obtained at $<4 \times 10^6$/mouse as described above. Similarly, donor-derived $CD4^+$ T cells and $CD8^+$ T cells were collected from spleen mononuclear cells in mDC- or rDC-treated recipients of allogeneic BMS ($<3 \times 10^7$/ mouse or $<2 \times 10^7$/mouse) at $<10^7$/mouse or $<3 \times 10^6$/mouse, respectively. Cells were then rested in medium containing murine IL-2 (10 U/ml) for 3 days and used for subsequent experiments.

In Vivo Behavior of rDCs in Mice

rDCs ($H\text{-}2^b$) were labeled with CFSE (Molecular Probes, Eugene, OR) and injected i.v. into the recipients ($H\text{-}2^d$) of BMS ($H\text{-}2^b$) at 1.5×10^6/mouse. Spleen mononuclear cells were collected at various intervals and analyzed by flow cytometry. For preparation of in vivo infused rDCs, host-mismatched rDCs were injected into recipients of third-party allogeneic BMS because it is difficult to obtain the injected rDCs from syngeneic recipients with a high purity. In brief, rDCs ($H\text{-}2^d$) were collected from spleen mononuclear cells in rDC ($H\text{-}2^d$)-treated recipients ($H\text{-}2^b$) of allogeneic BMS ($H\text{-}2^d$) 5 days after transplantation using anti-$I\text{-}K^d$ mAb plus goat anti-mouse IgG mAb-conjugated immunomagnetic beads, and assayed for phenotype and allogeneic T cell ability.

Flow Cytometry

Cells were stained with fluorescein-conjugated mAbs to CD3, CD4, CD8, CD11c, CD25, CD40, CD80, CD86, CD152, CD154, I-K, I-A/I-E, and isotype-matched control mAb (all from BD PharMingen). Alternatively, intracellular analysis of the expression of cytokines was performed according to the manufacturer's instructions with some modification. In brief, $CD4^+$ T cells were stimulated with platebound anti-CD3 mAb (10 μg/ml, BD PharMingen) plus soluble anti-CD28 mAb (10 μg/ml, BD PharMingen) for 6 hr. 2 μM monensin (Sigma) was added for the last 5 hr of culture. Cells were collected, washed, fixed, saponin-permeabilized (fix/perm solution, BD PharMingen), and stained with fluorescein-conjugated mAbs to IL-2, IL-4, IL-10, and IFN-γ (all from BD PharMingen). Analysis of fluorescence staining was performed with a FACScan flow cytometer and CELLQuest Software, and data are expressed as percent mean positive cells or mean fluorescence intensity (MFI).

Allogeneic Mixed Leukocyte Reaction

Unprimed or primed $CD4^+$ T cells (2×10^5) were cultured in 96-well plates (Coster, Cambridge, MA) with irradiated (15 Gy) allogeneic mDCs, rDCs, or D_3-conditioned DCs (10^3-2×10^5) at various T cell:DC ratios in the presence or absence of murine IL-2 (100 U/ml) for 3 days. In another experiment, primed $CD4^+$ T cells (2×10^5) obtained from the spleen of recipients of allogeneic BMS were cultured with irradiated mDCs (2×10^4 or 4×10^4) at various T cell:DC ratios in the presence or absence of mDCs (8×10^4) or rDCs (2×10^3-8×10^4) for 3 days. Proliferation was measured by [^{3}H]thymidine (Amersham Life Science, Buckinghamshire, UK) incorporation.

Cytotoxicity Assay

In vivo primed $CD8^+$ T cells were cultured with $Na_2{}^{51}CrO_4$ (NEN Life Science Products, Boston, MA)-labeled P815 cells (10^4), EL4 cells (10^4, RIKEN Cell Bank), or Con A blasts (10^4) (Sato et al., 1999) for 4 hr at various effector cell to target cell ratios (E/T ratios). The radioactivity of the supernatants was measured, and the percent specific lysis was calculated (Sato et al., 1999).

Enzyme-Linked Immunosorbent Assay

Mice were anesthetized and exsanguinated; their blood was clotted on ice for 10 min. Serum was then separated by centrifugation and stored at −80°C for further analysis and was measured in duplicate wells. The murine IFN-γ, TNF-α, and IL-12p40 were measured using ELISA kits purchased from BioSource (Camarillo, CA).

Measurement of cAMP Concentration

Unprimed or primed $CD4^+$ T cells (10^6) were isolated as described above, and the concentration of intracellular cAMP was determined by enzyme immunoassay (BIOMOL Research Laboratories, Plymouth Meeting, PA).

Immunoblotting

Total cell lysates were collected from isolated $CD4^+$ T cells (4×10^6). Protein samples were fractionated by 12% SDS-PAGE, transferred onto PVDF membranes (Millipore, Bedford, MA), and probed with anti-$p27^{kip1}$Ab (Transduction Laboratories, Lexington, KY) plus horseradish peroxidase-conjugated secondary Abs (Santa Cruz Biotech, Santa Cruz, CA). Blots were visualized by enhanced chemiluminescence (New England Biolabs, Beverly, MA) (Sato et al., 1999).

Analysis of Tr Cell Function

Unprimed $CD4^+CD25^+$ T cells were isolated from spleen $CD4^+$ T cells in normal mice ($H\text{-}2^b$) with anti-CD25 mAb plus sheep anti-rat IgG mAb-conjugated immunomagnetic beads. $I\text{-}K^{b+}CD4^+CD25^+$ T cells were also selected from spleen $I\text{-}K^{b+}CD4^+$ T cells in mDC- or rDC-treated recipients of allogeneic BMS on the indicated days as described above, and they were typically >90% pure as indicated by FACS analysis. $CD4^+$ T cells (10^5, $H\text{-}2^b$) were cultured with or without mDCs (10^4, $H\text{-}2^d$ or $H\text{-}2^a$) in the presence or absence of different numbers of unprimed $CD4^+CD25^+$ T cells or $I\text{-}K^{b+}CD4^+CD25^+$ T cells (2×10^4– 10^5) in 96-well plates. For Transwell experiments, $I\text{-}K^{b+}CD4^+CD25^+$ T cells (10^6) plus mDCs (10^5, $H\text{-}2^d$) were either added directly to the coculture of $CD4^+$ T cells (10^6, $H\text{-}2^b$) with mDCs (10^5, $H\text{-}2^d$) in 24-well plates (Coster) or were placed separately in 24-well Transwell cell culture chambers (Millicell, Milipore, Bedford, MA) in the same well for 4 days. Following depletion of mDCs, total T cells (10^5/well) were transferred to 96-well plates. The proliferation of activated T cells was evaluated on day 5 based on [^{3}H]thymidine incorporation.

Statistical Analysis

Data are expressed as mean values ± standard deviation (SD). All analyses for statistically significant differences were performed with Student's paired t test or Mann-Whitney's U test. P values <0.01 were considered significant.

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Estrogen receptor β mediates the inhibitory effect of estradiol on vascular smooth muscle cell proliferation

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Abstract

Objectives: It has been demonstrated that 17β-estradiol (E2) has an inhibitory effect on the proliferation of vascular smooth muscle cells (VSMCs) through an estrogen receptor (ER)-dependent pathway. Both ER subtypes, classical ER (ERα) and the newly identified ER subtype (ERβ), are expressed in VSMCs. However, it remains unknown which receptor plays the critical role in the inhibitory effect on VSMC proliferation. **Methods and results:** We constructed replication-deficient adenoviruses bearing the coding region of human ERα, ERβ, and the dominant-negative form of ERβ (designated AxCAERα, AxCAERβ, and AxCADNERβ, respectively). Prior to infection with the adenoviruses, 100 nmol/l E2 attenuated DNA synthesis by up to 14% and transactivated the estrogen-induced expression of the desired mRNA in rat VSMCs. This was accompanied by increased transcriptional activity of estrogen responsive element in response to E2, and the increase was comparable between AxCAERα and AxCAERβ. When VSMCs were infected with AxCAERβ at a multiplicity of infection of 5 or higher, DNA synthesis as well as cell number decreased by 50% in response to E2, and the effect was abolished by co-infection with AxCADNERβ. In contrast, when VSMCs were infected with AxCAERα, the reduction in DNA synthesis was minimal. **Conclusions:** Our results indicate that ERβ is more potent than ERα in the inhibitory effect on VSMC proliferation.

Keywords: Atherosclerosis; Gene expression; Hormones; Receptors; Smooth muscle

1. Introduction

The proliferation of vascular smooth muscle cells (VSMCs) is a common feature associated with vascular proliferative disorders such as atherosclerosis and restenosis after balloon angioplasty [1]. Inhibition of VSMC growth is thus one therapeutic target for the prevention of vascular diseases. Estrogen exhibits a variety of actions on the vascular wall that could be involved in its atheroprotective effects [2,3]. These include the stimulation of nitric oxide production by endothelial cells [4] and the inhibition of VSMC proliferation [5–8]. However, results from recent randomized double-blind trials, which were conducted to evaluate the effect of hormone replacement therapy (HRT) in primary prevention [9] and in secondary prevention [10], have failed to show a protective effect of HRT on cardiovascular disease. These conflicting data might result from the prothrombotic effects of estrogen [11], which could abolish the beneficial effects of estrogen on vascular function. Additionally, progestin, combined with estrogen to decrease the risk of endometrial cancer during HRT, might exert prothrombotic and proinflammat-

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ory effects. So far, the protective effects of estrogen alone on cardiovascular diseases remain unknown.

Most of the effects of estrogen are thought to be mediated by the estrogen receptor (ER), a member of the intra-nuclear receptor family. A new subtype of ER, ERβ, was discovered in 1996 [12], and has a somewhat different expression and localization patterns and transcriptional activity in reproductive and non-reproductive organs from those of classical ERα [13]. The ER subtypes may provide a clue to answering the question of why estrogen exerts differential effects in various cells and tissues; that is, estrogen stimulates proliferation in MCF-7 breast cancer cells [14] and osteoblastic cells [15], but inhibits proliferation in VSMCs. Morey et al. showed that, in VSMCs, the growth inhibitory effect of estrogen can be blocked by the nonspecific estrogen receptor antagonists tamoxifen [6] and ICI 182,780 [8]. However, it remains unknown which ER subtype mediates the growth inhibitory effect of estrogen in VSMCs, where both ER subtypes are expressed [16–18].

Also, in vivo studies using genetically engineered mice have provided insufficient information on this issue. Estrogen inhibites VSMC proliferation of the medial area in response to vascular injury in ERα knockout mice [19] as well as in ERβ knockout [20] and double knockout mice [21]. In contrast, estrogen has no detectable effect on VSMC proliferation in fully null ERα knockout mice [22], suggesting that a splice variant of the ERα gene in the previous knockout mice lines plays a role. However, some points remain unclear in the study. Would the function of a splice variant, scarcely expressed in the vascular wall, really be as efficient as that of wild-type ERα? VSMC proliferation is inhibited in newly generated ERα knockout mice in an estrogen-independent manner as compared to wild-type mice [22]. This result suggests that ERα could exert ligand-independent VSMC proliferation, an interesting, but not established, concept.

In the rat carotid injury model, ERβ is predominantly expressed after injury [23], and the isoflavone phytoestrogen genistein, which showed a 20-fold higher binding affinity to ERβ than to ERα, exhibited a vasculoprotective effect. Taken together, ERβ might be a main mediator for the estrogen-mediated vasculoprotective effect. In the present study, to clarify which ER subtype plays the pivotal role in the inhibitory effect of estrogen on VSMC proliferation, we used adenovirus vectors to transfer ER subtypes into VSMCs. As reported previously, estradiol (E2) attenuates DNA synthesis dose-dependently. Adenovirus-mediated overexpression of ERβ in VSMCs augments growth inhibition in a ligand-dependent manner.

2. Methods

2.1. Cell culture

Rat VSMCs were harvested from the aortae of 8-week-old Wistar male rats by enzymatic dissociation according to the modified method of Chamley et al. [24]. All of the experimental protocols were approved by the Animal Research Committee of the University of Tokyo. Human aortic VSMCs were purchased from Clonetics (Cat. #CC-2571). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Medical Laboratory, Tokyo, Japan) supplemented with 10% fetal bovine serum (Intergen Co., Purchase, NY, USA), 25 mM HEPES (pH 7.4), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Rat VSMCs at six to 10 passages were used in the experiments. At the time of the experiments, we used dextran-coated charcoal-stripped FBS (DCC-FBS) and phenol red-free RPMI1640 medium for rat VSMCs and M199 medium for human VSMCs to avoid contamination with steroids and estrogen receptor agonist. All dishes used in this study were purchased from Asahi Techno Glass Co., Ltd., Tokyo.

2.2. Construction of adenovirus vector carrying estrogen receptor subtypes and transfer into VSMCs

Replication-deficient adenovirus vectors carrying the CMV-IE enhancer, chicken β-actin promoter, and the coding region of human ERα, ERβ, or the dominant-negative form of ERβ [25] were constructed by use of an adenovirus expression vector kit (Takara Shuzo Co., Kyoto, Japan) as described before [26], and are denoted AxCAERα, AxCAERβ, and AxCADNERβ, respectively. VSMCs were exposed to different multiplicities of infection (MOI) of either AxCAERα, AxCAERβ, AxCADNERβ, or a replication-deficient recombinant adenovirus carrying the *Escherichia coli* β-galactosidase gene (AxCALacZ) for 2 h in DMEM with 5% FBS. The cells were then rinsed with phosphate-buffered saline once, and used for the experiments.

2.3. RNA isolation, reverse transcription polymerase chain reaction (RT-PCR), and Northern blot analysis

For RT-PCR, total RNA was prepared from VSMCs and, as a positive control, rat ovary, using Isogen (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Then, 1 μg total RNA was reverse transcribed into cDNA, and 1/20 of the product was amplified for 35 cycles. Negative control RT-PCR reactions were performed by omitting reverse transcriptase. The primer pairs used in PCR were: CTAAGAAGAATAGCCCCGCC (forward, +1126 to +1145) and CAGACCAGACCAATCATCAGG (reverse, +1402 to +1382) for rat ERα (GenBank, accession number NM_012689), and CGACTGAGCACAAGCCCA-AATG (forward, +76 to +97) and ACGCCGTAATGAT-ACCCAGATG (reverse, +353 to +332) for rat ERβ (GenBank, accession number AB012721). Both PCR products were subsequently sequenced, and were used as the probes for rat ERα and ERβ.

For Northern blotting, VSMCs were plated on 10 cm diameter dishes, and infected with adenovirus bearing either ER subtype at 70–90% confluence. At 24 h after infection, VSMCs were harvested using ISOGEN. The RNA was fractionated on 1.3% formaldehyde-agarose gel and transferred to nylon filters (Hybond-N; Amersham Life Science Inc.). The filters were hybridized at 68 °C for 2 h with a random-primed ^{32}P-labeled human ER cDNA probe in QuikHyb solution (Stratagene) and autoradiographed. The products digested by EcoRI and PVUII from human ERα plasmid and EcoRI from human ERβ plasmid were used as the human ERα and human ERβ probe, respectively.

2.4. Western blot analysis

Cells were washed quickly with phosphate-buffered saline twice, and lysed in RIPA buffer: 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors cocktail (Complete, Mini; Boehringer Mannheim). The samples were separated on 12% SDS–PAGE, electroblotted onto nitrocellulose membranes, and immunoblotted with anti-ERα polyclonal antibody (H-184; Santa Cruz, 1:1000 dilution), anti-ERβ monoclonal antibody, CWK-F12 (kindly provided by Dr. Benita S. Katzenellenbogen, Department of Molecular and Integrative Physiology, University of Illinois College of Medicine, 1:1000 dilution), or cyclin A polyclonal antibody (C-19; Santa Cruz, 1:1000 dilution). Antibody was detected with a horseradish peroxidase-linked secondary antibody using an enhanced chemiluminescence system (Amersham Life Science Inc.).

2.5. Transfection and luciferase assays

We used the ERE-TK-Luc reporter plasmid and a firefly luciferase reporter vector as previously described [24]. VSMCs were transfected with ERE-TK-Luc reporter plasmid and pRL-SV40 control plasmid using FuGENE6 (Roche) for 24 h according to the manufacturer's instructions. Then, VSMCs were incubated in phenol-red-free RPMI1640 containing 0.1% DCC-FBS for 24 h, and exposed to 1–100 nmol/l E2 (water-soluble 17β-estradiol; Sigma–Aldrich Japan), 10–1000 nmol/l ICI 182,780 or vehicle, β-cyclodextrin solution (Sigma) as a vehicle for water-soluble E2, for an additional 24 h. We measured two kinds of luciferase activity using a dual-luciferase reporter assay system (Promega) according to the manufacture's protocol, and the ratio of firefly luciferase activity to that of Renilla luciferase in each sample was used as a measure of normalized luciferase activity. Each experiment was repeated at least three times.

2.6. Measurement of [^{3}H]thymidine incorporation into DNA of VSMCs

VSMCs seeded onto 24-well tissue culture plates were grown until 70–90% confluence, and then made quiescent by culturing them in phenol-red-free RPMI1640 medium (Gibco) for 24 h. Then, the cells were stimulated with 5% DCC-FBS in the presence of E2 (water-soluble 17β-estradiol; Sigma–Aldrich Japan) or vehicle for 24 h, followed by pulse-labeling with 1 μCi/ml [^{3}H]thymidine for 3 h. [^{3}H]Thymidine incorporated into DNA was determined as previously described [5].

2.7. Number of VSMCs

VSMCs were seeded onto six-well multiplates and cultured until a confluent state was obtained. After infection of VSMCs with adenovirus vectors, the medium was replaced with phenol-red-free RPMI1640 to arrest the growth. After 24 h, the medium was replaced again with phenol-red-free RPMI1640 containing 5% DCC-FBS with E2 or vehicle. After incubation for 48 h, the cells were trypsinized and suspended. Then the number of cells was determined using a Coulter Counter (model ZM, Coulter Electronics, Hialeah, FL, USA).

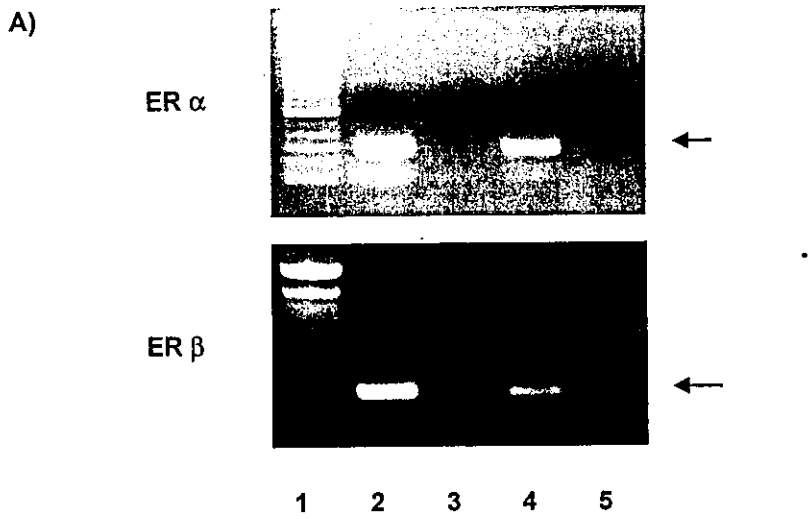
2.8. Statistical analysis

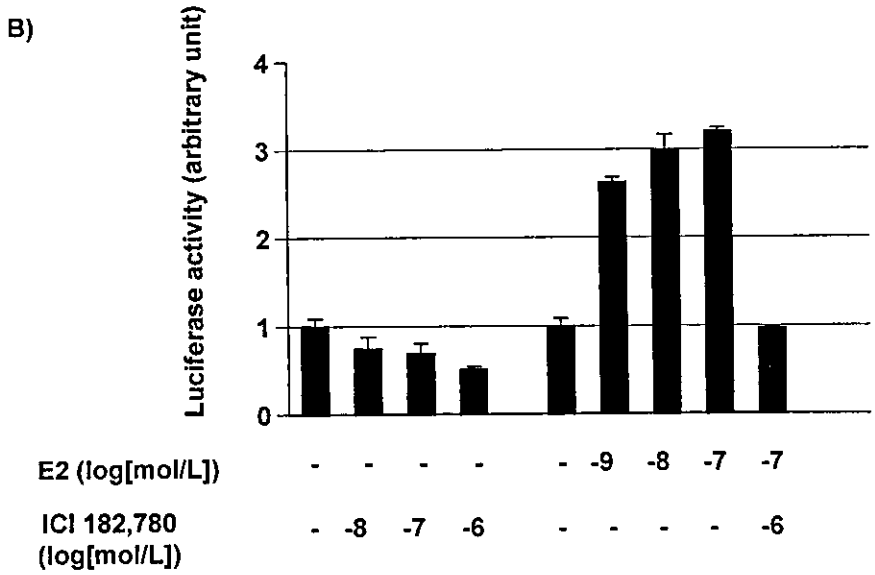
The dose–response effect of E2 or adenoviruses on DNA synthesis in VSMCs and the luciferase activity in E2-treated VSMCs were analyzed using one-way ANOVA. If a statistically significant effect was found, Newman–Keuls' test was performed to isolate the difference between groups. A value of $P<0.05$ was considered statistically significant. All data in the text and figures are expressed as mean±S.E.

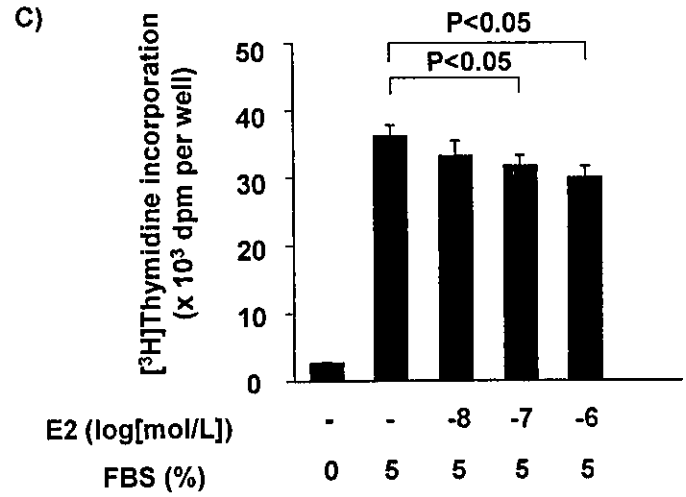
3. Results

3.1. Endogenous expression of ER subtypes in VSMCs and effect of E2 on VSMC growth

To investigate the endogenous expression of ER in rat VSMCs, RT-PCR amplification was performed. Both rat ERα and ERβ were expressed in VSMCs (Fig. 1A). Next, we examined the transcriptional activity of endogenous ER by means of the luciferase activity of the ERE reporter plasmid, and the inhibitory effect of E2 on VSMC proliferation by evaluating DNA synthesis. E2 at 1–100 nmol/l augmented the luciferase activity of ERE by approximately three-fold compared to vehicle, and this increase was abolished by the nonselective pure ER antagonist ICI 182,780 (AstraZeneca) (Fig. 1B). At these concentrations, E2 inhibited the proliferation of VSMCs dose-dependently (Fig. 1C). In the absence of E2, ICI 182,780 inhibited the luciferase activity dose-dependently by up to 50% compared to vehicle (Fig. 1B), but did not influence thymidine incorporation into VSMCs at concentrations of 10–1000 nmol/l (data not shown). This result indicates that there may be some leakage of estrogenic activity from cell


A)
ER α
ER β
1 2 3 4 5


B)
Luciferase activity (arbitrary unit)
0
1
2
3
4
E2 (log[mol/L]) - - - - - -9 -8 -7 -7
ICI 182,780 (log[mol/L]) - -8 -7 -6 - - - - -6


C)
[³H]Thymidine incorporation (x 10³ dpm per well)
P<0.05
P<0.05
0
10
20
30
40
50
E2 (log[mol/L]) - - -8 -7 -6
FBS (%) 0 5 5 5 5

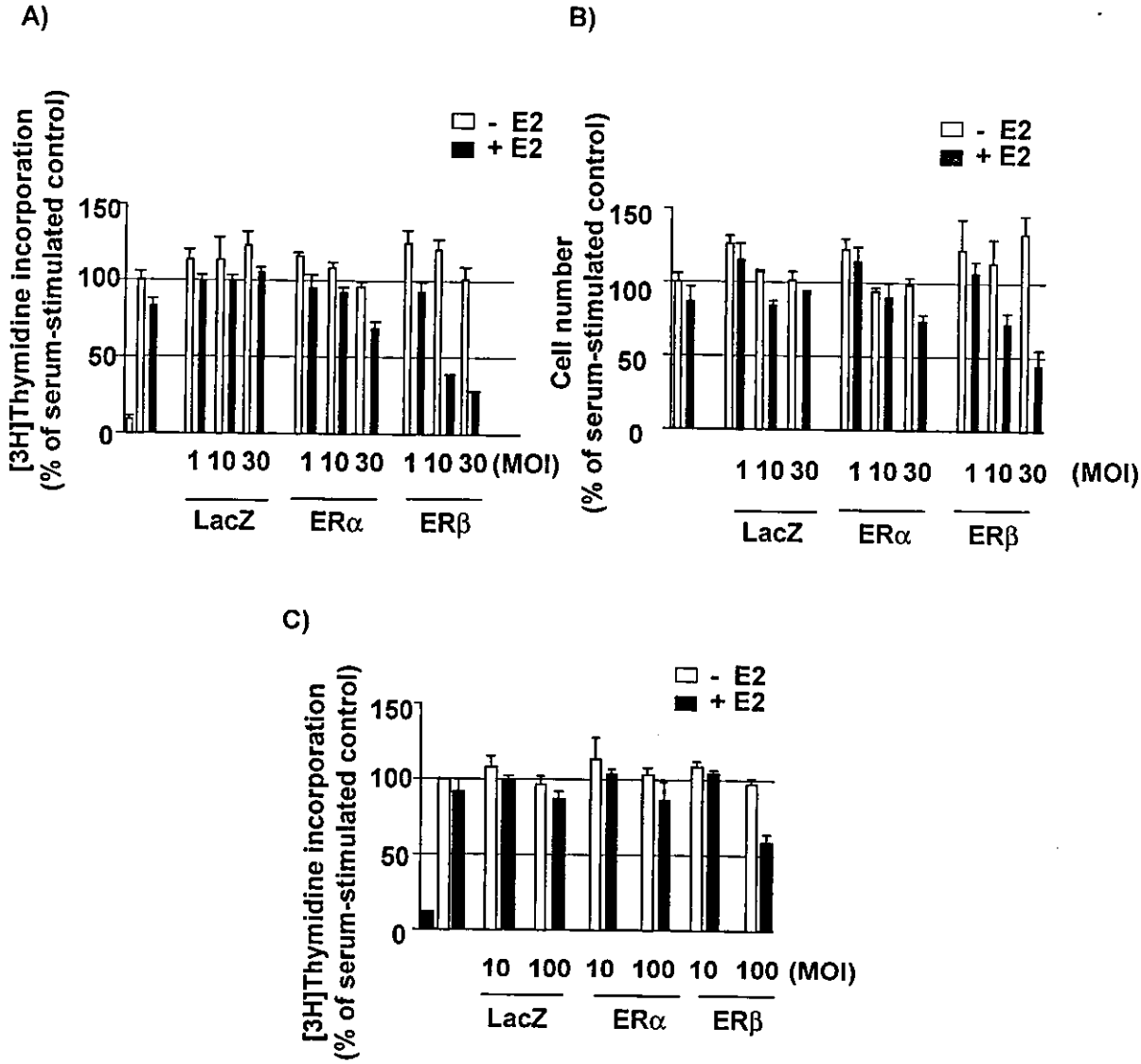


Fig. 2. Inhibition of VSMC growth by adenovirus-mediated transfer of ER gene. (A) Rat VSMCs seeded onto a 24-well plate were exposed to DMEM containing either AxCALacZ, AxCAERα, or AxCAERβ (1, 10, and 30 MOI, increasing left to right) for 2 h, and were serum-deprived for 24 h. [^{3}H]Thymidine incorporation into DNA was determined after 24 h of stimulation with 5% DCC-FBS in the presence or absence of 100 nmol/l E2, respectively. The three left-sided bars indicate non-infected VSMCs serum free, 5% DCC-FBS in the absence of E2, and 5% DCC-FBS in the presence of 100 nmol/l E2, respectively. Results are shown as mean±S.E. (n=4). Similar results were obtained in three independent experiments. (B) VSMCs seeded onto a six-well plate were exposed to DMEM containing either AxCALacZ, AxCAERα, AxCAERβ, or AxCAERDNβ (1, 10 and 30 MOI, increasing left to right) for 2 h, and were serum-deprived for 24 h. Cell numbers were counted after 48 h of stimulation with 5% DCC-FBS in the presence or absence of 100 nmol/l E2. The two left-sided bars indicate non-infected VSMCs with 5% DCC-FBS in the absence of E2 and with 5% DCC-FBS in the presence of 100 nmol/l E2, respectively. Results are shown as mean±S.E. (n=3). Similar results were obtained in three independent experiments. (C) Human aortic VSMCs seeded onto a 24-well plate were exposed to DMEM containing either AxCALacZ, AxCAERα, or AxCAERβ (10, and 100 MOI, increasing left to right) for 2 h, and were serum-deprived for 24 h. [^{3}H]Thymidine incorporation into DNA was determined after 24 h of stimulation with 20% DCC-FBS in the presence or absence of 100 nmol/l E2, respectively. The three left-sided bars indicate non-infected VSMCs serum free, 20% DCC-FBS in the absence of E2, and 20% DCC-FBS in the presence of 100 nmol/l E2, respectively. Results are shown as mean±S.E. (n=3). *P<0.05 vs. E2 (−). Similar results were obtained in three independent experiments.

Fig. 1. The endogenous expression of ER subtypes, transcriptional activity of ER, and inhibitory effect of E2 on DNA synthesis in rat VSMCs. (A) RT-PCR was performed using the cDNA of rat ovary as a positive control with (lane 2) or without reverse transcriptase (lane 3) and that of rat VSMCs with (lane 4) or without reverse transcriptase (lanes 5). A single band of predicted size (277 bp for ERα and 278 bp for ERβ, indicated by an arrow) was detected in lane 2 and lane 4. Lane 1 shows the molecular weight marker. (B) VSMCs were transfected with luciferase reporter plasmid containing ERE and pRL-SV40 control plasmid. Twenty-four hours after transfection, the cells were treated with 1–100 nmol/l E2 and/or 10–1000 nmol/l ICI182,780 for 24 h. The values were normalized to the vehicle treatment. Results are shown as mean±S.E. (n=3). *P<0.01 vs. E2 (−). (C) Serum-starved VSMCs were stimulated with 5% DCC-FBS in the absence or presence of 10–1000 nmol/l 17β-estradiol for 24 h. [^{3}H]Thymidine incorporation into DNA was determined by pulse-labeling for the last 3 h of incubation. Results are shown as mean±S.E. (n=6).

culture dishes [27,28] detected in the luciferase assays, but the activity is not strong enough to influence VSMC proliferation.

3.2. Effect of adenovirus-mediated transfer of the ER subtype gene on growth of VSMCs

To examine the effect of ERα and ERβ gene transfer into VSMCs, we constructed a replication-deficient adenovirus carrying the ER gene, AxCAERα, AxCAERβ, or AxCADNERβ. When AxCALacZ was introduced into VSMCs at more than 30 MOI, DNA synthesis was reduced in a MOI-dependent manner (data not shown). Therefore, we examined DNA synthesis at 30 MOI or less, at which the adenovirus itself did not affect DNA synthesis. When AxCAERβ was introduced into VSMCs, DNA synthesis did not change in the absence of E2. However, in the presence of 100 nmol/l E2, DNA synthesis of VSMCs infected with AxCAERβ decreased strongly compared to that of VSMCs treated with vehicle, in a MOI-dependent manner (Fig. 2A). In contrast, VSMCs infected with AxCAERα at 10 MOI or less did not show an additional reduction in DNA synthesis in the presence of E2, although an inhibitory effect was seen in VSMCs infected with AxCAERα at 30 or higher MOI (Fig. 2A and data not shown). In parallel, the increase in VSMC number stimu-

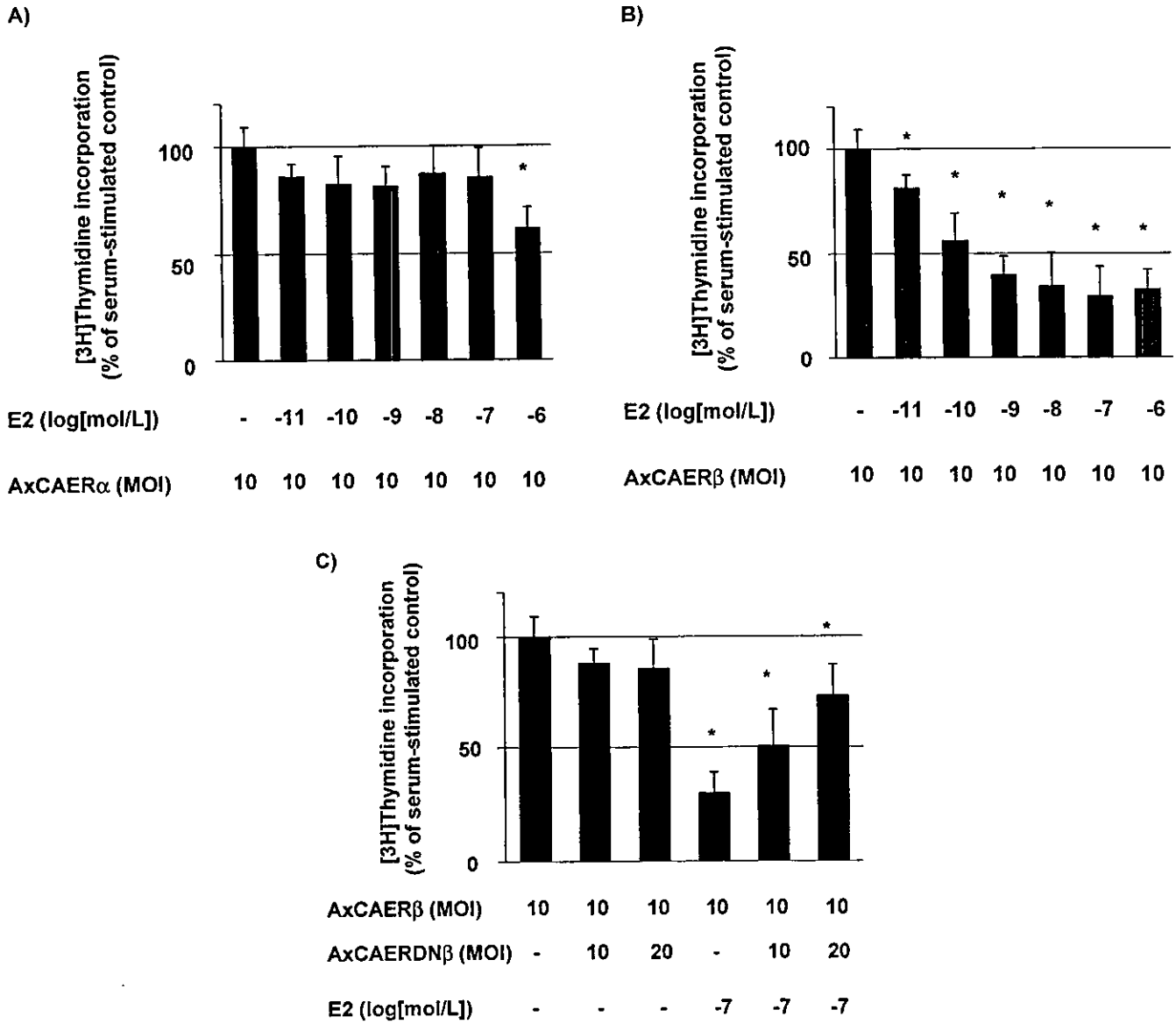


Fig. 3. Dose–response of E2 and receptor dependence of the inhibitory effect of DNA synthesis on adenovirus-mediated transfer of ER genes. VSMCs seeded onto a 24-well plate were exposed to DMEM containing 10 MOI of AxCAERα (A) or AxCAERβ (B) for 2 h, or 10 MOI of AxCAERβ and the indicated MOI of AxCADNERβ (C). After infection, VSMCs were serum-deprived for 24 h. [^{3}H]Thymidine incorporation into DNA was determined 24 h after stimulation with 5% DCC-FBS in the absence or presence of the indicated concentrations of E2. Results are shown as mean±S.E. (n=4).

lated with 5% DCC-FBS for 48 h was attenuated in VSMCs infected with AxCAERβ at 10 and 30 MOI in the presence of E2, but it was not significant in VSMCs infected with AxCALacZ or AxCAERα (Fig. 2B). To exclude the possibility that the findings might be specific for rat VSMCs, we tested human aortic VSMCs and found that the results were comparable in human aortic VSMCs (Fig. 2C). Also, in VSMCs infected with AxCAERβ at 10 MOI, DNA synthesis was significantly inhibited by 0.01–1000 nmol/l E2 in a concentration-dependent manner (Fig. 3B). In contrast, this inhibitory effect was not observed in VSMCs infected with AxCAERα, except in the presence of 1 μmol/l E2 (Fig. 3A). To examine whether the inhibitory effect in VSMCs overexpressing ERβ is actually mediated through ERβ, AxCADNERβ was co-infected with AxCAERβ. The ~70% reduction in DNA synthesis that was observed when AxCAERβ alone was infected was attenuated by co-infection of AxCADNERβ MOI-dependently (Fig. 3C). We also examined the effect of ERα overexpression on ERβ-mediated inhibition of VSMCs. However, AxCAERα at up to 10 MOI did not influence the growth inhibition exerted by AxCAERβ at 10 MOI in the presence of 100 nmol/l E2 (data not shown).

3.3. Production of ER genes and transcriptional activity of ERE in VSMCs infected with ER genes

We examined the mRNA expression of human ERα, ERβ and DNERβ by Northern blot analysis (Fig. 4A and data not shown). Neither ERα nor ERβ mRNA was seen in non-infected VSMCs (data not shown), although both were detected by RT-PCR. Infection of VSMCs with AxCAERα or AxCAERβ induced mRNA expression abundantly in a MOI-dependent manner (Fig. 4A). Similar results were obtained when the membranes were hybridized with the probes for rat ERα and ERβ, indicating that the mRNA expression of endogenous ER was undetectable by Northern blot analysis. Production of the ERα and ERβ protein was confirmed by Western blot analysis (Fig. 4B). The bands corresponding to ERα (65 kD) or ERβ (55 kD) were seen in VSMCs infected with AxCAERα, or VSMCs infected with AxCAERβ, respectively (Fig. 4B) and also in MCF-7 cells which were used as a positive control (data not shown). In parallel with the mRNA expression, the protein expression of the ER subtype was undetectable in non-transfected VSMCs and was increased by overexpression MOI-dependently. We also checked the protein level of both ER subtypes in non-infected cells after the addition of E2. However, E2 did not affect the protein level of either ER subtype under our experimental conditions (data not shown).

To check whether overexpressed ER functions as a transcription factor in VSMCs, the transcriptional activity of ERE was examined (Fig. 5A). VSMCs infected with AxCAERα or AxCAERβ at 10 MOI showed a significant increase in transcriptional activity in the presence of E2 ($P<0.01$ vs. VSMCs infected with 10 MOI AxCALacZ), indicating that both subtypes can work as transcription factors. The results in COS-7 cells (Fig. 5B), in which no endogenous ER is expressed, are clear-cut and suggest that adenovirus infection can induce ERα and ERβ to a similar extent in terms of transcriptional activity. Compared with COS-7 cells, the additional activity caused by ER overexpression was small in VSMCs. The increase, however, was completely abolished by co-infection with AxCADNERβ, suggesting that the transcriptional activity both in non-infected and infected VSMCs in response to E2 was specific for ER.

3.4. Effect of E2 on cyclin A expression

The expression of cyclin A protein in VSMCs was

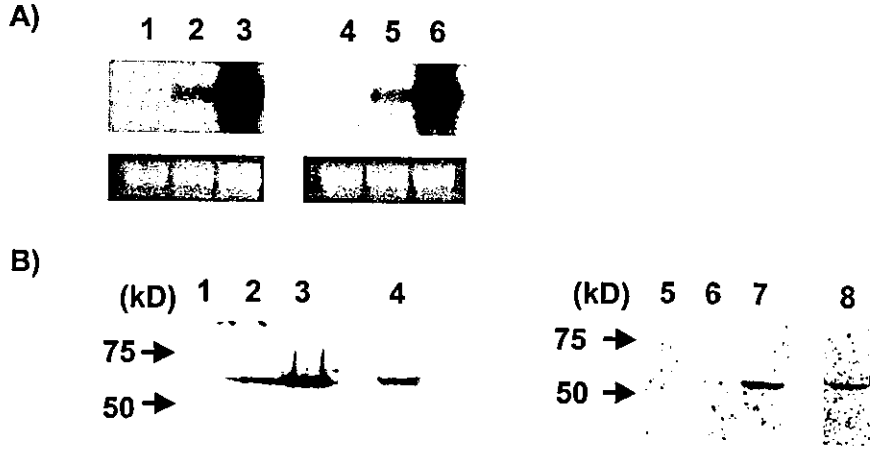


Fig. 4. Induction of ER mRNA and protein in VSMCs by adenovirus-mediated transfer of ER genes. (A) VSMCs were infected without (lanes 1 and 4), or with 10 and 100 MOI of AxCAERα (lanes 2 and 3, respectively) or AxCAERβ (lanes 5 and 6, respectively). Total RNA was extracted from VSMCs, and Northern blot analysis was performed with 15 μg total RNA per lane. The membrane was hybridized to ^{32}P-labeled ERα or β (upper lane). The 18S RNA is shown as the loading control (lower lane). (B) VSMCs were infected without (lanes 1 and 5) or with 10 and 100 MOI of AxCAERα (lanes 2 and 3, respectively) or AxCAERβ (lanes 6 and 7, respectively). Positive controls are shown in lane 4 (MCF-7 cells) and lane 8 (rat ovary). Western blot analysis was performed with 40 μg of protein per lane by using an anti-ERα polyclonal antibody or anti-ERβ monoclonal antibody.

examined 18 h after the addition of 100 nmol/l E2. The expression was increased by the addition of serum and was attenuated in VSMCs infected with AxCAERβ in the presence of E2. In contrast, E2 did not significantly inhibit cyclin A protein expression in VSMCs infected with AxCALacZ or AxCAERα (Fig. 6).

4. Discussion

Our previous work and several articles by other investigators have clearly demonstrated that E2 inhibits the proliferation of VSMCs [5–8]. Recently, it was reported that ER antagonists, ICI182,780 [8] and tamoxifen [6], antagonized the inhibitory effect of estrogen, indicating that the effect was mediated by ER. However, these inhibitors are non-selective for the ER subtype, and tamoxifen exerts a partial agonistic effect in some tissues [29]. Thus, it remained unclear which receptor is involved in the inhibitory effect of estrogen on VSMC proliferation.

There are several reports focusing on the effect of estrogen on cell proliferation using adenoviruses carrying ER into the breast cancer cell line or the pituitary lactrope cell line [30–32]. In MDA-MB 231 cells, an ER-negative human breast cancer cell line, overexpression of ERα inhibited proliferation hormone-dependently, whereas that of ERβ inhibited proliferation ligand-independently [31]. Also, overexpression of wild-type ERα in a pituitary lactrope cell line inhibited proliferation and induced apoptosis [32]. In contrast to these reports, overexpression of the dominant-negative form of ERα inhibited the proliferation of MCF-7 cells, in which endogenous ERα was expressed, and the proliferation was increased in an estrogen-dependent manner [30]. Although the question of ERα overexpression resulting in growth inhibition depending on the cell line is unresolved, the use of recombinant adenoviruses in this study enabled us to induce ERα and ERβ abundantly in VSMCs in which the endogenous expression of both ER subtypes was low.

The present study demonstrates direct evidence that ERβ is involved in the control of VSMC proliferation. The inhibitory effect of ERβ overexpression was restored by co-infection of dominant negative ERβ, indicating that this phenomenon actually resulted from signaling via ERβ. This dominant negative form of ERβ has an inhibitory effect on the transcriptional activity of both wild-type ERα and ERβ, as demonstrated by Ogawa et al., who originally made these constructs [25]. They made the C-terminal truncated ERβ, and showed that this dominant negative form of ERβ can bind to both wild-type ERα and ERβ. Accordingly, the dominant negative ERβ we used can inhibit not only the homodimerization of wild-type ER, but also heterodimerization of ERα and ERβ. The downstream effect was unknown, although competition for ERE binding, formation of inactive heterodimers with wild-type ER and specific transcriptional silencing could be assumed.

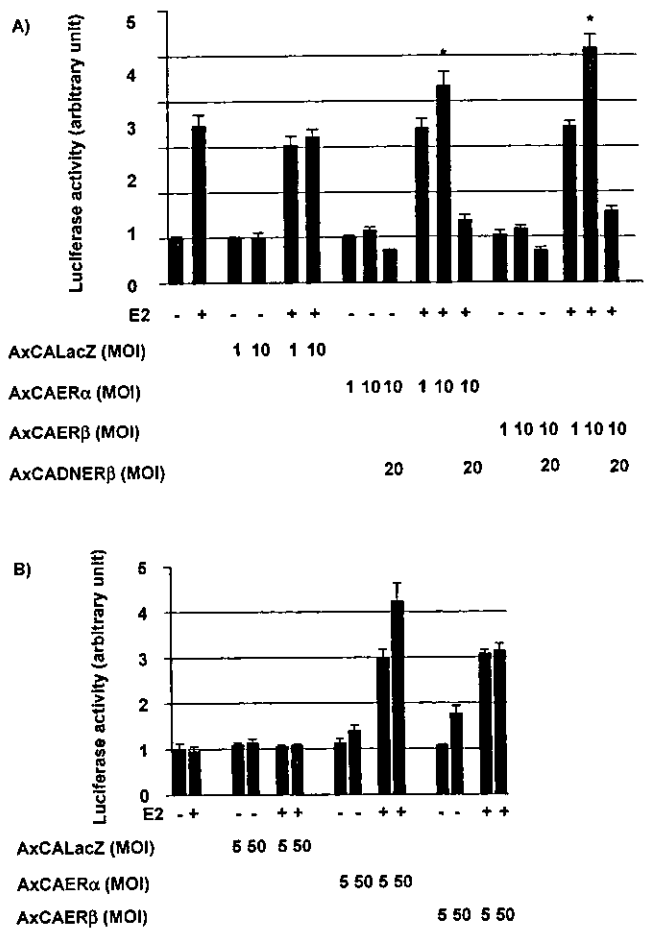


Fig. 5. Influence of overexpression of ERs on promoter activity of ER responsive enhancer elements in VSMCs and COS-7 cells. (A) VSMCs were infected with AxCALacZ, AxCAERα, AxCAERβ, or AxCADNERβ for 2 h, and were then transfected with luciferase reporter plasmids. Twenty-four hours after transfection, the cells were treated with or without 100 nmol/l E2 for 24 h. *$P<0.01$ vs. VSMCs infected with 10 MOI of AxCALacZ with E2. (B) COS-7 cells were infected with AxCALacZ, AxCAERα, or AxCAERβ for 2 h, and were then transfected with luciferase reporter plasmids. Twenty-four hours after transfection, the cells were treated with or without 100 nmol/l E2 for 24 h. The values were normalized to those in non-infected VSMCs without E2. Results are shown as mean±S.E. ($n=3$).

Surprisingly, the inhibitory effect of E2 was seen even at 10 pmol/l in VSMCs infected with ERβ. On the other hand, what is the role of ERα in VSMC proliferation? When ERα was infected into growth-arrested VSMCs, no proliferative response to E2 was seen (data not shown). Also, when both ERα and ERβ were co-infected into VSMCs, the inhibitory effect of ERβ was not affected. These results indicate that ERα does not show stimulatory effects or antagonize ERβ in terms of VSMC growth. When VSMCs were infected with AxCAERα at a higher MOI (30 or 100 MOI), an inhibitory effect appeared (Fig. 2A and data not shown). Taken together, ERα may have a weak inhibitory effect on VSMC proliferation.

The divergent effects of the ER subtypes may be

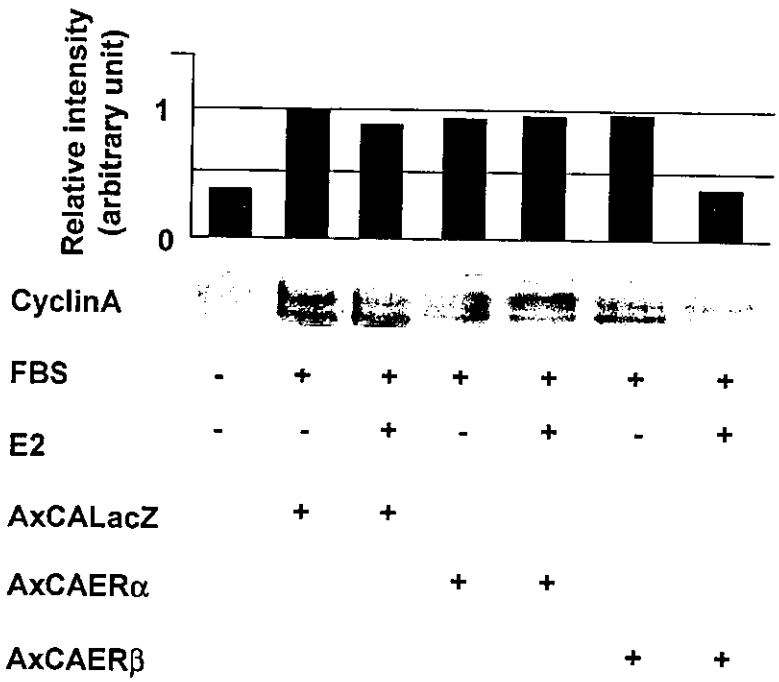


Fig. 6. Downregulation of cyclin A protein by E2 in VSMCs infected with AxCAERβ. VSMCs seeded onto 10 cm dishes were exposed to DMEM containing 10 MOI of AxCALacZ, AxCAERα, or AxCAERβ for 2 h. After infection, VSMCs were serum-deprived for 24 h. Samples were harvested 18 h after stimulation with 5% DCC-FBS in the absence or presence of 100 nmol/l E2. Western blot analysis was performed with 30 μg of protein per lane by using an anti-cyclin A polyclonal antibody. In the upper panel, levels of cyclin A protein expression in the membrane were measured by densitometry, and were plotted in comparison with that in serum-stimulated VSMCs without E2. Similar results were obtained in three independent experiments.

explained by the differential induction of estrogen response genes [33], or they may be due, in part, to the differential recruitment of transcriptional co-factors. A difference in ligand-binding affinity has also been reported [34]. Recently, it was also reported that ERβ, but not ERα, binds MAD2, a cell cycle spindle assembly checkpoint protein. The interaction is not altered by the absence or presence of E2, and thus ERβ is thought to function as a component of the spindle checkpoint assembly, not as a transcriptional factor [35]. With respect to cell growth, c-myc proto-oncogene expression was decreased in breast cancer cells infected with ERα, but was not changed in cells infected with ERβ, although the transcriptional activity is similar in cells infected with different ER subtypes [31]. In our study, there is also a discrepancy between the ERE luciferase activity and thymidine incorporation in terms of dose-dependency and the differential roles of ER subtypes. The reason for this is unknown, but similar findings have been reported [31]. We can put forward an hypothesis: some machinery, such as the co-factor for ER, would be limited in VSMCs, and the overexpression of ER could increase the ERE transcription activity to a small extent. By contrast, the signaling pathway mediating growth inhibition might manipulate some response gene that did not contain the typical ERE or non-genomic factors [6,36]. Thus we checked a cell cycle regulated gene, cyclin A. This molecule is important in the G1/S transition and in the S and G2/M phases of the cell cycle and plays a critical role in DNA replication [37]. Although the direct interaction between ERβ and cyclin A could not be clarified, cyclin A might be one of the specific response genes for ERβ in VSMCs. With respect to signaling pathways, it was reported that E2 had an inhibitory effect on VSMC proliferation via the inhibition of mitogen-activated protein kinase (MAPK) activity [6], an increase in the expression of MAPK phosphatase-1 and the activity of two Src homology 2 domain-containing cytosolic tyrosine phosphatases [38], or the cyclic AMP–adenosine pathway [7]. These signaling pathways are attributable, in part, to the non-genomic action of E2 [6,38]. We have tested whether the inhibition of MAPK activity could be involved in the inhibition of VSMC growth. However, under our study conditions, E2 did not affect ERK activity regardless of infection. Further investigations are required to clarify the specific signaling pathway by which ER subtypes exert differential effects on VSMC proliferation.

The in vivo relevance of our findings should be discussed. Studies on vascular injury using genetically engineered mice, such as ERα knockout [19], ERβ knockout [20], ERα and β double knockout mice [21] and fully null ERα knockout [22], are not yet conclusive in addressing the role of ER subtypes. In rats, ERβ is predominantly expressed in the aorta [39] or after injury to the carotid artery [23], and might play a more important role. To understand the more exact mechanism of action of ER in the vascular wall, we should make an effort to resolve this