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Pentoxifylline Functions As an Adjuvant In Vivo to Enhance T Cell Immune Responses by Inhibiting Activation-Induced Death

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Modalities for inducing long-lasting immune responses are essential components of vaccine design. Most currently available immunological adjuvants empirically used for this purpose cause some inflammation, limiting clinical acceptability. We show that pentoxifylline (PF), a phosphodiesterase (PDE) inhibitor in common clinical use, enhances long-term persistence of T cell responses, including protective responses to a bacterial immunogen, Salmonella typhimurium, via a cAMP-dependent protein kinase A-mediated effect on T cells if given to mice for a brief period during immunization. PF inhibits activation-mediated loss of superantigen-reactive CD4 as well as CD8 T cells in vivo without significantly affecting their activation, and inhibits activation-induced death and caspase induction in stimulated CD4 as well as CD8 T cells in vitro without preventing the induction of activation markers. Consistent with this ability to prevent activation-induced death in not only CD4 but also CD8 T cells, PF also enhances the persistence of CD8 T cell responses in vivo. Thus, specific inhibition of activation-induced T cell apoptosis transiently during immune priming is likely to enhance the persistence of CD4 and CD8 T cell responses to vaccination, and pharmacological modulators of the cAMP pathway already in clinical use can be used for this purpose as immunological adjuvants. The Journal of Immunology, 2002, 169: 4262–4272.

In response to infections, the immune system must not only provide immediate protective efficacy but also generate long-term memory responses. During Ag-mediated T cell proliferation and differentiation, there is a long-term effect on at least some of the responding cells or their progeny, to convert them into memory cells capable of responding more easily upon antigenic reexposure (1). Because T cell activation also commits the T cell to a pathway of apoptotic activation-induced cell death (AICD) (2), most effector T cells will die (3), and the few that escape and survive will persist as memory T cells (4). A simple prediction of the resultant model of memory generation in which all activated T cells first differentiate into effectors and some of those persist as memory (5) is that inhibiting apoptosis during T cell priming is likely to be sufficient to enhance T cell memory.

In contrast, there is an alternate model in which complete stimulation of T cells would lead to terminal effector differentiation, while partial but still above-threshold triggering would cause differentiation directly into a memory state (6). In such a model suggested by data implicating differential signals (7) and pathways (6) in generating memory vs effector T cells, alternate pathways may be responsible for the generation of short-lived effectors vs long-lived memory cells. The existence of long-lived effector cells (8) complicates these interpretations further.

On this uncertain background it is not surprising that, while adjuvants have been used for a century for generating long-lived immune responses, their use is largely empirical. They commonly evoke inflammation, which has been thought to induce the co-stimulatory signals on APCs required for inducing T cell memory (9). Recent data suggest that adjuvants may be critical for inhibiting T cell apoptosis (10). We have shown earlier that the presence of pentoxifylline (PF) during priming of human CD4 T cells in vitro against alloantigenic target APCs inhibited the primary proliferative T cell responses but enhanced secondary proliferative responses and reduced the frequency of apoptosis in activated T cells (11, 12). However, it was unclear whether modulation of secondary proliferative responses of T cells by PF, measured over a short time frame in vitro, reflected physiologically relevant modulation of the induction of immune T cell responses in vivo. We now show evidence addressing this potential use of PF as an immune adjuvant for both CD4 and CD8 T cell responses in vivo, probably through its effects on AICD.

Materials and Methods

Allo-priming assays in vitro

C57BL/6 splenocytes were used as responders and BALB/c splenocytes (irradiated at 1000 rad or irradiated and fixed with 0.003% parafomaldehyde) were used as stimulators. For primary proliferative alloresponses,
T cell immune responses to immunization in vivo

BALB/c or C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were immunized with the Ags indicated. All animal experiments were done under approval from the Institutional Animal Ethics Committee. Pure protein Ags were used in a single i.p. dose of 1 mg per mouse (native Ags) and 300 μg per mouse (maleylated Ags), while in CFA they were given as a single s.c. dose of 100 μg per mouse. Proteins were maleylated as previously described (13). For all immunization, C57BL/6 mice were immunized s.c. with a single dose of gamma-irradiated (500 rad) BALB/c spleen cells (10–30 million cells per mouse). For immunization with Salmonella typhimurium (Stm), mice were given a single dose of 10^6 CFUs of the auxotrophic aroA mutant of Stm (Stm-aroA) per mouse i.p. as previously described (14). Immune mice were treated as appropriate with various pharmacological modulators i.p. daily from day −2 to day +5 of immunization. The doses used were arrived at after preliminary titration and were 1 ± 2 μg per mouse per day for dibutyryl cAMP (dbcAMP), 10 μg per mouse per day for Rolipram (Rm), and 10 nmol per mouse per day for Rp-8-Br-cAMPs (RpBrcAMPs).

For measuring immune response recall in vitro in bulk assays, splenic cells taken on the days indicated after immunization were stimulated in vitro with titrated Ag doses. At 3 days, induced levels of IFN-γ were estimated in the culture supernatants using commercial enzyme immunoassays (R&D Systems, Minneapolis, MN). In replicate cultures, proliferation was estimated as above at 72–96 h. For cells from Stm-aroA-immunized mice, sonicated aqueous extract of Stm was used as the recall Ag (14).

Clonal responder frequency and burst size analysis of Ag-specific precursor cells in the various groups of immunized mice was estimated by limiting dilution analysis (LDA) as reported earlier (12). Briefly, C37BL/6 mice were immunized s.c. with maleyl-OVA in CFA (100 μg per mouse) with or without PF cover as above. After 8 wk, titrating numbers of lymph node cells from various groups of mice were added to a constant number of gamma-irradiated spleen cells from immunized syngeneic mice as APCs, with or without maleyl-OVA (100 μg/ml). Of the wells plated per responder dose, those wells showing a response at least 3-fold that of the background value without maleyl-OVA were scored positive and the responder frequency was calculated as described (12). The response was considered clonal at the responder cell number where the proportion of positively responding wells was <37% (12). At responder cell concentrations showing clonal frequencies, the average cpm values of responding (vs nonresponding) wells were used as an estimate of the average clonal burst size of responding single T cells.

In vitro tracking of allosreactive CD4 and CD8 T cells, B6.Thy.1 congenic mice were immunized s.c. with irradiated BALB/c spleen cells with or without PF cover, and 7 wk later lymph node cells from individual immunized or unimmunized mice were labeled with 10 μM CFSE (Molecular Probes, Eugene, OR) for 15 min at 37°C and restimulated with gamma-irradiated BALB/c spleen cells as APCs. Four days later, liver cells were stained for Thy-1.1 vs CD4 or CD8, and the degree of CFSE dilution on gated Thy-1.1-bearing responder CD4 or CD8 cells in unstimulated and stimulated cultures from individual mice was determined flow cytometrically. The frequencies of CD4 or CD8 cells undergoing various numbers of divisions were estimated using the proliferation platform of FlowJo software (TreeStar, San Jose, CA).

Bacterial clearance assays

T cells purified from splenic cells by passage over nylon-wool columns were transferred i.p. into naive syngeneic recipients (10^7 T cells per mouse), which were challenged with 100 CFUs i.p. of a virulent strain of Stm, Stm-754 (15), and 24 h later the bacterial load in their spleens was estimated by titrating splenic lysates on Salmonella-Shigella-agar plates for bacterial CFU counting.

**Induction of superantigen-mediated T cell activation and loss in vivo**

MHC-matched (H-2b) mouse strains CBA/J and CBA/CaJ, which differ in their ms superantigen status, were used. CBA/CaJ mice given 1 × 10^6 CBA/J spleen cells i.p. were treated with either PBS or PF as above. Splenic cells from these recipients, as well as from control untreated CBA/J and CBA/CaJ mice, were stained with anti-TCR-V66 (BD Biosciences, Franklin Lakes, NJ) for flow cytometric analysis. Also, these spleen cells were stimulated in vitro on day 7 postinjection with titrated doses of gammapa-irradiated CBA/J spleen APCs for 3 days and proliferative responses were measured.

In cell transfers in the converse direction, CBA/J mice were given CBA/ Caja spleen cells i.v. (3 × 10^5 cells per mouse) and were treated with either PBS or PF daily from day −2 to the day of analysis. At indicated time points postinjection, splenic cells from recipient mice as well as from control CBA/J and CBA/Caj mice were stained with either anti-CD4 or anti-CD8 together with anti-TCR-V66 (BD Biosciences) for two-color flow cytometric analysis.

Flow cytometric analyses were done on a BD LSR (BD Biosciences), an Elite ESP (Beckman Coulter, Fullerton, CA), or a Blyte (Bio-Rad, Hemel Hampstead, U.K.) flow cytometer. Data were analyzed using either WinMDI shareware or FlowJo software (TreeStar). In some experiments, the CBA/CaJ spleen cells were labeled with CFSE before transfer, and staining was done with anti-CD4, anti-CD8, and anti-TCR-V66 for multicolor flow cytometry. For detection of induction of activation markers on injected responder T cells, recipient spleen cells were taken at 48 h after injection and stained for TCR-V66 vs CD49 or CD95L (BD Biosciences) for two-color flow cytometry.

**CD8 T cell responses**

Female C57BL/6 mice were immunized with 1 × 10^5 male spleen cells i.p. and treated with either PBS or PF as above. At day 60 postimmunization (p.i.), spleen cells were stimulated in vitro for 6 days with male APCs and then were used as effector cells in various E:T ratios to lyse 51 Cr-loaded target RMA-S cells (H-2b) incubated with either an irrelevant H-2D^d-binding influenza hemagglutinin (H1N1) virus–LCMV-derived peptide (KAYVNFATM; pLCMV) or with the H-2D^d-binding H-Y peptide (KCRSRNRQYL (16); pHY) in a 4-h cytotoxicity assay to determine the percentage of cytotoxicity.

**Results**

**PF enhances marine CD4 T cell priming**

We have previously observed that PF inhibits primary allospecific proliferation of human CD4 T cells in vitro (11, 12). Primary allostimulated proliferation of mouse T cells is also similarly affected by PF (Fig. 1a). In comparison to unprimed cells, primed cells show enhanced secondary proliferative responses when re-stimulated with fresh stimulators. Significantly, the presence of PF during priming enhanced this secondary alloproliferative response (Fig. 1b). The stimulators used were irradiated and lightly fixed to ensure that the effect of PF was directly on the responding T cells. The cells proliferating in these assays were confirmed to be CD4 by inhibition with anti-CD4 mAb (data not shown).

We next examined whether this enhancement of allo-priming by PF in vitro could be extrapolated to Ag-specific T cell responses in vivo by immunizing mice with the protein Ag OVA without adjuvant. Ag-recalled T cell proliferative responses could not be detected at day 60 in mice immunized with a single dose of OVA in PBS (Fig. 1c). In contrast, if the mice were treated daily with PF from day −2 to day +5 of immunization, T cell responses could be recalled very efficiently at this time (Fig. 1c). PF exhibited this
5000 cpm in b, 4–OVA in PBS and treated with either PBS or PF daily from day concentrations of OVA at day 60 p.i. from BALB/c mice immunized with

Proliferative responses of splenic cells stimulated in vitro with titrated numbers of irradiated BALB/c APCs. Proliferative responses of splenic cells stimulated in vitro with titrated concentrations of OVA at day 60 p.i. from BALB/c mice immunized with OVA in PBS and treated with PBS, PF, dbcAMP, or Rm daily from day −2 to day +5 of immunization. Background levels of [3H]thymidine incorporation in the absence of Ag were 4000–6000 in a and 6000–8000 cpm in b. The data are representative of three to five independent experiments.

FIGURE 1. PF enhances T cell memory commitment in vitro and in vivo. a, The alloresponse of C57BL/6 mouse splenic T cells to gamma-irradiated BALB/c spleen cell APCs in the presence of either PBS or PF (100 μg/ml). b, Proliferative responses of C57BL/6 responder cells cultured with no stimulus (−/−), or with irradiated and fixed BALB/c stimulator APCs in the absence (+/−) or presence (+/+ ) of PF (100 μg/ml) and then restimulated with titrated numbers of irradiated BALB/c APCs. c, Proliferative responses of splenic cells stimulated in vitro with titrated concentrations of OVA at day 60 p.i. from BALB/c mice immunized with OVA in PBS and treated with either PBS or PF daily from day −2 to day +5 of immunization. Background levels of [3H]thymidine incorporation in the absence of antigenic stimulation were 1000–2000 cpm in a, 3000–5000 cpm in b, and 5000–7500 cpm in c. The results are representative of 4–10 independent experiments.

effect in OVA immunization with adjuvants such as alum or CFA as well (data not shown).

Immune priming in vivo by PF is mediated through protein kinase A (PKA)

Mice were immunized with maleylated conalbumin (CA), which is delivered specifically to scavenger receptors on APCs and function as better immunogens than native CA (13, 17), and treated as above with PBS, PF, Rm, an inhibitor of the PDE4 isoform prominetly found in T cells (18), or the cell-permeable cAMP agonist analog dbcAMP. When the recall T cell responses of these mice were analyzed at 42 days p.i., transient treatment with PF, Rm, or dbcAMP resulted in significantly higher proliferative responses than PBS treatment yielded (Fig. 2a). We next tested the effects of PKA inhibition by a diastereoisomer of cAMP, RpBrcAMPs (19), on PF-mediated enhancement of T cell priming. When T cell proliferative responses of maleyl-CA-immunized mice were analyzed at 42 days p.i., it was observed that, while transient PF treatment led to enhanced recall responses, simultaneous treatment with the PKA inhibitor RpBrcAMPs blocked this enhanced commitment (Fig. 2b). However, treatment with the PKA inhibitor alone did not modify T cell recall responses.

PF treatment enhances T cell memory functional in vivo against a bacterial immunogen

The effect of PF on immunization with a bacterial immunogen was examined next. We immunized mice with live Stm-aroA bacteria as described previously (14), along with PBS or PF treatment as above. Recall proliferative and cytokine responses were evoked from the spleen cells of these mice in vitro with a sonicated extract of Stm 45 days later. Mice immunized under PF cover showed a significant increase in the proliferative responses (Fig. 3a), which we have confirmed to be from CD4 T cells by blocking with anti-CD4 Ab (data not shown). Similarly, IFN-γ levels were significantly higher in recall cultures from mice immunized under PF cover (Fig. 3b). The levels of other T cell cytokines such as IL-10 were also enhanced in these cultures (data not shown).

To examine the functionality of these PF-enhanced anti-Stm T cell responses in vivo, T cells were purified from the spleens of Stm-aroA-immune mice at day 45 p.i. and transferred into naive syngeneic recipients, which were then challenged with a live virulent strain of Stm. Splenic clearance estimates of injected bacteria in these mice 24 h later showed that, while recipients of immune T cells showed some enhanced clearance over mice receiving non-immune T cells, PF treatment of donors resulted in a further increase in bacterial clearance (Fig. 3c).
Inhibition of postactivation T cell loss in vivo by PF

We next began testing the effects of PF on T cell activation and AICD in vivo, using the well-characterized model of superantigen-mediated activation and deletion of T cells bearing certain TCR-Vβ elements (20, 21), in the MHC-matched (H-2k) mls-disparate mouse strains CBA/J (mls-stimulator) and CBA/CaJ (mls-responder). We transferred mls-responder T cells into mls-stimulator recipients with or without PF treatment. The frequency of TCR-Vβ6-bearing cells was higher in PF-treated mice than in untreated mice on day 7, in both CD4 and CD8 T cell populations (Fig. 4a). Supernatigen-mediated T cell apoptosis has been reported to be dependent on initial proliferation (22), and the very high frequencies of TCR-Vβ6-bearing cells in PF-treated recipients (Fig. 4a) indicated that PF may not inhibit T cell proliferation in vivo and may instead enhance survival.

The effect of PF on proliferation and loss of TCR-Vβ6-bearing cells was further examined by marking the input cells from mls-responder mice with the fluorescent dye CFSE before being transferred into mls-stimulator recipients as before. By 72 h posttransfer, CFSE-labeled TCR-Vβ6-bearing cells had proliferated to the point of losing the fluorescent label irrespective of PF treatment (Fig. 4b). By 7 days posttransfer, there was significant reduction in the frequency of TCR-Vβ6-bearing cells in the PBS-treated recipients, while these cells persisted at virtually unchanged levels in PF-treated mice (Fig. 4b). Thus, while PF inhibited TCR-Vβ6-bearing T cell loss in vivo, it did not appear to slow down their activation-induced proliferation to any significant degree.

Because TCR-Vβ6-bearing cells lost CFSE label by 72 h, we examined the effect of PF on their rate of proliferation by sampling shorter time points. At 24 h posttransfer, CFSE-labeled TCR-Vβ6-bearing cells could be detected in mls-responder CBA/CaJ recipients as well as in mls-stimulator CBA/J recipients with or without PF treatment (Fig. 5a). However, while no significant proliferation had as yet begun at this time, both CD4 and CD8 TCR-Vβ6-bearing cells had already become larger than their counterparts in mls-responder mice in both PF-treated and saline-treated mice (Fig. 5a). By 48 h posttransfer, TCR-Vβ6-bearing CD4 and CD8 cells had begun to proliferate, and PF treatment made no difference in the number of cell divisions they went through by this time (Fig. 5b). We also examined whether PF modified the induction of cell surface activation markers on TCR-Vβ6-bearing T cells upon injection into mls-stimulator mice. The enhancement of CD95L and CD122 (data not shown). Thus, mls-driven T cell activation appeared unaffected by PF in vivo.

The issue of the reactivity of T cells activated under PF cover was next addressed. We injected mls-stimulator splenic APCs into mls-responder mice, with or without PF treatment. TCR-Vβ6-bearing T cells in these groups of mice were followed flow cytometrically at days 0, 3, 7, or 14 postinjection. Significant loss of TCR-Vβ6-bearing cells was seen in mls-responder mice after injection of mls-stimulator APCs, and this loss was prevented by PF in vivo (Fig. 6a). However, TCR-Vβ6-bearing cells in mice given PF in vivo did show an increase in size at early time points (Fig. 6a). One week after cell injection, the responses of the splenic T cells from the mls-responder recipients to mls-stimulator APCs in vitro were determined. While normal mls-responder cells proliferated well, cells from mls-responder mice that had been given mls-stimulator APCs in vivo did not (Fig. 6b). However, mls-responder mice given PF in addition to mls-stimulator APCs in vivo showed an excellent maintenance of mls-1 reactivity (Fig. 6b). Thus, PF-mediated protection of responding T cells is not accompanied by induction of anergy.
**Inhibition of AICD in vitro by PF for both CD4 and CD8 T cells**

The mls-based system used above established that PF inhibited the loss of T cells in vivo consequent to activation. Therefore, a direct measurement of AICD was next undertaken. We stimulated normal mouse lymph node cells in vitro with an anti-CD3 mAb in the presence or absence of PF. The cultured cells were stained for either CD4 or CD8 vs annexin V to detect apoptotic cells 4 days later. While anti-CD3 mAb induced substantial apoptosis in both CD4 and CD8 T cells, the presence of PF significantly inhibited the apoptosis (Fig. 7). Thus, PF protected not only CD4 but also CD8 T cells against AICD. PF also inhibited the anti-CD3-induced activation of caspases, the cysteine aspartyl proteases that mediate many AICD pathways, in these T cells at 48 h (Fig. 7).

**PF enhances murine CD8 T cell priming**

Finally, we tested for the potential enhancement of CD8 T cell memory by PF in vivo using immunization for the male-specific Ag H-Y (16). Female mice were primed with a single dose of syngeneic male spleen cells either with or without PF cover, and H-Y-specific cytotoxic T cell CTL precursors were assayed 60 days p.i. Mice immunized under PBS cover showed poor CD8 T cell responsiveness by day 60 p.i. (Fig. 8). In contrast, if the mice were transiently treated with PF, H-Y-specific CTL responses could be recalled very efficiently even at day 60 p.i. (Fig. 8).

**PF increases the frequency but not clonal burst size of Ag-specific T cells**

We next examined whether PF cover in immunized mice enhanced the frequency and/or the clonal burst size of Ag-specific T cells. C57BL/6 mice (three mice per group) were immunized s.c. with maleyl-OVA with or without PF cover from day 1 to day 5 of immunization. Seven weeks later, LDA was done with maleyl-OVA stimulation of lymph node cells to estimate the frequencies and clonal burst sizes of the memory CD4 T cells. PF treatment significantly increased the clonal frequency of maleyl-OVA-specific T cells (Fig. 9a). In contrast, clonal burst size as indicated by the proliferation seen in responding wells at clonal frequency (<37%) showed no difference between cultures from mice treated with saline or PF (Fig. 9a).

We next identified responding T cells in recall cultures in vitro by labeling lymph node cells from alloimmunized mice with CFSE before putting them in culture with allostimulator APCs. B6.Thy-1.1 congenic mice (three mice per group) were immunized s.c. with gamma-irradiated BALB/c spleen cells with or without PF cover. Seven weeks later, lymph node cells were labeled with CFSE and either kept in culture without stimulation or stimulated with gamma-irradiated BALB/c spleen cells as APCs. After 4 days in culture, live cells were stained for Thy-1.1 and CD4 or CD8, and flow cytometric analysis was done. Thy-1.1-bearing responder CD4 or CD8 cells were gated from unstimulated or stimulated...
FIGURE 5. PF does not inhibit activation and proliferation of CD4 and CD8 T cells in vivo. CBA/CaJ or CBA/J mice were given CFSE-labeled CBA/CaJ spleen cells i.v. and were treated with either saline or PF from day -1 to the day of analysis. Splenic cells from recipient mice were stained for flow cytometry at 24 h (a) or 48 h (b) postinjection. Data are shown as contour plots of gated CD4 or CD8 cells for TCR-Vβ6 vs CFSE fluorescence (a and b). Contour plots of gated TCR-Vβ6-bearing CD4 or CD8 cells for cell size (forward scatter) vs CFSE are also shown in a, while the numbers of cell divisions undergone by the TCR-Vβ6-bearing CD4 or CD8 cells were calculated and are plotted as shown for PF- or saline-treated mice in b. c, CBA/J mice were given CBA/CaJ spleen cells i.v. and were treated with either PBS or PF from day -1 to day 2 postinjection. At 48 h postinjection, splenic cells from recipient mice as well as from control CBA/CaJ mice (shaded curves) were stained with anti-TCR-Vβ6 vs anti-CD95L or anti-CD44. TCR-Vβ6-bearing cells were gated, and their levels of CD95L or CD44 are shown as single-color histograms. Data are representative of two to four independent experiments.
cultures from individual mice, and the degree of CFSE dilution was examined in two-color analyses of CFSE vs cell size (Fig. 9b).

While the frequency of large cells undergoing division is greater in the PF-treated immune mice than in the saline-treated immune mice, the extent of proliferation of individual responding cells does not appear to be different. When the frequencies of CD4 and CD8 cells undergoing various numbers of divisions were estimated, it could be observed that, while the frequencies of responding cells were higher in PF-treated mice at every cell division, neither CD4 nor CD8 T cells from mice immunized under PF cover have undergone clonal division at any more rapid rate than cells from mice immunized without PF cover (Fig. 9c).

Thus, the frequency, but not the clonal burst size, of Ag-specific proliferation-competent T

FIGURE 6. Activation of T cells under PF cover does not lead to anergic tolerance. a, CBA/CaJ mice given CBA/J spleen cell APCs were treated with either PBS (–PF) or PF (+PF). Contour plots of spleen cells at days 3, 7, and 14 after APC administration are shown for TCR-Vβ6 vs cell size. The profile for CBA/CaJ mice not given any CBA/J APCs is shown (day 0) for comparison. Figures in boxes indicate the frequencies of TCR-Vβ6-bearing cells. b, CBA/CaJ mice given CBA/J spleen cell APCs were treated with either PBS or PF. Proliferation responses of splenic cells from these mice, as well as from control untreated (con) CBA/J and CBA/CaJ mice, stimulated in vitro on day 7 postinjection with titrated doses of gamma-irradiated CBA/J spleen cell APCs, are shown. Background levels of [3H]thymidine incorporation in the absence of stimulation were between 4500 and 6000 cpm. Data are representative of three separate experiments.

FIGURE 7. PF protects CD4 and CD8 T cells against activation-induced death. Splenic cells from BALB/c mice were activated in vitro with either no stimulus (shaded curves) or with anti-CD3 in the presence (thick lines) or absence (thin lines) of PF (100 µg/ml). Flow cytometric analysis using anti-CD4 or anti-CD8 staining vs VAD-fmk-Flu and vs annexin V was done 48 and 96 h later, respectively. The annexin V and VAD-fmk-Flu profiles of the gated CD4 or CD8 cells are shown as single-color histograms. The data are representative of 5–8 independent experiments.

FIGURE 8. PF enhances commitment to CD8 T cell memory in vivo. Female C57BL/6 mice immunized with male APCs were treated with either PBS or PF from day −2 to day +5 of immunization. Cytotoxicity levels obtained in assays done at day 60 p.i. in the E:T ratios indicated against MHC-matched RMA-S cells loaded with either an irrelevant LCMV-derived peptide (KAVYNFATM; pLCMV) or with the H-2D<sup>b</sup>-binding H-Y peptide (KCSRNRQYL; pHY) are shown as indicated. The data are representative of three separate experiments.
Discussion

We have shown previously that PF enhances the secondary responsiveness of human alloreactive CD4 T cells if they are primed in vitro in the presence of PF (11). This effect of PF was directly on the T cells because it was seen even if fixed stimulators were used, and it was mediated by an increase in the secondary responder cell frequency, accompanied by a reduction in apoptotic cell death of the activated T cells (12). These data suggested the hypothesis that if PF were present during primary activation of T cells, it would lead to an enhancement of T cell memory. We have now tested this hypothesis in vivo in mice and have found that PF does indeed achieve this result, indicating that PF can work as a pharmacological immune adjuvant.

To begin with, we have confirmed that PF has the same effect on allo-priming of mouse T cells in vitro that we have reported it has on human T cell priming (11, 12). The presence of PF during allo-priming in vitro enhances the resultant secondary allore sponsiveness of T cells, an effect likely to be directly on T cell signaling, because the allostimulators used were fixed and show no evidence of APC activation or cytokine secretion from them (data not shown).

In vivo, we have used PF treatment in a clinically used dose range for 5 days into primary immunization, starting from just before immunization. Thus, exposure to PF is transient and restricted to only the first few days while T cell priming in vivo is taking place. Thereafter, mice were maintained for varying periods of time before being tested for persistence of recall responses. The initial experiments used single doses of pure protein Ags such as OVA or CA without any adjuvant at all because persistence of immune responses is poor in the absence of adjuvant, to see whether PF could function on its own to enhance the persistence of recall T cell responses. In some experiments, we have used the maleylated forms of these protein Ags as immunogens, because they show enhanced immunogenicity without providing any Ag-nonspecific adjuvant activity (13, 17). We have also observed this adjuvant effect of PF during immunization with adjuvants such as alum or CFA. In all these situations, transient PF treatment early during immunization resulted in enhanced persistence of recall CD4 T cell proliferative responses in the immunized mice, establishing that PF could indeed function in vivo as an immune adjuvant.

PF is an inhibitor of PDEs, and most of its functions are thought to be mediated by enhanced cAMP levels as a result of reduced degradation of cAMP via PDE (23). This would predict that other modalities of enhancing cAMP levels would have effects similar to that of PF. PDE4 is a prominent PDE isoform in T cells and Rm is a specific inhibitor of PDE4 (18) that is structurally unrelated to PF. Transient treatment during immunization with either Rm or the

FIGURE 9. PF enhances memory T cell frequencies rather than their clonal burst size or kinetics of response. a, C57BL/6 mice were immunized s.c. with maleyl-OVA-CFA with or without PF cover. Seven weeks later, LDA was done with maleyl-OVA stimulation of lymph node cells to estimate the frequencies and clonal burst sizes of the memory CD4 T cells as shown. Clonal burst size is indicated as the proliferation seen in responding wells at clonal frequency (<37%). [3H]Thymidine incorporation in non-responding wells is also shown for comparison. b, Lymph node cells from B6.Thy-1.1 congenic mice immunized 7 wk earlier with gamma-irradiated BALB/c spleen cells s.c. with or without PF cover were labeled with CFSE, cultured with or without gamma-irradiated BALB/c spleen cells as APCs for 96 h, and stained for flow cytometric analysis. The degree of CFSE dilution is shown in two-color analyses of CFSE vs cell size of gated Thy-1.1-bearing responder CD4 or CD8 cells from unstimulated or stimulated cultures as indicated. c, The frequencies of either CD4 or CD8 cells undergoing various numbers of divisions are plotted for the experiment done in b. The data are from three mice per group (mean ± SE). Data are representative of three to eight independent experiments.
cell-permeable analog of cAMP, dbcAMP, showed the same kind of enhancement in T cell recall responses as that shown by PF treatment. Thus, enhanced cAMP levels for a few days during T cell priming in vivo is sufficient to enhance the persistence of T cell recall responses.

The major pathway of signal transduction by cAMP is mediated through the cAMP-dependent PKA, although there are PKA-independent pathways suggested as well. The PKA inhibitor we have used, RpBrcAMPs, inhibits release of the catalytic subunits of PKA from the regulatory subunits, preventing their activation (19). Inhibition of the PF-mediated immune enhancement by simultaneous treatment with the PKA inhibitor provides evidence that the immune adjuvant effect of PF is indeed mediated through cAMP-dependent PKA activation.

The data discussed so far deal with adjuvant-free immunization with pure proteins and with immune responses read out in vitro as Ag-induced proliferation. However, in vitro recall responses do not necessarily indicate functional capabilities in vivo. Therefore, it was essential to examine whether the PF-mediated immune enhancement observed extended to immune protection in vivo against infections. For this purpose, we have tested the effect of PF on immunization with a Salmonella strain Stm-aroA, a vaccine vector (24, 25) we have worked with previously (14).

The results with Stm immunization and PF usage make three points. First, there is a clear PF-mediated enhancement of the proliferative recall responses induced by Stm-aroA immunization. Second, this enhancement extends to the generation of T cell cytokines as well, specifically IFN-γ, the cytokine crucially responsible for providing protective T cell immunity against facultative intracellular pathogens such as Stm (26). This is significant in light of conflicting indirect reports about whether PF affects the induction of a Th1 cytokine, IFN-γ, in CD4 T cell responses (27, 28). In fact, we observe no major PF-mediated alteration in the balance of cytokine profiles of T cells primed in vivo under PF cover (data not shown). Third, adoptive transfer experiments show that the anti-Stm T cells generated under PF cover also show enhanced functionality in vivo in clearing Stm infection. Thus, PF is clearly useful as an agent to enhance CD4 T cell responses to vaccination in vivo.

While our data show that this effect of PF is mediated through PKA, it was not clear what downstream event in T cell activation was being targeted. Our preliminary data in the system using human T cell allo-priming in vitro had indicated that the induction of activation-mediated T cell death could be reduced by PF (12). To examine whether this was also the case in vivo, it was necessary to track responding T cells in vivo. Because T cells responding to protein immunogens in normal mice are difficult to identify and to track phenotypically, we used a well-established model of inducing activation and death in T cells bearing specific TCR-Vβ segments via superantigens (20, 29).

In the mls-based model we have used, a superantigenic protein, v-sag-7, coded by the integrated retroviral gene Mtv-7, leads to activation of TCR-Vβ-6-expressing T cells. In a mouse strain that expresses Mtv-7 endogenously, such as CBA/J, the TCR-Vβ-6-bearing T cells are deleted in the thymus during development and are not seen in the peripheral lymphoid tissues (29). However, while a related strain, CBA/CaJ, is syngeneic to CBA/J in other respects, it does not have the Mtv-7 integration and therefore shows a significant component of TCR-Vβ-6-expressing T cells in peripheral lymphoid organs. When mature CBA/CaJ TCR-Vβ-6-bearing T cells were injected into mls-responder CBA/J mice, PF had no effect on the resultant activation (as indicated by the expression of activation markers such as CD44 and CD95) and extensive proliferation (as indicated by dilution of the fluorescent cytoplasm marker, carboxyfluorescein) of these cells, but it prevented the cell loss that followed.

An interesting point here is the divergence of the effect of PF on T cell proliferation in vitro and in vivo. While PF is inhibitory for T cell proliferation in vitro, it does not appear to have the same consequence in vivo. Although we do not have direct evidence of an explanation of this difference, it may be noted that PF specifically inhibits the induction of the transcription factor c-rel in T cells, which is crucial for the induction of IL-2 transcription (30), and the absence of IL-2 is known to lead to a far greater compromising of T cell proliferation in vitro than in vivo (31, 32).

An additional issue was the reactivity status of T cells that were activated under PF cover so that their loss was prevented. This is a particularly significant issue in light of previous reports that elevating cAMP leads to T cell anergy rather than to enhanced memory (33, 34). It was necessary to confirm that T cells protected from deletion by PF remained Ag responsive rather than becoming anergic. We find that, while injection of mls-stimulator APCs into mls-responder mice caused tolerance in the responder mice, in part by deletion and in part by anergy as reported earlier (20, 35, 36), concomitant PF treatment not only increased the frequency of mls-reactive T cells surviving deletion but also maintained their superantigen responsiveness. Previous reports regarding the induction of anergy by increased cAMP have used secondary T cells, most commonly as stable T cell clones (33), and while primary T cells have been used in one of these reports, the anergy induced in them has been the result of blockade of CD40-CD40 ligand interaction rather than by enhancement of cAMP levels (34). Long-term T cell clones may show quite different signaling pathways in comparison to naive T cells, particularly naive T cells in vivo, and these data together may indicate an interesting difference between priming vs restimulation of Ag-specific T cells.

However, our data show that T cell activation and proliferation in vivo are not affected by PF, nor is there any evidence for induction of T cell anergy due to PF cover. Instead, our data so far suggested that PF could be directly inhibiting AICD of T cells. In vitro experiments demonstrate that anti-CD3 mAb-mediated induction of apoptosis, as detected both by the activation of caspases in the T cells and by the membrane reorganization characteristic of apoptosis (i.e., the exposure of the phosphatidylserine polar head groups detected by binding of annexin V), is inhibited by PF, establishing the ability of PF to inhibit AICD in T cells.

Are these two properties of PF (i.e., enhancing the persistence of recall T cell responses and inhibiting T cell apoptosis) causally related? Interestingly, while CD4 and CD8 T cells may differ in their requirements for memory commitment and in the AICD pathways they follow (37, 38), PF nonetheless inhibited the loss in vivo of activated mls-reactive CD8 T cells and inhibited anti-CD3-induced AICD in them in vitro. If inhibition of apoptosis is sufficient for causing enhanced T cell priming, CD8 T cells should also show PF-mediated increases in the persistence of recall responses. This hypothesis was borne out when the effect of PF on persistence of CD8 T cell responses was tested, reinforcing the probability of a causal relationship between inhibition of T cell AICD and enhancement of persistence of T cell responsiveness.

One prediction of our explanation is that PF treatment during immunization would lead to an increase in the memory T cell frequency rather than in the efficiency of their response. We have shown previously that, in human CD4 T cells allo-primed in vitro, PF cover during priming leads to an increase in the frequency but
not the clonal burst size of Ag-specific secondary T cells. We have now also shown that memory T cells, both CD4 and CD8, generated in vivo by immunization under PF cover, show an increase in frequency but not in clonal burst size. We have used LDA assays with maleyl-OVA immunization for this purpose, as well as tracking proliferating primed alloresponder cells in vitro by CFSE dilution. In preliminary experiments, we have also examined the frequencies and number of cell divisions undergone by maleyl-OVA-specific CD4 T cells from mice immunized >6 wk earlier with or without PF cover, and we find that, while the frequencies of responding cells are increased by PF cover, the modal number of cell divisions responding T cells undergo is not different. In other experiments, we have also estimated the frequency of Ag-specific T cells surviving in vivo without taking recourse to in vitro challenge, by using pigeon cytochrome c as an immunogen in H-2b mice, where the I-E\(^{b}\)-restricted CD4 T cells responding to the immunodominant peptide mainly use TCR-V\(^{11}\) and TCR-V\(^{11}\), and PF treatment significantly increases this frequency as early as 1 wk after immunization (data not shown). 

Together, all these data confirm that PF causes an increase in the frequency of surviving Ag-specific T cells, rather than any increase in their burst size, supporting the probability that PF leads to an enhancement of memory T cell frequency through inhibition of AICD during priming.

While our data appear to support a linear model of memory T cell generation in which all activated T cells first differentiate into effectors and some of these persist as memory by escaping AICD (4, 5), it is not clear whether all activated T cells rescued from apoptosis would necessarily enter the memory pathway, or whether qualitatively distinct signaling pathways are needed for memory differentiation (6, 7). Further analysis of the phenotype of the Ag-specific T cells triggered under cover of PF would allow this issue to be addressed. Also, the characterization of the T cell death pathway affected by PF would allow a new approach to the relationship between death and memory in T cells.

Thus, these data show that transient PF treatment early during immunization generates longer-lasting T cell memory in vivo via a CAMP-PKA-mediated pathway by inhibiting AICD-mediated T cell loss rather than inhibiting T cell activation per se. In passing, it is curious that the CAMP-dependent signaling pathway, which is critical for long-term neural memory (39), can also apparently contribute to T cell memory. Therefore, our observations identify a novel class of immunological adjuvants for vaccination, especially because PF enhances immune memory in both CD4 and CD8 T cells without altering the T cell cytokine balance. PF is commonly used clinically, and its doses used here are within the clinical range (23, 40). The CAMP-dependent pathway has been extensively analyzed, making it possible to find other potential drugs even more efficient at enhancing immune memory.

References


