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Drug-Induced Expansion and Differentiation of Vγ9Vδ2 T Cells In Vivo: The Role of Exogenous IL-2[1]

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Human Vγ9Vδ2 T cells recognize nonpeptidic Ags generated by the 1-deoxy-D-xylulose 5-phosphate (many eubacteria, algae, plants, and Apicomplexa) and mevalonate (eukaryotes, archaebacteria, and certain eubacteria) pathways of isoprenoid synthesis. The potent Vγ9Vδ2 T cell reactivity 1) against certain cancer cells or 2) induced by infectious agents indicates that therapeutic augmentations of Vγ9Vδ2 T cell activities may be clinically beneficial. The functional characteristics of Vγ9Vδ2 T cells from *Macaca fascicularis* (cynomolgus monkey) are very similar to those from *Homo sapiens*. We have found that the i.v. administration of nitrogen-containing bisphosphonate or pyrophosphomonoester drugs into cynomolgus monkeys combined with s.c. low-dose (6×10^5 U/animal) IL-2 induces a large pool of $CD27^+$ and $CD27^-$ effector/memory T cells in the peripheral blood of treated animals. The administration of these drugs in the absence of IL-2 is substantially less effective, indicating the importance of additional exogenous costimuli. Shortly after the costimulatory IL-2 treatment, only γδ (but not αβ) T cells expressed the CD69 activation marker, indicating that Vγ9Vδ2 T lymphocytes are more responsive to low-dose IL-2 than αβ T cells. Up to 100-fold increases in the numbers of peripheral blood Vγ9Vδ2 T cells were observed in animals receiving the γδ stimulatory drug plus IL-2. Moreover, the expanded Vγ9Vδ2 T cells were potent Th1 effectors capable of releasing large amounts of IFN-γ. These results may be relevant for designing novel (or modifying current) immunotherapeutic trials with nitrogen-containing bisphosphonate or pyrophosphomonoester drugs. *The Journal of Immunology,* **2005, 175: 1593–1598.**

T lymphocytes expressing the γδ TCR are involved in both innate and adaptive immune responses. The primate Vγ9Vδ2 T cell subset (constitutes the majority of circulating γδ T cells in *Homo sapiens*) can polyclonally respond to a variety nonpeptidic Ags (NpAgs)[3] generated as precursors of >20,000 known natural isoprenoids. Many of these molecules were first isolated from mycobacteria (1–5). Phosphostim (bromohydrin pyrophosphate (BrHPP)), a small synthetic pyrophosphomonoester (PP-ME) molecule analogous to natural NpAgs (6), is currently in a Phase I clinical trial in renal carcinoma. In vitro stimulation with BrHPP directly triggers T cells expressing the Vγ9Vδ2 TCR and requires neither Ag uptake/processing, nor MHC class I or II expression.

Nitrogen-containing bisphosphonate (N-BP) drugs such as pamidronate disodium (PAM) or zoledronic acid (ZOL) inhibit the bone-destroying activity of osteoclasts. They are approved for the treatment of osteoporosis, bone metastases, tumor-induced hypercalcemia, and myeloma bone disease and can induce the accumulation of Vγ9Vδ2 T cell stimulatory precursors of isoprenoid biosynthesis (7, 8). The recognition of NpAgs of endogenous or exogenous origin allows Vγ9Vδ2 T cells to respond polyclonally and very quickly to infected or transformed cells (9, 10).

Vγ9Vδ2 T lymphocytes proliferate in response to HIV-infected cells and can exert a powerful cytotoxic activity against HIV-infected targets (10, 11). Also, Vγ9Vδ2 T cells produce the HIV inhibitory β-chemokines and α-defensins (12, 13). Vγ9Vδ2 T cells reduce the viability of intracellular and extracellular *Mycobacterium tuberculosis* through granulysin-dependent pathways (14, 15). Adaptive immune responses of Vγ9Vδ2 T cells were observed during mycobacterial infections (16, 17), and bacille Calmette-Guérin (BCG) vaccination was shown to enhance human Vγ9Vδ2 T cell responsiveness to mycobacteria (18). In addition, Vγ9Vδ2 T cells have been repeatedly shown to kill a broad range of lymphoma or myeloma cell lines (7, 19, 20). Interestingly, intravesical BCG therapy in patients with superficial bladder carcinoma increases the representation of γδ T cells among tumor-infiltrating lymphocytes (21). Thus, it is possible that boosting Vγ9Vδ2 T cell activities may be of therapeutic value in some cancers and infectious diseases.

We have studied two distinct subsets of Vγ9Vδ2 T cells defined by a loss of CD27 expression in functionally differentiated cells (22). Naive and central memory $CD27^+$ Vγ9Vδ2 T cells express lymph node homing receptors, abound in lymph nodes, and lack immediate effector functions (23). Conversely, effector/memory $CD27^-$ Vγ9Vδ2 T cells are represented poorly in the lymph nodes but are plentiful at sites of inflammation, probably due to specific homing receptor expression. Circulating Vγ9Vδ2 T effectors are

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[3] Abbreviations used in this paper: NpAg, nonpeptidic Ag; BrHPP, bromohydrin pyrophosphate, phosphostim; PP-ME, pyrophosphomonoester; N-BP, nitrogen-containing bisphosphonate; PAM, pamidronate disodium; ZOL, zoledronic acid; BCG, bacille Calmette-Guérin; EI, expansion index; EpoxP, epoxymethylbutyl pyrophosphate; DPG, 2,3-diphospho-D-glyceric acid; IPP, isopentenylpyrophosphate.

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significantly reduced in patients with acute pulmonary tuberculosis or with active HIV disease. This is reflected by a reduced frequency of IFN-γ-producing cells after in vitro stimulation with NpAgs from mycobacteria (22, 24). In addition, only a low number of patients with hematological malignancies display Vγ9Vδ2 T cell responses to NpAgs in vitro, perhaps due to immunosuppression by chemotherapy and/or underlying disease (25). Thus, augmenting the function/numbers of Vγ9Vδ2 T effectors could be beneficial in many of the clinical situations described above. For that reason, we have tried to develop a method for inducing effector/memory Vγ9Vδ2 T cell responses in vivo. Macaque and human Vγ9Vδ2 TCRs share several conserved motifs (26). Similar to human Vγ9Vδ2 T cells, rhesus monkey γδ T cells are stimulated when exposed to prenyl pyrophosphate, N-BPs, and alkylamine Ags (27). Sequencing of the rhesus monkey Vγ9Vδ2 TCR revealed a strong sequence homology to the human Vγ9Vδ2 TCR with preserved important sequence motifs (26–28). Because rodents lack the humans specificity of Vγ9Vδ2 T cells, we used a nonhuman primate model in this study. Our data confirm that macaques may provide a suitable animal model for human γδ T cell responses. Specifically, we observed that s.c. injections of low doses of IL-2 combined with i.v. administration of aminobisphosphonate or PP-ME drugs substantially expand the pool of Vγ9Vδ2 Th1 effectors in vivo.

Materials and Methods

Animals

The in vitro study was performed using blood samples collected from 56 cynomolgus monkeys (*Macaca fascicularis*). The animals were born and maintained in a closed colony established in 1981 at the Consiglio Nazionale delle Ricerche (Italy) Breeding Center in Rome and appeared healthy at the time of experimentation. When handling the animals, they were anesthetized by 10 mg/kg ketamine hydrochloride injected i.m. Blood samples were obtained from the femoral vein. During the study, the animals were housed in stainless steel primate cages in indoor facilities and were fed a standard monkey diet, supplemented with fresh fruit. The ethical committee of the Italian Ministry of Health approved this research.

Human mAbs cross-reactive with cynomolgus monkeys

To define the phenotype of simian mononuclear cell populations, a series of mAbs against human CD Ags and TCR were tested for cross-reactivity with the corresponding monkey cell surface receptors. The mAbs used in this study were as follows: anti-monkey CD3 biotin conjugate mAb (clone FN-18 obtained from Biosource) with streptavidin PE-cychrome 5 detection, anti-human Vδ2 (clone 15D obtained from Pierce), anti-human CD25 (clone M-A251 obtained from BD Pharmingen), anti-human CD69 (clone FN50 obtained from DakoCytomation), anti-human CD4 and anti-human CD8 (clone L200 and RPA-T8, respectively, obtained from BD Biosciences), and anti-human CD27 (clone CLB-27/1 obtained from Caltag Laboratories). Isotype-matched mAbs (BD Biosciences) were used in all experiments as controls.

Cell preparations and in vitro stimulations

Animal PBMCs were isolated from heparinized blood by Ficoll-Hypaque (Pharmacia Biotech) and cultured at 2×10^6 cells/ml in complete medium (RPMI 1640 medium, 10% v/v heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). PBMCs were stimulated in vitro for 10 days in the presence of different NpAgs listed in Table I. All in vitro NpAg stimulations were performed in the presence of 100 U/ml rIL-2 (Boehringer Mannheim). After a 7-day culture period, a half of the culture supernatant was replaced by fresh complete medium with 100 U/ml IL-2. The expansion and activation of Vγ9Vδ2 T cells after 10 days of culture were determined by three-color FACS analysis using TCR-Vδ2, anti-CD25, and anti-CD69 mAbs coupled to FITC or PE, respectively, and anti-monkey CD3 biotin-conjugated mAb. The Vδ2 expansion index (EI) was calculated by dividing the absolute number of Vδ2 T cells in stimulated cultures by the absolute number of Vδ2 T cells in unstimulated cultures.

In vivo treatments

For the in vivo administration of NpAgs, two *M. fascicularis* (nos. 113 and 122; an average body weight of 6.5 kg) received an i.v. injection of epoxymethylbutyl pyrophosphate (EpoxP) (10 mg/kg, following a protocol established by Innate Pharma), two animals (nos. 135 and 251; an average body weight of 4.6 kg) received an i.v. injection of 2,3-diphosphoglyceric acid (DPG) (10 mg/kg, as previously described (17)), and two control animals (nos. 243 and 246; an average body weight of 3.0 kg) received physiological solution (placebo). For the combined administration of s.c. IL-2, six macaques with an average body weight of 6.9 kg were selected. Two macaques (nos. 164 and 179) received an i.v. injection of BrHPP (90 mg/kg diluted in 50 ml of physiological solution and slowly injected for 30 min according to a protocol established by Innate Pharma), two macaques (nos. 191 and 195) received an i.v. injection of PAM (2 mg/kg diluted in 50 ml of physiological solution, slowly administered for 30 min; this dose is twice higher than a typical human dose; the bisphosphonate infusions in *H. sapiens* are spread over 1 h, but we had to reduce the time period to 30 min to avoid additional anesthetic injections of ketamine hydrochloride). Two control animals (nos. 127 and 169) received i.v. injections of physiological solution (placebo). The animals also received s.c. injections of IL-2 (6×10^5 U; Proleukin; Chiron) every day for 4 days, beginning on the same day as the BrHPP, PAM, or placebo injections. Body weight, body temperature, and hematological values were monitored in all 12 animals during the study. No influence of the treatments was noted.

Flow cytometry analyses were performed before the treatment and 1, 4, 7, 10, and 31 days after the in vivo treatment. Percentages of γδ T cells expansion and activation were determined by three-color FACS analysis using anti-Vδ2, anti-CD3, anti-CD69, anti-CD25, and anti-CD27 mAbs. PBMCs collected before and 1, 4, 7, and 10 days after in vivo treatment with phosphoantigens plus IL-2 were restimulated in vitro with isopentenylpyrophosphate (IPP) (30 μg/ml), BrHPP (0.16 μg/ml), and EpoxP (1.64 μg/ml), and after 24 h, the culture supernatants were collected for IFN-γ detection by ELISA (monkey IFN- γ ELISA kit; BioSource International).

Results

Monkey γδ T cell distribution and in vitro reactivity to NpAgs

T cell subset distribution in the peripheral blood from cynomolgus monkeys was analyzed using human mAbs cross-reactive with corresponding simian Ags. In comparison with the human peripheral blood, an increased number of CD8 T cells and a reduced frequency of CD4 T cells were observed in *M. fascicularis*, resulting in an inversed CD4:CD8 ratio (the human CD4:CD8 ratio = 1.8, whereas the cynomolgus CD4:CD8 ratio = 0.3). Similarly, an increased number of Vδ1 T cells and a reduced frequency of Vδ2 T cells were observed in *M. fascicularis*, resulting in an inversed

Table I. *Nonpeptidic Ags and bisphosphonates used to stimulate simian Vγ9Vδ2 T cells*

Acronym	Name	Source	In Vitro Concentration (μg/ml)	In Vivo Concentration (mg/kg)
IPP	Isopentenylpyrophosphate	Sigma-Aldrich	30	NT[a]
DPG	2,3-Diphospho-D-glyceric acid	Sigma-Aldrich	150	10
EpoxP	Epoxymethylbutyl pyrophosphate	Innate Pharma	1.64	10
BrHPP	Bromohydrazine pyrophosphate (phosphostim)	Innate Pharma	160	90
PAM	Pamidronate disodium	Novartis	10	2

[a] NT, not tested.

Vδ2:Vδ1 ratio (the human Vδ2:Vδ1 ratio = 2.9, whereas the cynomolgus Vδ2:Vδ1 ratio = 0.6). Thus, the T cell number regulatory mechanisms may be somewhat different in human and nonhuman primates. The expression of the Vδ2 TCR and the CD69 activation marker was analyzed in 12 *M. fascicularis* after 10 days of in vitro culture with IL-2 in the presence or absence of different NpAgs. Similar to *H. sapiens*, all tested stimulatory molecules listed in Table I were able to activate peripheral blood Vγ9Vδ2 T cells in *M. fascicularis* (data not shown). Fig. 1*A* shows a representative experiment illustrating cynomolgus Vγ9Vδ2 T cell reactivity to EpoxP. CD69 activation marker was expressed by 18% of γδ T cells after 10 days of culture with IL-2, by 23% of γδ T cells in the presence of EpoxP alone, and by 83% of γδ T cells in the presence of EpoxP plus IL-2. However, only in the presence of EpoxP plus IL-2, the expansion of $CD69^+V\delta2^+$ T cells occurred (0.2% (at the initiation of in vitro culture) vs 8.5% (day 10 with EpoxP + IL-2) vs 0.7% (day 10, IL-2 only) and vs 0.4% (day 10, EpoxP alone)). Fig. 1*B* shows the distribution of Vδ2 EI in 41 cynomolgus monkeys. The animals with the EI < 2 were considered "low responders" and those with the EI > 3 "good responders." In this population, eight animals were low responders (19.5%), pointing out the inequality of NpAg reactivities among individual animals.

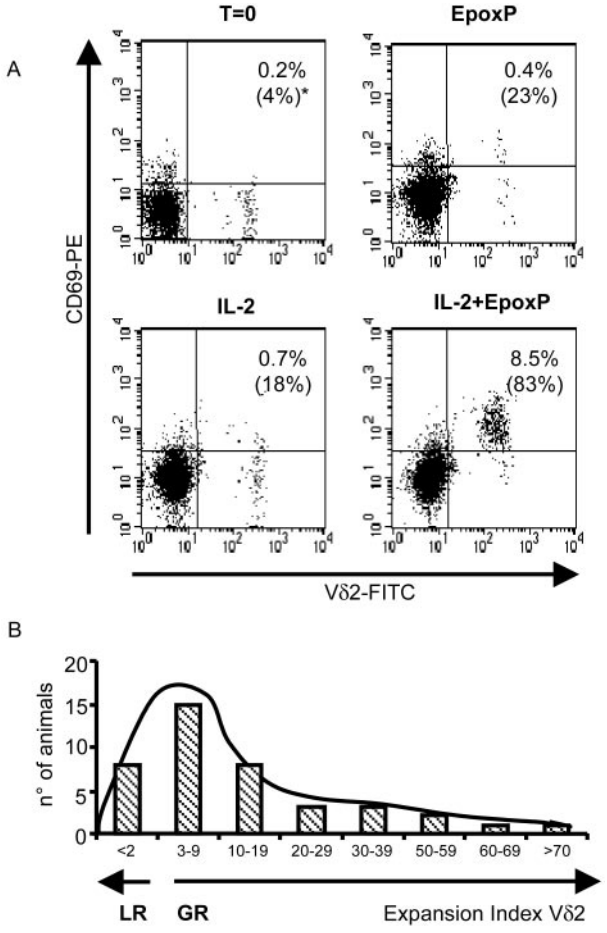

FIGURE 1. In vitro responses to EpoxP in *M. fascicularis*. The percentages of $CD69^+$ Vδ2 T cells were determined by two-color FACS analyses using anti-human CD69 PE-conjugated and anti-human Vδ2 FITC-conjugated mAbs before ($t = 0$) and after 10-day cultures of simian PBMCs in the presence of IL-2 with or without EpoxP and with EpoxP alone (1.64 μg/ml). *A*, Flow cytometry analyses of representative macaque PBMCs. *B*, The frequency and magnitude of EpoxP responsiveness measured in 41 *M. fascicularis*. PBMCs were incubated with IL-2 in the presence or absence of EpoxP. After 10 days of culture, the Vδ2 T cell expansion was measured by flow cytometry using anti-human Vδ2 FITC-conjugated and anti-monkey CD3 biotin-conjugated mAbs. The EI was calculated as follows: the absolute number of $V\delta2^+$ T cells after stimulation divided by the absolute number of $V\delta2^+$ T cells before stimulation. The percentage of $CD69^+$ cells among Vδ2 T cells is reported in parentheses.

In vivo reactivity to NpAgs

The i.v. administration of NpAgs alone caused no adverse effects but failed to reproduce the same extent of responses observed in vitro (Fig. 2, *left*). A slight increase of Vδ2 T cells was observed in animal no. 135 following the DPG administration and in animal no. 113 following the EpoxP administration. To improve the in vivo reactivity to NpAgs, six animals were treated with IL-2 (6×10^5 U injected s.c.). Because the total number of animals enrolled has been limited by ethical and logistic (availability of animals and space) constraints on the number of animals enrolled, in this particular experiment, we did not include animals treated with drugs in the absence of IL-2. However, the in vitro and in vivo influence of the relative IL-2 deficit was addressed in other experiments. For example, Fig. 1 shows that γδ T cells exposed to similar drugs do not expand in vitro in the absence of exogenous IL-2. Fig. 2 illustrates that drug-exposed γδ T cells fail to expand in vivo without the presence of exogenous IL-2. Thus, these results are compatible with the idea that under the given conditions, the drug exposure does not generate sufficient amounts of IL-2 to support the Vγ9Vδ2 T cell expansion and the supplementation with exogenous IL-2 is a sine qua non for substantial expansions to occur. On the first day of IL-2 treatment, two animals (nos. 164 and 179) were simultaneously treated with BrHPP and two animals (nos. 191 and 195) with PAM. A substantial expansion of Vγ9Vδ2 T cells was observed 4 days after the drug application in the BrHPP-treated animals (nos. 164 and 179) and in one of PAM-treated animal (no. 195). The Vδ2 T cell numbers in 1 μl of blood increased from 13 to 195 in animal no. 164, from 22 to 5182 in

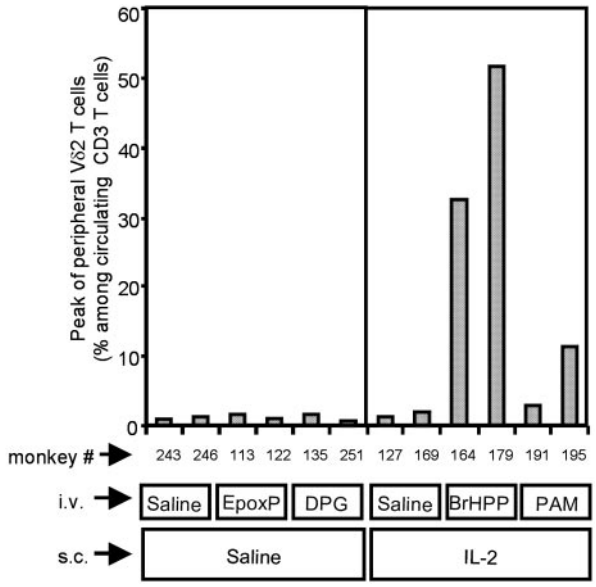

FIGURE 2. IL-2 dependence of *M. fascicularis* Vγ9Vδ2 T cell responses in vivo. Eight animals received an i.v. administration of NpAgs (EpoxP, DPG, BrHPP, or PAM) with or without s.c. administrations of IL-2. Four control animals received a placebo saline solution injected i.v. The percentage of Vδ2 T cells was determined by FACS before and after treatments with NpAgs at different time points (0, 24 h, and days 4, 7, 10, 14, 21, and 28). The given value indicates the highest measured percentage of Vδ2 T cells.

animal no. 179, and from 50 to 265 in animal no. 195. This expansion was only transient because the Vδ2 T cell numbers were back to normal levels 3 days after stopping the IL-2 treatment (23, 26, and 177 for animal nos. 164, 179, and 195, respectively). No adverse reactions were observed in the treated animals, and no effect of IL-2 treatment alone was noted (Figs. 2 and 3). Because CD69 is an early activation marker expressed on the surface of Vγ9Vδ2 T cells (Fig. 1), we analyzed the expression of CD69 on PBMCs from IL-2-treated animals shortly after the treatment. Fig. 4*A* shows that 10–20% of Vδ2 T cells were activated 24 h after the initiation of treatment. Interestingly, Vδ2 T lymphocytes were also activated in the control animals receiving IL-2 alone (nos. 127 and 169), whereas no significant activation of αβ T cells from treated or untreated animals (Fig. 4*B*) was observed, suggesting that the sensitivity of αβ T lymphocytes to low doses of s.c. IL-2 may be lower than that of γδ T cells.

Effector/memory Vγ9Vδ2 T cell subsets and IFN-γ production after in vivo treatment with NpAgs

We have analyzed the expression of CD27, a costimulatory molecule that is expressed by naive and memory Vγ9Vδ2 T cells but is absent on the cell surface of effector Vγ9Vδ2 T cells. Most simian circulating Vγ9Vδ2 T cells are $CD27^+$ cells, regardless of the treatment with IL-2 (Fig. 5). The combined treatment with IL-2 plus NpAgs expanded mainly $CD27^+$ memory cells (8.9-, 41.5-, or 9.6-fold in animal nos. 164, 179, or 195, respectively). Interestingly, the small fraction of circulating $CD27^-$ effector Vγ9Vδ2 T cells was also increased (8.5-, 9.3- or 3.5- fold in animal nos. 164, 179, or 195, respectively).

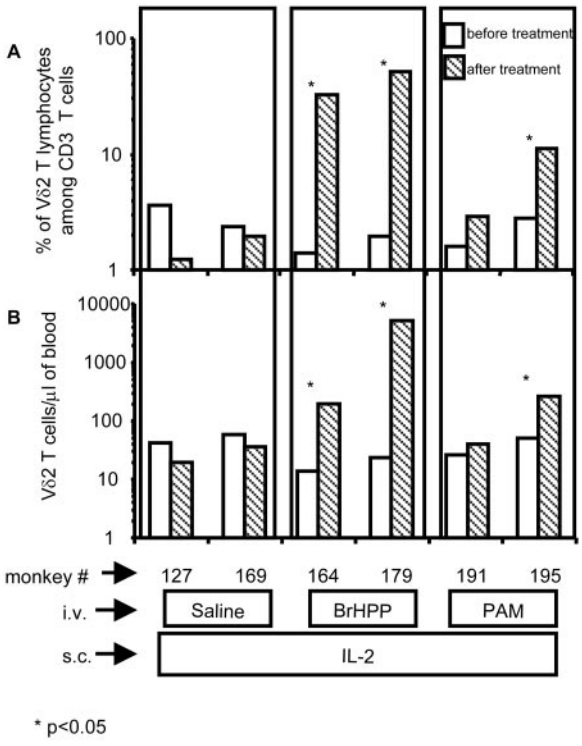

FIGURE 3. In vivo responses to Vγ9Vδ2 T cell stimulatory molecules in *M. fascicularis*. Six animals received s.c. IL-2 (6×10^5 U/dose) administered daily for 4 days. In addition to the IL-2 treatment, two animals (nos. 164 and 179) received BrHPP (90 mg/kg), and two animals (nos. 191 and 195) received PAM (2 mg/kg) injections. The values indicate the percentage of Vδ2 T cells among $CD3^+$ T cells (*A*) and the absolute Vδ2 T cell numbers/1 μl of blood (*B*) 4 days after the initial treatment.

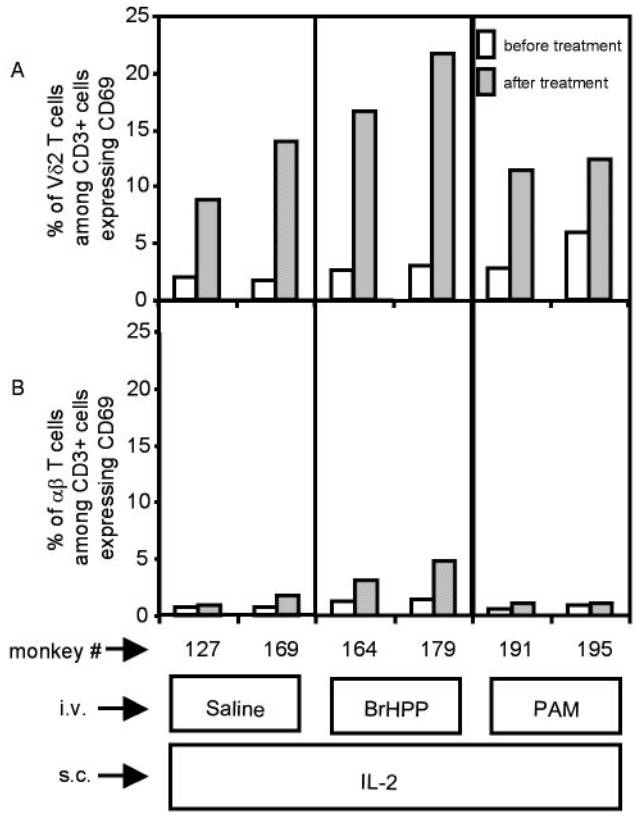

FIGURE 4. CD69 expression on T cells after s.c. administration of IL-2. The expression of CD69 molecules on γδ T cells (*A*) and on αβ T cells (*B*) was analyzed by flow cytometry before (□) and 24 h after (▨) the initial IL-2 treatment (6×10^5 U/dose).

Because both $CD27^+$ and $CD27^-$ effector/memory Vγ9Vδ2 T cells may release different levels of Th1 cytokines (22, 23), we measured the production of IFN-γ by Vδ2 T cells restimulated by NpAgs in vitro at the same time points. There was a log increase (12.8-fold maximum) in IFN-γ production in animal no. 179 treated with IL-2 + BrHPP, and in the animal no. 195 treated with IL-2 +PAM, the increase was even higher (56.1-fold maximum). A moderate increase was also observed in the animal no. 164 (treated with IL-2 + BrHPP, 2.3-fold maximum) and in animal no. 191 (treated with IL-2 + PAM, 8.8-fold maximum). No substantial changes were observed in controls animals (1.4- and 1.1-fold increases in nos. 127 and 169, respectively). No production of IL-4 and IL-10 by Vδ2 T cells was detected (data not shown). These results also support the idea that the increase in Vδ2 effector Th1 cells after the administration of NpAgs in vivo is IL-2 dependent.

Discussion

In this study, cynomolgus monkeys were used to assess the in vivo response of Vγ9Vδ2 T cells to NpAgs. The observed responses to NpAgs were not uniform among the tested animals. Some animals could be classified as very high responders, some as nonresponders, and many animals displayed intermediate responses. The broad range of individual Vγ9Vδ2 T cell responsiveness in this model system somewhat contrasts with the situation in putatively healthy *Homo sapiens*, where very strong responses to NpAgs predominate, whereas weak responses are relatively rare and nonresponsiveness practically doesn't exist in healthy individuals. These relatively high reactivities of human Vγ9Vδ2 T cells to NpAgs may be induced by environmental stimuli or subclinical infections (17). Healthy human low responders are rare and are particularly

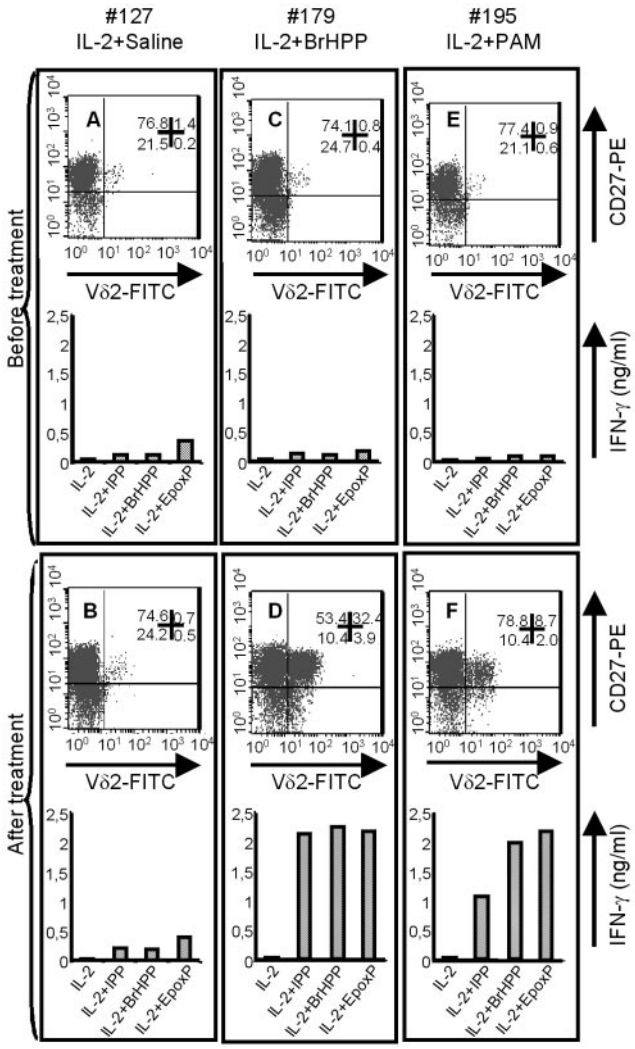

FIGURE 5. The induction of effector/memory phenotype in the Vγ9Vδ2 T cell subset and IFN-γ production after the treatment with NpAgs and IL-2 in vivo. The expression of CD27 molecules on γδ T cells was measured by flow cytometry before (*A*, *C*, and *E*) and after (*B*, *D*, and *F*) in vivo treatments with NpAgs (no. 179 with BrHPP and no. 195 with PAM). This allows discriminating between $CD27^+$ central memory and $CD27^-$ effector Vγ9Vδ2 T cells. At the same time points, PBMCs from the experimental and control animals were restimulated in vitro with IPP, BrHPP, or EpoxP, and the supernatants were collected after 24 h of culture. The production of IFN-γ (pg/ml) was measured in the culture supernatants by ELISA. The figure illustrates representative experiments.

found among newborns (29), elderly persons (30), and immunocompromised hosts (22, 31, 32). It is conceivable that a regular "environmental boosting" with NpAgs may be required to maintain the natural immune activity mediated by γδ T lymphocytes. Vγ9Vδ2 T cells recognize NpAgs, including synthetic N-BPs such as PAM. PAM, which is currently used in the clinical treatment of bone metastases, can induce in vivo Vγ9Vδ2 T cell activation in cancer patients and antimicrobial activity in humanized SCID mice (33, 34). Another N-BP, ZOL, was shown to induce Vγ9Vδ2 T cell maturation, resulting in an IFN-γ-producing effector phenotype in cancer patients treated for bone metastases (35).

In the current study, we observed that the sole i.v. administration of NpAgs had no adverse effects in *M. fascicularis* but failed to reproduce the marked reactivity observed in vitro. Thus, we have developed a new protocol of NpAg administration in vivo using IL-2 costimulation. Our results indicate that the IL-2 administration is a strict requirement for Vγ9Vδ2 T cell expansion in vivo. Wilhelm et al. (25) reported a partial in vivo γδ T cell response to PAM administered with IL-2 in non-Hodgkin's lymphoma or multiple myeloma patients. Most patients developed fever and postinfusional thrombophlebitis. Because i.v. IL-2 infusions have been associated with frequent undesirable side effects, s.c. IL-2 administrations have become preferable. During our pilot study assessing s.c. injections of low doses of IL-2, we observed up to 2 log increases in circulating Vγ9Vδ2 T cells without any obvious adverse effects. Moreover, only γδ, but not αβ, T cells were expressing the CD69 activation marker shortly after the IL-2 treatment, suggesting that γδ T lymphocytes may be more sensitive to low doses of s.c. IL-2 than αβ T cells. This is compatible with the finding that short-term exposures to IL-2 in vitro induce the expression of CD69, CD25, and HLA-DR on human γδ, but not αβ, T cells (36). Comparable amounts of the IL-2Rα-chain are expressed on γδ and αβ T cells, whereas the relative density of the IL-2Rβ-chain is more than twice as high on γδ cells, a feature which they share with NK cells.

The response was apparently stronger in the animals receiving BrHPP in comparison with the PAM-treated animals. This difference may be due to the individual animal variability in terms of Vγ9Vδ2 T cell responsiveness to NpAgs, which clearly exists (see Fig. 1*B*). Also, the magnitude of in vivo responses to BrHPP and PAM drugs may be influenced by different pharmacologic properties of PP-ME and N-PB drugs. For example, PP-ME molecules appear to be interacting directly with the Vγ9Vδ2 TCR, whereas the activity of Vγ9Vδ2 T cell stimulatory N-BPs may be less direct and is likely to be associated with the accumulation of the endogenous PP-MEs, IPP, and/or its isomer 3,3-dimethylallyl pyrophosphate (8). Nevertheless, we have observed recently that ZOL is able to activate γδ T cells in vitro at micromolar concentrations and is highly effective in vivo (35). Thus, ZOL represents a promising candidate for future N-BP + IL-2-combined treatments in vivo.

Two subsets of human Vγ9Vδ2 T cells have been identified on the basis of CD27 expression—naive and central memory $CD27^+$ cells and effector $CD27^-$ cells (22, 23). Interestingly, the in vivo combined treatment with Vγ9Vδ2 T cell-stimulatory drugs and IL-2 was able to induce both $CD27^-$ and $CD27^+$ Vγ9Vδ2 effector/memory T cell subsets in the peripheral blood of treated macaques. Peripheral Vγ9Vδ2 T cell numbers increased up to 2 logs, and the Th1 effector function of these cells was confirmed by showing their ability to release large amounts of IFN-γ.

Although murine γδ T cells have been considered to lack long-term memory, analyses in human and nonhuman primates have suggested that Vγ9Vδ2 T cells provide both primary and recall responses to mycobacteria (16, 18). *Mycobacterium bovis* BCG vaccination has been shown to augment human γδ T cell responsiveness to mycobacteria (18). Moreover, primary and recall expansions of Vγ9Vδ2 T cells could be induced by BCG vaccination in a macaque model (16). Also, we have observed that both human and simian Vγ9Vδ2 T cells are primed in vivo by either tuberculosis disease or i.v. NpAgs administration (17). Thus, the Vγ9Vδ2 T cell subset appears to have a unique ability to contribute both to innate immunity by providing the readily available $CD27^-$ effectors and to adaptive immune responses by supplying the CD27+ memory pool.

In summary, our data indicate that either PP-ME or N-BP drugs in combination with low doses of IL-2 could be used relatively safely for boosting the potent antiviral (12, 37), antimycobacterial

(14, 15), and antitumor (7, 20) responses of Vγ9Vδ2 T lymphocytes. This provides a method for increasing the number of effector/memory Vγ9Vδ2 T cells in vivo with a direct application in the area of immunotherapy of cancer and infectious diseases.

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Disclosures

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