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Article

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Antibacterial effect of human V γ 2V δ 2 T cells in vivo

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V γ 2V δ 2 cells, a class of T cells found only in primates, are reactive to nonpeptide organophosphate and alkylamine antigens secreted by bacteria and parasites. These cells make up 2–5% percent of human peripheral blood T cells but expand to make up 8–60% of peripheral blood T cells during bacterial and parasitic infections. We show here, using a chimeric severe combined immunodeficiency (SCID) mouse (hu-SCID) model, that human V γ 2V δ 2 T cells mediate resistance to extracellular gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli* and *Morganella morganii*) bacteria, as assessed by survival, body weight, bacterial loads, and histopathology. Surprisingly, this bacterial resistance was evident 1 day after infection, and bacteria were cleared well before $\gamma\delta$ T cell expansion was detected 6 days after infection. Decreased resistance in V δ 2 T cell-depleted hu-SCID mice correlated with decreased serum IFN- γ titers. Intravenous treatment of infected, reconstituted hu-SCID mice with pamidronate, a human V γ 2V δ 2 T cell-specific aminobisphosphonate antigen, markedly increased the in vivo antibacterial effect of V γ 2V δ 2 T cells. Therefore, this large pool of antigen-specific, yet immediately reactive memory human V γ 2V δ 2 T cells is likely to be an important mediator of resistance against extracellular bacterial infection and may bridge the gap between innate and acquired immunity.

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Introduction

Studies in mice suggest that $\gamma\delta$ T cells have diverse functions, mediating host defense in preventing lethal herpes simplex type 1-induced encephalitis (1) and providing protective immunity against malaria (2) and antibacterial effects in tuberculosis (3) and listeriosis (4). $\gamma\delta$ T cells also play a role in immunoregulation by ameliorating the severity of adjuvant arthritis (5), and enhancing immune tolerance in several model systems (6–9).

Several lines of evidence also implicate $\gamma\delta$ T cells as being important in human bacterial infections. Polyclonal V γ 2V δ 2 T cells expand in vivo, typically to comprise 8–60% of all peripheral blood T cells during a variety of bacterial diseases such as tuberculosis (mean, 14%) (10), salmonellosis (mean, 18%) (11), tularemia (mean, 31%) (12), brucellosis (mean, 29%) (13), listeriosis (mean, 12%) (14), and ehrlichiosis (mean, 57%) (15). However, there is no direct in vivo evidence showing antibacterial effects of human V γ 2V δ 2 T cells.

Found only in primates, human V γ 2V δ 2 T cells recognize, in a T cell receptor-dependent, MHC- and CD1-unrestricted manner, nonpeptide alkylamine and organophosphate antigens secreted by bacteria (16–18). The human pathogens *Morganella morganii* (18), *Escherichia coli*, *Yersinia enterocolitica* (19, 20), *Salmonella typhimurium* (11, 21), and *Listeria monocytogenes* (22, 23) produce the antigenic alkylamines isobutylamine (IBA; *S. typhimurium*, *Y. enterocolitica*, *E. coli*, *M.*

morganii), isoamylamine, and *n*-butylamine (*L. monocytogenes*), and these bacterial infections cause in vivo or in vitro $\gamma\delta$ T cell expansions in humans. In contrast to human $\gamma\delta$ T cells, murine $\gamma\delta$ T cells do not respond to nonpeptide alkylamine or organophosphate antigens; therefore, it is not at all certain that the biological functions of mouse $\gamma\delta$ T cells are the same as those in primates. Furthermore, the in vivo functions in humans of these alkylamines and organophosphate antigens are unknown. Here, using a chimeric SCID mouse model, we provide evidence that human V γ 2V δ 2 T cells are important mediators of natural immunity against extracellular bacterial infection. Furthermore, treatment or prophylaxis of human bacterial infections with V γ 2V δ 2-specific nonpeptide antigens either in vitro or in vivo can markedly enhance $\gamma\delta$ T cell-mediated antibacterial effects.

Methods

Ab and antigen reagents. The following mAb ascites were used against T cell antigens: control mAb (P3), pan- $\gamma\delta$ TCR (anti-TCR δ 1), V δ 1 (A13), V δ 1/J δ 1 (δ TCS1), V δ 2 (BB3), V γ 2 (7A5), and CD3 (OKT3). The specificity of these mAbs is reviewed in Porcelli et al. (24). Other reagents were purchased as follows: FITC-conjugated (Fab')₂ goat anti-mouse IgG (catalog number AMI4708; BioSource International, Camarillo, California, USA), IBA (catalog number I-3634; Sigma Chemi-

cal Co., St. Louis, Missouri, USA), pamidronate and etidronate (Novartis Pharmaceutical Corp., East Hanover, New Jersey, USA), mouse anti-human IFN- γ (catalog number 554698; PharMingen, San Diego, California, USA), mouse anti-human TNF- α (catalog number 18630D; PharMingen), and human TNF- α and IFN- γ (recombinant; National Cancer Institute, Frederick, Maryland, USA).

PBMCs. Human PBMCs obtained from random healthy donor leukopacks (Dana Farber Cancer Institute, Boston, Massachusetts, USA) were isolated by Ficoll-Hypaque centrifugation (Pharmacia Corp., Peapack, New Jersey, USA). PBMCs were screened for reactivity to V γ 2V δ 2 T cell antigens by culturing them at 1×10^6 cells per well in 24-well flat-bottom plates with or without IBA (0.4 mM) or pamidronate (5 μ M) in RPMI medium containing 10% FBS, 2 mM glutamine, 1 nM 2-mercaptoethanol, 100 IU of penicillin, and 100 IU of streptomycin at 37°C. On day 3, IL-2 was added to a concentration of 0.5 nM, and on day 10–12 the cells were counted and analyzed by flow cytometry using TCR V gene-specific mAbs. Before expansion in culture, the percentage of V δ 2 T cells ranged from 1 to 5% of T cells. PBMCs responding to IBA and pamidronate, as assessed by a 10- to 40-fold expansion of V γ 2V δ 2 T cells (around 90% of screened donors responded), were cryopreserved in FBS containing 10% dimethyl sulfoxide at -196°C until use.

SCID mice. All animal experimental protocols were approved by the internal review board of Brigham and Women's Hospital and Harvard Medical School. Homozygous C.B-Igh-1^b/Gbms-Prkdc(SCID)-Lyst(beige)N7 (SCID-beige) male mice or C.B-17 scid/scid (SCID) male mice, 5–6 weeks old, were purchased from Taconic Farms (Germantown, New York, USA) and maintained in microisolator cages. Animals were fed autoclaved food and water, and all manipulations were performed under laminar flow. One day prior to PBMC inoculation, SCID mice were injected with intraperitoneal rabbit anti-asialo GM1 Ab (25 μ l/mouse; Wako Chemicals USA Inc. Richmond, Virginia, USA), which depletes murine natural killer (NK) cells (25). Prior to PBMC engraftment, SCID mice were irradiated (3 Gy γ irradiation, ¹³⁷Cs source), which allows a high level of functional engraftment of human PBMCs (26). Since successful reconstitution of human PBMCs can be achieved in SCID-beige mice, which are deficient in NK cells, monocytes, and granulocytes, these animals were not treated with anti-asialo GM1 Ab and irradiation.

Treatment of PBMCs with V γ 2V δ 2-specific antigens and engraftment of SCID mice. PBMCs were cultivated in RPMI with either IBA (1 mM) for 24 hours at 37°C or 10 μ M of pamidronate for 30 minutes at room temperature. Then all cells were collected and washed twice with RPMI medium. Depletion of V γ 2V δ 2 T cells was performed using either mouse anti-human V δ 2 Ab (BB3) or P3, an isotype-matched mock control, and goat anti-mouse IgG Dynabeads M-450 (cat-

alog number 110.06; Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. For most deletions, P3, an isotype-matched control mAb, was substituted for the anti-V δ 2 mAb. More than 95% of V δ 2 T cells were depleted, as confirmed by surface marker staining and flow cytometry. We screened several donors by two-color fluorescence and found that 100% of V δ 2-bearing T cells also expressed V γ 2. Depletion of V γ 2 δ 2 T cells using anti-V δ 2 (BB3) mAb in these donors abrogated antibacterial effects in vivo. Since not every donor was screened by two-color fluorescence, we cannot formally rule out antibacterial effects mediated by V δ 2 T cells paired with V γ 1 in other donors. However, because this type of $\gamma\delta$ T cell is exceedingly rare and does not react with nonpeptide antigens, such an effect is highly unlikely. Groups of five SCID mice were injected with either 0.5 ml intraperitoneally or 0.1 ml intravenously RPMI medium containing 3×10^7 human PBMCs under aseptic conditions. In vivo activation of human V γ 2V δ 2 T cells was performed as follows. Two hours after PBMC reconstitution and *M. morganii* infection, each SCID mouse was given either an intraperitoneal injection of 1.0 ml pamidronate (20 μ M, 7.38 μ g/mouse) or an intravenous injection of 0.1 ml pamidronate (100 μ M, 10 mg/kg body weight). An equivalent amount of etidronate was given as a control. Pamidronate, but not etidronate, induced IL-2 release from a V γ 2V δ 2 TCR transfectant, demonstrating that its recognition is TCR-dependent. Neither pamidronate nor etidronate had any direct effects on bacterial growth in vitro (data not shown).

Bacterial infection. *M. morganii* (formerly known as *Proteus morganii*) strain 235 (National Collection of Type Cultures, London, United Kingdom), *E. coli* (number 25922; American Type Culture Collection, Manassas, Virginia, USA), or *Staphylococcus aureus* (number 25923; American Type Culture Collection) were grown in LB broth at 37°C until the culture reached early stationary phase. Freshly grown *M. morganii* was used. *E. coli* and *S. aureus* were divided into aliquots (1 ml/vial) and stored in LB broth containing 10% glycerol at -80°C until use. Prior to infection, bacteria were washed once with 30 ml of PBS (three washes for *S. aureus*) and plated on LB agar to determine number of CFUs. Each SCID mouse was inoculated with either 0.5 ml intraperitoneal or 0.1 ml intravenous PBS containing the indicated bacterial CFUs (3×10^7 *M. morganii*, 5×10^6 to 3×10^7 *E. coli*, or 2×10^6 *S. aureus*). At the indicated timepoints after infection, peritoneal lavage, liver (right median lobe), lung (right lobes), and spleen were harvested, and the organs were individually homogenized in 3 ml of PBS. There was no significant difference between groups in the weight of the same organ to be homogenized. Each organ was processed separately; the organs were not pooled. Bacteria were enumerated by plating serial tenfold dilutions in water on LB agar plates. After overnight culture at 37°C, bacterial colonies were counted.

Stimulation of PBMCs with dead bacteria in vitro. PBMCs were washed twice after a 24-hour preincubation with 1 mM IBA. Dead *E. coli* or *M.morganii* (inactivated at 56°C for 2 hours) were added to each well. Final concentrations were: *E. coli*, 5×10^5 CFU/ml, *M.morganii*, 5×10^6 CFU/ml. At the indicated timepoints, the culture supernatant was collected for analysis of IFN- γ levels by ELISA, and PBMCs were analyzed by flow cytometry to quantitate V γ 2V δ 2 T cell expansion.

Detection of human IFN- γ in the sera of reconstituted SCID mice and tissue culture supernatant. Human IFN- γ ELISA was performed according to procedures recommended by the manufacturer (catalog number 2613KI; PharMingen). There were no Ab cross-reactions with mouse IFN- γ , according to the manufacturer. The detection limit of the assay was 4.7 pg/ml.

Intracellular cytokine staining. Human PBMCs harvested from the peritoneal lavage of hu-SCID mice were washed twice with RPMI medium containing antibiotics. The cells were cultured in RPMI medium containing monensin (GolgiStop; PharMingen) for 4 hours at 37°C, which enhanced intracellular cytokine accumulation. Cells were washed with PBS and stained with surface marker, either AlexaFluor-conjugated 488 IgG control Ab or pan-TCR δ 1 (antibodies were purified and conjugated by our laboratory). After two washes, cells were fixed with 2% formaldehyde in PBS and permeabilized with 0.5% (wt/vol) saponin (PharMingen). Intracellular IFN- γ was stained with phycoerythrin-conjugated Ab (catalog number 18905A; PharMingen) in saponin buffer. After two washes, cells were resuspended in PBS and analyzed using a FACS flow cytometer (Becton Dickinson and Co., Franklin Lakes, New Jersey, USA) and FlowJo software (Tree Star Inc., San Carlos, California, USA).

Monocyte bactericidal assay. The assay was performed as previously described (27). After depletion or mock depletion of V γ 2V δ 2 T cells, human PBMCs (1×10^6 cells) in 1 ml RPMI 1640 containing either 1 mM IBA or 10 μ M pamidronate were cultivated in 24-well tissue culture plates for 4 days in the presence or absence of either anti-IFN- γ Ab (end concentration: 1 μ g/ml) or IFN- γ (end concentration: 100 IU/ml). The cells were washed three times with balanced salt solution (phosphate-buffered BSS) and resuspended in 0.65 ml BSS. Fifty microliters of ice-cold normal human serum and 0.3 ml of *E. coli* or *M.morganii* (7.5×10^6 CFU/ml, middle stationary phase) were added to each well. After cultivation for 20 minutes at 37°C, cells were washed 4–6 times with 2 ml of ice-cold BSS and then centrifuged at 200 g for 8 minutes. One plate was designated for time 0, and Triton X-100 (1.5% in PBS) was added to release bacteria from the monocytes. Other plates were designated for 60 minutes (*E. coli*) and 120 minutes (*M.morganii*): BSS (950 μ l) and normal human serum (50 μ l) were added to each well and incubation was continued for 60 minutes or 120 minutes at 37°C to allow for monocyte killing of bacteria prior to adding Triton X-100. Bacteria were diluted serially with water, plated on LB agar, and cultivated overnight at 37°C.

Histopathology. Mouse tissues were visualized histologically from formalin-fixed and paraffin-embedded hematoxylin and eosin.

Statistics. Values were expressed as mean \pm SEM of the respective test or control group. Statistical significance was calculated between control and test groups using the Student's two-tailed *t* test, and among different groups by ANOVA. Data were representative of two to four experiments.

Results

Depletion of V δ 2 T cells exacerbates bacterial infection. To determine whether human $\gamma\delta$ T cells mediate antibacterial effects in vivo, we reconstituted SCID mice with human PBMCs that were either mock-depleted or depleted of $\gamma\delta$ T cells, and challenged the mice with *E. coli* or *M.morganii*, two gram-negative bacteria that are important causes of urinary tract infections and urosepsis. SCID mice receiving a lethal dose of *E. coli* (1×10^7 CFUs) alone all died of infection within 2 days, whereas SCID mice receiving a lethal dose of bacteria and human PBMCs all survived (Figure 1a). These data suggest that human PBMCs play a crucial role in protecting against bacterial infection, and that residual mouse immune cells have negligible effects. This hu-SCID infection model enables us to study further the antibacterial function of human V γ 2V δ 2 T cells. Six of ten SCID mice reconstituted with human PBMCs depleted of V δ 2 T cells died of infection, whereas only one of ten SCID mice reconstituted with human PBMCs that had been mock-depleted of V δ 2 T cells died ($P = 0.0307$, Figure 1b). In addition, in contrast to the SCID mice reconstituted with mock-depleted human PBMCs, SCID mice reconstituted with human PBMCs depleted of V δ 2 T cells had up to a 2 log₁₀ increase in bacterial CFUs in the peritoneal lavage 5 days after infection (Figure 1c). These results strongly suggest that human V γ 2V δ 2 T cells mediate antibacterial activity against *E. coli* and *M.morganii*.

Antibacterial effects of V γ 2V δ 2 T cells are enhanced by live bacterial products. Bacteria such as *M.morganii* (18), *E. coli* (21), *Y. enterocolitica* (21), *S. typhimurium* (11, 21), and *L. monocytogenes* (22, 23) stimulate V γ 2V δ 2 T cell expansion, and secrete IBA and other alkylamine antigens at concentrations up to 4 mM, whereas dead bacteria do not produce IBA (18). IBA is recognized in vitro by human V γ 2V δ 2 T cells in a TCR-dependent, MHC- and CD1-unrestricted manner (18). However, the in vivo biological functions of alkylamine antigens are unknown. We speculated that these alkylamine antigens, which are secreted by live bacteria and cannot be recognized by mouse $\gamma\delta$ T cells, regulate the antibacterial effects of human V γ 2V δ 2 T cells in vivo.

To determine whether stimulation of PBMCs with the live bacterial product IBA could enhance the antibacterial effect of PBMCs in vivo, we reconstituted SCID mice with IBA-pretreated (1 mM) PBMCs that had been washed prior to inoculation. SCID mice reconstituted with IBA-pretreated PBMCs had a lower

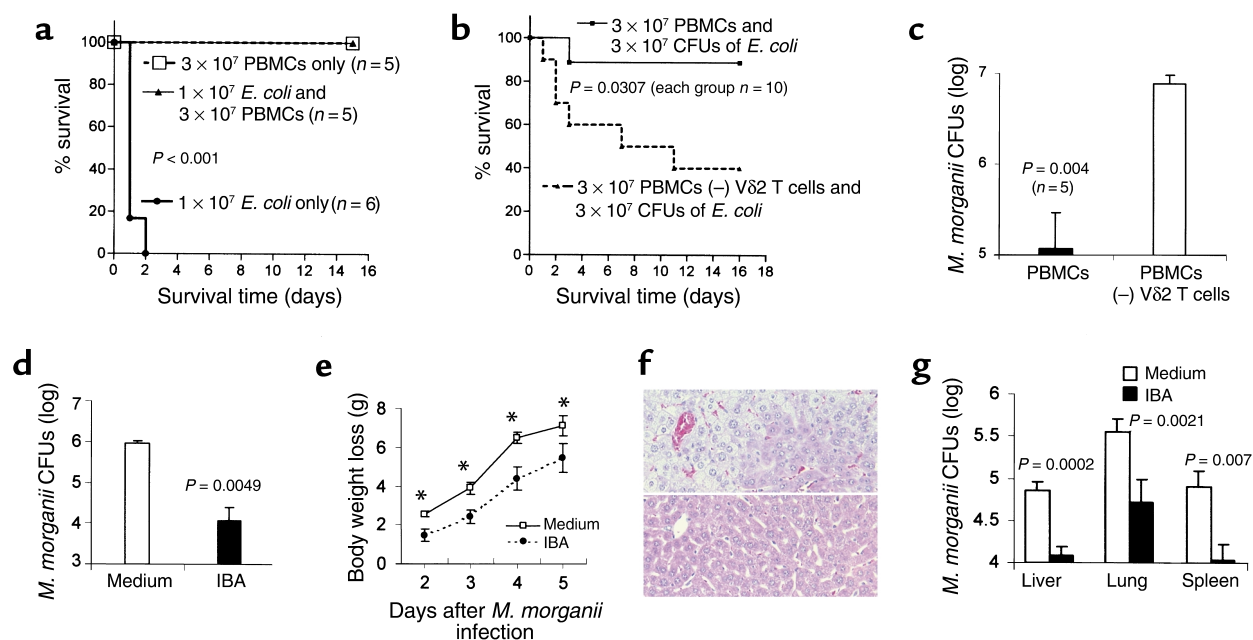


Figure 1

Depletion of V δ 2 T cells exacerbated bacterial infection (a–c), whereas live bacterial product augmented the antibacterial effects of V γ 2V δ 2 T cells (d–g). (a) All SCID-beige mice infected with a lethal dose of *E. coli* (1×10^7 CFUs, administered intraperitoneally) were dead within 2 days, whereas all mice reconstituted with human PBMCs survived this lethal infection. (b) Six of ten SCID-beige mice reconstituted with human PBMCs depleted of V δ 2 T cells died of *E. coli* infection (3×10^7 CFUs, administered intraperitoneally); in contrast, only one of ten mice receiving mock-depleted PBMCs died. (c) Reconstitution of SCID mice with intraperitoneal PBMCs depleted of V δ 2 T cells resulted in higher bacterial loads in the peritoneal lavages of these mice 5 days after intraperitoneal inoculation of *M. morgani* (3×10^7 CFUs). (d) SCID mice ($n = 5$ for each group) reconstituted intraperitoneally with PBMCs pretreated with IBA, a natural V γ 2V δ 2 T cell-specific antigen secreted by bacteria, had lower numbers of bacterial CFUs in the peritoneal lavage (e) less loss of body weight (* $P < 0.05$), and (f) markedly less liver degeneration than those reconstituted intraperitoneally with medium-pretreated PBMCs 5 days after intraperitoneal infection with 3×10^7 CFUs of *M. morgani* ($\times 200$, hematoxylin and eosin staining). Bottom panel: IBA group. Top panel: medium control. (g) SCID mice reconstituted intraperitoneally with IBA-pretreated PBMCs (IBA) had lower numbers of bacterial CFUs than those receiving medium-pretreated PBMCs (Medium) 5 days after intravenous infection with 3×10^7 CFUs of *M. morgani*.

number of bacterial CFUs (nearly 2 log₁₀ fewer), lost less body weight, and had milder hepatocyte degeneration than those reconstituted with mock-pretreated PBMCs (Figure 1, d–f). In vitro, human V γ 2V δ 2 T cells expanded and produced cytokines in response to stimulation only with IBA, not with dead bacteria and LPS (our unpublished observations).

IBA did not activate $\alpha\beta$ T cells, NK cells, NK T cells, or monocytes, and did not show toxicity at concentrations of less than 2 mM (data not shown). Thus, the live bacterial product IBA specifically activated V γ 2V δ 2 T cells, which enhanced the antibacterial activity of human PBMCs in vivo.

Intraperitoneal inoculation with both PBMCs and *M. morgani* might be regarded as creating only local and not systemic antibacterial effects. We therefore reconstituted mice with PBMCs intraperitoneally and inoculated with *M. morgani* intravenously. SCID mice receiving IBA-pretreated PBMCs had up to tenfold fewer bacterial CFUs than those receiving medium-pretreated PBMCs (Figure 1g), indicating that the PBMCs were mediating a systemic antibacterial effect. Thus, in this model, human V γ 2V δ 2 T cells play a very important role against bacterial infection, and this effect is augmented in vivo by the live bacterial product IBA.

Treatment with V γ 2V δ 2 T cell-specific antigens in vivo enhanced the antibacterial effect of PBMCs. Since the natural V γ 2V δ 2 T cell-specific antigen IBA enhanced the antibacterial effect of PBMCs in vivo, we speculated that pharmaceutical V γ 2V δ 2 T cell-specific antigens (28, 29) such as pamidronate, alendronate, or risedronate, may have similar effects. Like IBA, these potent aminobisphosphonate antigens stimulate V γ 2V δ 2 T cells in a TCR-dependent, MHC- and CD1-unrestricted manner (30, 31), and have a proven track record of safety (32, 33).

We reconstituted SCID mice intraperitoneally with untreated PBMCs, and infected the mice intravenously with *M. morgani*. Two hours after infection, when the mice were clinically ill, pamidronate was injected either intravenously (Figure 2a) or intraperitoneally (data not shown), and the mice were sacrificed on day 4 to quantitate bacterial CFUs in their organs. Reconstituted SCID mice treated with the antigen pamidronate appeared much healthier and had up to 2.5 log₁₀ fewer bacterial CFUs in their spleens than those treated with the antigenically inactive bisphosphonate etidronate (Figure 2a). We also treated the reconstituted SCID mice with pamidronate 2 hours prior to *M. morgani* infection, and produced similar results (data not

shown). Neither pamidronate nor etidronate had direct antibacterial effects *in vitro*, and neither drug was toxic to cultured human PBMCs at a concentration fivefold higher than that used in this study. Thus, treatment of reconstituted SCID mice that were clinically ill from *M. morgani* infection, with an antigen specific for V γ 2V δ 2 T cells, had a remarkable systemic antibacterial effect.

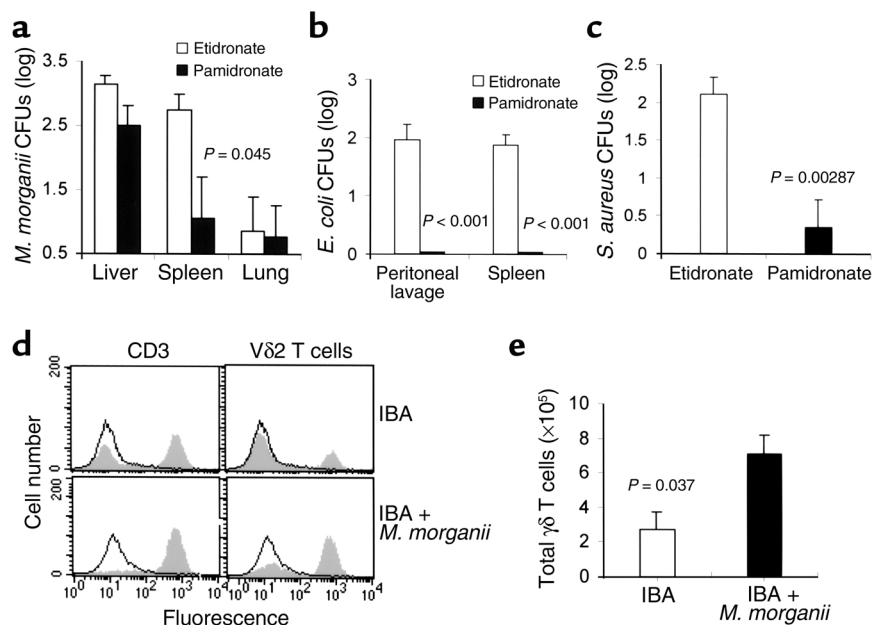
Previous studies have demonstrated that human $\gamma\delta$ T cells are expanded *in vivo* and *in vitro* by a variety of gram-positive and gram-negative bacterial infections (reviewed in ref. 34), prompting us to investigate the protective role of human $\gamma\delta$ T cells against other bacteria. Pretreatment of PBMCs with pamidronate rendered these PBMCs up to 100-fold more effective in mediating antibacterial effects in reconstituted SCID mice infected with gram-negative *E. coli* (Figure 2b) or gram-positive *S. aureus* (Figure 2c), than infected SCID mice reconstituted with PBMCs pretreated with etidronate. Taken together, these data strongly suggest that pamidronate and other V γ 2V δ 2 T cell antigens might be useful as prophylaxis or treatment *in vivo* for both gram-positive and gram-negative extracellular bacterial infection.

Expansion of human $\gamma\delta$ T cells in SCID mice. To examine the fate of T cells in this *in vivo* model, SCID mice reconstituted with IBA-pretreated PBMCs were mock-infected or infected with *M. morgani*, and then sacrificed on day 7 for determination of the number of peritoneal $\gamma\delta$ T cells by flow cytometry. $\gamma\delta$ T cells had not expanded in the peritoneal cavity by days 3–4 after infection (data not shown). By 7 days after infection, human V γ 2V δ 2 T cells in the peritoneal lavage of the

infected hu-SCID mice comprised 72% of CD3⁺ T cells (Figure 2d). There were an average of 7.2×10^5 human $\gamma\delta$ T cells in the peritoneal cavities of the infected mice, compared with 2.9×10^5 cells in the uninfected mice (Figure 2, d and e). Human V γ 2V δ 2 T cells in the blood of the infected hu-SCID mice comprised 19.42% of CD3⁺ T cells, compared with 7.3% in the blood of the mock-infected mice. The V γ 2V δ 2 T cells were also detectable from other organs of infected SCID mice, such as liver, spleen, and bone marrow, by 7 days after infection. In contrast, it was difficult to find these cells in the organs of uninfected mice. Since the SCID mice received PBMCs that contained approximately 2.5×10^5 $\gamma\delta$ T cells (approximately 2% of CD3⁺ T cells), we estimate that there was an increase of less than 2.5-fold in absolute numbers of human $\gamma\delta$ T cells in infected mice by 7 days after infection, compared with uninfected mice. By 12 days after infection, most human PBMCs had migrated to the mouse spleen. There was a mean total of 13.4×10^6 human V γ 2V δ 2 T cells found in the spleen of each infected hu-SCID mouse, whereas a mean of only 1.8×10^6 $\gamma\delta$ T cells were found in the spleens of mock-infected hu-SCID mice. Similar results were observed in expansion of V γ 2V δ 2 T cells when mice were intravenously reconstituted with PBMCs and intraperitoneally infected with *M. morgani*, or vice versa, or both were inoculated intraperitoneally. Thus, bacterial infection resulted in the expansion of $\gamma\delta$ T cells in this *in vivo* hu-SCID mouse model, reflecting the expansion seen in peripheral blood during infection in man.

Figure 2

Treatment with aminobisphosphonate antigen but not bisphosphonate *in vivo* enhanced the antibacterial effect of PBMCs (a–c); human V γ 2V δ 2 T cells expanded in SCID mice (d, e). SCID mice intraperitoneally reconstituted with PBMCs and treated with intravenous pamidronate (10 mg/kg body weight), a V γ 2V δ 2 T cell-specific aminobisphosphonate antigen, 2 hours after infection with intravenous *M. morgani* (3×10^7 CFUs), had fewer liver and spleen CFUs than did reconstituted, infected SCID mice treated with etidronate (10 mg/kg body weight), an antigenically inactive bisphosphonate analogue of pamidronate. (a) Four days after infection, SCID mice ($n = 5$) reconstituted intraperitoneally with PBMCs pretreated with pamidronate had lower numbers of CFUs than those reconstituted with etidronate-pretreated PBMCs 27 hours after intraperitoneal infection with *E. coli* (5×10^6 CFUs) (b) or 17 hours after infection with *S. aureus* (2×10^6 CFUs) (c); $n = 5$ for each group. (d) Flow cytometry profile, and (e) absolute numbers of V γ 2V δ 2 T cells in the peritoneal lavage: Human V γ 2V δ 2 T cells in the peritoneal lavage of SCID mice ($n = 5$ in each group) reconstituted intraperitoneally with IBA-pretreated PBMCs were expanded by day 7 after intraperitoneal infection with 3×10^7 CFUs of *M. morgani* (IBA + *M. morgani*), compared with peritoneal lavage V γ 2V δ 2 T cells from reconstituted SCID mice that were mock-infected (IBA). Each SCID mouse was reconstituted with PBMCs that contained 2.5×10^5 $\gamma\delta$ T cells. Data were representative of three experiments.



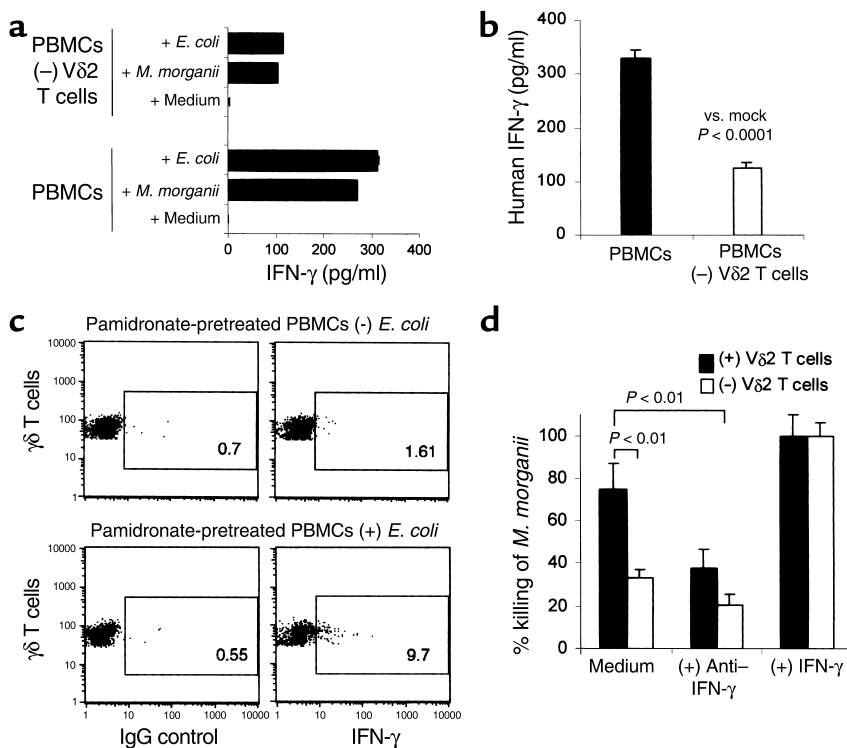


Figure 3

Vδ2 T cells and IFN-γ play a crucial role in monocyte-mediated killing of extracellular bacteria. IBA-pretreated PBMCs exposed in vitro to dead bacteria for 17 hours induced γδ T cell-dependent IFN-γ secretion (a). At 17 hours after intraperitoneal infection with *E. coli* (5×10^6 CFUs), SCID mice ($n = 5$ in each group) reconstituted intraperitoneally with IBA-pretreated, mock-depleted PBMCs had higher levels of serum human IFN-γ than did mice reconstituted with IBA-pretreated PBMCs depleted of Vδ2 T cells (b). Intracellular IFN-γ was produced by 9.7% of γδ T cells harvested from the peritoneal lavage of SCID mice that were reconstituted intraperitoneally with pamidronate-pretreated PBMCs (or IBA-pretreated PBMCs, data not shown) and infected intraperitoneally with *E. coli* (5×10^6 CFUs) (c, bottom), while only 1.6% of γδ T cells harvested from reconstituted and mock-infected SCID mice produced IFN-γ (c, top). Monocytes from PBMC cultures that were depleted of Vδ2 T cells killed less than half as many bacteria than did monocytes from mock-depleted cultures. Inclusion of neutralizing mAbs to IFN-γ in the culture abrogated the γδ T cell-dependent monocyte-mediated killing of *M. morgani*. Addition of IFN-γ to the Vδ2 T cell-depleted culture completely reconstituted the antibacterial effect lost by depletion of Vδ2 T cells (d). Use of pamidronate-pretreated PBMCs resulted in data similar to that obtained using IBA-pretreated PBMCs (data not shown). Data were representative of two to four experiments.

IFN-γ production is partially dependent on Vδ2 T cells.

Exposure of Vγ2Vδ2 T cells to bacteria or nonpeptide bacterial antigens results in secretion of IFN-γ, a cytokine that is critically important in controlling bacterial infections (35–37). Since we found that γδ T cells could mediate antibacterial effects in as few as 17 hours after infection (Figure 2c), we examined the role of Vγ2Vδ2 T cells in the IFN-γ response to bacteria. PBMCs were either mock-depleted or depleted of Vδ2 T cells, primed with one of the Vγ2Vδ2 T cell antigens (IBA or pamidronate), and exposed to either heat-killed *E. coli* or heat-killed *M. morgani*. After 17 hours, the IFN-γ levels in the supernatants were determined by ELISA. In response to bacteria, PBMCs depleted of Vδ2 T cells produced titers of IFN-γ that were threefold lower than

Crucial role of Vδ2 T cells and IFN-γ in monocyte-mediated killing of bacteria.

One of the dominant mechanisms for eliminating extracellular bacteria is dependent on monocyte-mediated killing, and IFN-γ is an important activator of monocytes (35–37). Therefore, we tested the ability of human γδ T cells to influence monocyte-mediated killing of *M. morgani* in vitro. Mock-depleted PBMCs or those depleted of Vδ2 T cells were cultured for 4 days in medium containing the live bacterial product IBA. Monocytes from these cultures were then analyzed for their ability to kill *M. morgani*. Monocytes from PBMC cultures that were mock-depleted of Vδ2 T cells killed more than twice as many bacteria as did monocytes from cultures depleted of Vδ2 T cells. Inclusion of neutralizing mAbs to IFN-γ during the 4-day

those of mock-depleted PBMCs, showing that optimum production of IFN-γ is critically dependent on Vδ2 T cells (Figure 3a).

These in vitro observations were confirmed in vivo, since SCID mice reconstituted with Vγ2Vδ2 T cell-specific antigen-pretreated PBMC had 3-fold higher levels of serum IFN-γ 17 hours postinfection with *E. coli*, as compared to SCID mice reconstituted with Vγ2Vδ2 T cell-specific antigen-pretreated PBMC that were first depleted of Vδ2 T cells (Figure 3b). *E. coli* infection of SCID mice reconstituted with the pretreated PBMCs induced 9.7% of γδ T cells recovered by peritoneal lavage to express intracellular IFN-γ, whereas only 1.6% of γδ T cells from the peritoneal lavage of mock-infected, reconstituted SCID mice expressed intracellular IFN-γ (Figure 3c). It is important to emphasize that neither pamidronate nor IBA had any effect on antibacterial activity or IFN-γ production in the absence of Vδ2 T cells, showing that in this experimental system, the actions of these antigens are Vδ2 T cell-dependent. The data suggest that live bacterial infection in vivo results in IFN-γ production by human Vγ2Vδ2 T cells. Taken together, these data also suggest that IFN-γ production that is at least partially dependent on Vδ2 T cells may be important in mediating antibacterial activity during the first day of bacterial infection in vivo.

culture abrogated the V δ 2 T cell-dependent monocyte-mediated killing of *M. organii* (Figure 3d), while the addition of IFN- γ to the cultures that were depleted of V δ 2 T cells completely reconstituted monocyte-mediated killing (Figure 3d). Similar results were obtained with *E. coli* (data not shown). These data suggest that IFN- γ is sufficient to reconstitute $\gamma\delta$ T cell-dependent antibacterial activity.

Discussion

The discovery that marked expansion of $\gamma\delta$ T cells occurs in the peripheral blood of patients with bacterial infection first raised the possibility that these T cells play a role in resistance to bacteria (11, 14). This *in vivo* expansion was repeated *in vitro* by mixing human PBMCs with bacterial extracts containing nonpeptide antigens that were found to be responsible for expansion of V γ 2V δ 2 T cells in a TCR-dependent manner (16–18, 38, 39). Furthermore, these expanded $\gamma\delta$ T cells kill target cells infected with bacteria (40, 41). Abundant evidence from mouse models also suggests that $\gamma\delta$ T cells play a very important role in bacterial infection. However, the fact that mouse $\gamma\delta$ T cells do not recognize nonpeptide alkylamine or organophosphate antigens secreted by live bacteria raises questions as to whether human $\gamma\delta$ T cells play a similar role. Furthermore, it is unknown whether these nonpeptide antigens regulate V γ 2V δ 2 T cell functions *in vivo*. To address these questions, we developed a chimeric hu-SCID model for studying the effects of human $\gamma\delta$ T cells against bacterial infection.

The hu-SCID model has been used widely, and has proven to be a powerful model for study of human cells and tissues (42–46). The fact that SCID mice receiving *E. coli* infection alone were all dead within 2 days, whereas inoculation with human PBMCs rescued all SCID mice from lethal infection (Figure 1a), demonstrates that inoculated human PBMCs give rise to antibacterial effects. Therefore, the data indicating that hu-SCID mice reconstituted with human PBMCs depleted of V δ 2 T cells had a lower survival rate (Figure 1b) and higher bacterial load (Figure 1c) most likely reflect the function of human V γ 2V δ 2 T cells *in vivo*. Depletion of V δ 2 T cells markedly decreased, but did not completely abrogate PBMC-mediated resistance to bacterial infection *in vivo* or monocyte-mediated bactericidal effects *in vitro* (Figures 1, 2 a–c, and 3d), suggesting that other immune cells contribute to antibacterial effects. In contrast to LPS and dead bacteria, the V γ 2V δ 2 T cell-specific antigen IBA did not activate monocytes, $\alpha\beta$ T cells, NK cells, or NK T cells (data not shown), suggesting that distinct antigens orchestrate the responses of different immunologically active cells in host resistance to bacterial infection.

There was no significant expansion of $\gamma\delta$ T cells *in vivo* without antigen pretreatment. Nonetheless, untreated PBMCs that were mock-depleted of V δ 2 T cells provided more protection against infection than did PBMCs that were depleted of V δ 2 T cells (Figure

1b). In general, we obtained better $\gamma\delta$ T cell-dependent protection when the PBMCs were pretreated with specific antigen, and therefore we performed most experiments using antigen pretreatment. Consistent with the more potent *in vivo* protection using primed PBMCs, our unpublished data suggest that $\gamma\delta$ T cells in humans may require exposure to dietary and ambient $\gamma\delta$ T cell antigens *in vivo* to enable optimum reactivity in response to bacterial stimulation (Kamath et al., our unpublished observations).

Consistent with the data in clinically infected humans (10–15), our SCID mouse model showed that V γ 2V δ 2 T cells started expanding from day 6 or 7 after bacterial infection to comprise 72% of CD3⁺ T cells in the peritoneal lavage (Figure 2d). Since it is possible that a high percentage of V γ 2V δ 2 T cells might result from a compositional change instead of a cellular expansion, we calculated the absolute numbers of V γ 2V δ 2 T cells harvested from the peritoneal lavage. There were up to 2.5-fold more V γ 2V δ 2 T cells in the peritoneal lavage of hu-SCID mice infected with bacteria than in the peritoneal lavage in uninfected hu-SCID mice (Figure 2e). Whereas the absolute numbers of V γ 2V δ 2 T cells in peritoneal lavage represented only a proportion of these cells, they were detectable in hu-SCID mouse liver, spleen, blood, bone marrow, and other organs of infected mice, suggesting the possibility that these $\gamma\delta$ T cells could have local effects, perhaps anti-inflammatory in nature, in these organs at 7 days after infection.

Whereas this *in vivo* expansion of human $\gamma\delta$ T cells in reconstituted, infected SCID mice did not occur until 7 days after infection (Figure 2, d and e), the antibacterial effect of human $\gamma\delta$ T cells in our studies was evident as soon as 17 hours after infection (Figure 2c), indicating that expansion of $\gamma\delta$ T cells is not required for an antibacterial response. These results are rather surprising, since it is logical to speculate that expansion of $\gamma\delta$ T cell numbers occurs for the purpose of subsequently eliminating bacteria. However, a previous report using a mouse model showed that $\gamma\delta$ T cell-mediated antibacterial effects against *E. coli* occur before $\gamma\delta$ T cell expansion (47).

Although only 3–15% of V γ 2V δ 2 T cells produced cytokines in response to stimulation with IBA (our unpublished observations), these $\gamma\delta$ T cells are much more efficient at cytokine production on a per-cell basis than are $\alpha\beta$ T cells (48). Furthermore, these V γ 2V δ 2 T cells, which are reactive to nonpeptide antigens, are 5- to 50-fold more numerous than $\alpha\beta$ T cells that are specific for any one peptide, and are thus likely to have a major antibacterial impact. *In vitro*, V γ 2V δ 2 T cells directly produced cytokines, and also were necessary for optimum IFN- γ production by PBMCs, which was critical for monocyte-mediated killing of bacteria (Figure 3, a, c, and d). *In vivo*, SCID mice reconstituted with human PBMCs depleted of V δ 2 T cells had lower serum levels of human IFN- γ (Figure 3b), higher mortality (Figure 1b), and higher bacterial loads (Figure 1c) than those receiving PBMCs mock-depleted of V δ 2 T cells.

Besides depletion of fresh V γ 2V δ 2 T cells from PBMCs, other approaches to demonstrate V γ 2V δ 2 T cell functions *in vivo* are to use either purified fresh human V γ 2V δ 2 T cells or V γ 2V δ 2 T cell lines and clones. However, the requirement for large amounts of human PBMCs for reconstitution, technical difficulties in purifying a small proportion of $\gamma\delta$ T cells from whole PBMCs by negative selection, and the unclear fate of $\gamma\delta$ T cells after positive selection make the use of purified V γ 2V δ 2 T cells less practical. V γ 2V δ 2 T cell lines and clones, unlike fresh human V γ 2V δ 2 T cells, might have different functions. In a mouse model, expanded mouse $\gamma\delta$ T cells kill monocytes during the late stages of infection (49, 50). In contrast, we found in our human studies that $\gamma\delta$ T cells, before they are expanded, are necessary for optimum activation of monocytes, illustrating the potential functional differences of expanded and recently stimulated $\gamma\delta$ T cells.

Consistent with mouse work, we found that *in vitro*, certain human V γ 2V δ 2 T cell lines and clones showed different cytokine profiles from fresh V γ 2V δ 2 T cells. These distinctions between fresh and expanded $\gamma\delta$ T cells may explain why transfer into SCID mice of expanded V γ 2V δ 2 T cell lines failed to mediate antibacterial activity (our unpublished observation). Perhaps these expanded $\gamma\delta$ T cells have a regulatory or anti-inflammatory function later in infection that is distinct from antibacterial activity (50, 51), or they are relatively refractory to antigenic stimulation (51) and lack CD28 (52). Alternatively, expanded $\gamma\delta$ T cells may be important in resistance to intracellular bacterial infections, which tend to last longer than extracellular infections.

V γ 2V δ 2 T cells may be considered part of the adaptive immune system, in that they have a memory phenotype, junctionally diverse TCRs that require gene rearrangement for their cell surface expression, and the ability to become either anergic or expanded, depending on the availability of costimulation (18, 53). On the other hand, V γ 2V δ 2 T cells also may be considered part of the innate immune response, since their frequently paired TCR variable region genes V γ 2 and V δ 2 reflect limited germline gene diversity. This V gene pairing enables each V γ 2V δ 2 TCR to immediately recognize families of unprocessed antigens with conserved molecular patterns, such as the alkylamines and prenyl pyrophosphates. This pattern recognition by the V γ 2V δ 2 TCR allows cytokine secretion by large numbers of memory $\gamma\delta$ T cells in response to a diverse array of bacterial species. These large numbers of memory T cells that are capable of responding within 2 hours to alkylamine and organophosphate antigens produced by microbes thus may bridge the gap between innate and adaptive immune responses.

Bacterial resistance to antibiotics used prophylactically and empirically is a serious problem that requires new approaches. Our data show that the synthetic aminobisphosphonate, pamidronate, a drug with a proven track record of safety (32, 33), provides prophylaxis and treatment against extracellular bacterial infec-

tion in a V γ 2V δ 2 T cell-dependent manner. Based on our findings, synthetic V γ 2V δ 2 T cell-specific antigens could potentially be used in humans to treat or prevent bacterial infection, as an alternative to antibiotics.

In summary, these data suggest that V γ 2V δ 2 T cells are likely to play an important role in mediating natural resistance to gram-positive and gram-negative extracellular bacterial infections in humans. Administration of antigens specifically recognized by this population of human $\gamma\delta$ T cells may be useful in the prevention and treatment of bacterial infection in humans.

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