results are consistent with this model, but in addition demonstrate that ongoing TCR triggering is required to sustain the signaling process. We have shown here that CD28 participates in the organization of the immune synapse by recruiting rafts into it (*23*). The fact that cross-linking of the rafts produced levels of costimulation comparable to that of CD28 engagement suggests that the costimulatory effect of CD28 in resting T cells is mediated to a large extent by its effect on raft redistribution.

Recent evidence suggests that membrane compartmentalization between rafts and nonrafts is required for efficient T cell activation (*24*). The recruitment of rafts to the site of TCR engagement may represent a general mechanism by which costimulation can increase the signaling process. This may result from increased recruitment of kinases and segregation of phosphorylated substrates from phosphatases (*25*).

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7. Human peripheral blood $CD4^+$ T cells were sorted by negative selection. Polystyrene Latex microsheres (Polysciences) were coated with various concentrations of anti-CD3 (clone TR66) in phosphate-buffered saline, followed by coating with anti-CD28 (clone CD28.1) at 10 μg/ml. T cells (5×10^4) were cultured with 10^5 beads in 200 μl of RPMI 10% fetal calf serum in round-bottom microplates. TCR down-regulation was measured after 4 hours and thymidine incorporation after 72 hours as described (*2*). Costimulation was not observed when anti-CD3 and anti-CD28 were presented on separate beads nor when antibodies to major histocompatibility complex (MHC) class I was used instead of anti-CD28 (A. Viola, unpublished data). To detect tyrosine phosphorylation, 10^6 T cells and 10^6 beads were centrifuged and the sediment was incubated for 5 min at 37°C in 100 μl of culture medium, followed by lysing for 30 min at 4°C in 1% NP40 buffer [20 mM tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA] in the presence of protease and phosphatase inhibitors [aprotinin (Sigma, 10 μg/ml), leupeptin (Sigma, 10 μg/ml), 1 mM Pefabloc-SC (Boehringer Mannheim), 50 mM NaF, 10 mM $Na_4P_2O_7$, and 1 mM $NaVO_4$]. Immunoblottings were performed as described with anti-phosphotyrosine mono-clonal antibody 4G10 (*26*).
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19. T cells were stimulated in tissue culture wells that had been coated with anti-CD3 at various concentrations followed by addition of antibodies to CD28 (CD28.1), CD59 (p282, Pharmingen), MHC class I (W6/32) (all at 10 μg/ml), or CTx (Sigma, 20 μg/ml).
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Transduction of Human $CD34^+$ Cells That Mediate Long-Term Engraftment of NOD/SCID Mice by HIV Vectors

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Efficient gene transfer into human hematopoietic stem cells (HSCs) is an important goal in the study of the hematopoietic system as well as for gene therapy of hematopoietic disorders. A lentiviral vector based on the human immunodeficiency virus (HIV) was able to transduce human $CD34^+$ cells capable of stable, long-term reconstitution of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. High-efficiency transduction occurred in the absence of cytokine stimulation and resulted in transgene expression in multiple lineages of human hematopoietic cells for up to 22 weeks after transplantation.

Human HSCs are an attractive target for gene therapy of inherited hematopoietic disorders as well as other acquired disorders because these cells have the ability to regenerate the entire hematopoietic system. A number of in vitro assays have been established to detect pluripotent human hematopoietic cells (*1*). However, these in vitro assays are unable to evaluate the long-term in vivo repopulating capacity that is a hallmark of HSCs. The NOD/SCID mouse (*2*) has been used to evaluate human HSCs in vivo (*3*). $CD34^+$ primitive cells that have the capacity to initiate long-term multilineage engraftment in these

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mice have been operationally defined as SCID-repopulating cells (SRCs) (*4*).

Retroviral vectors, derived from oncoretroviruses such as the murine leukemia virus (MLV), have been the most widely used vectors for gene transfer because the vector genome integrates into the chromosomes of target cells, resulting in stable expression of transgenes (*5*). Although retroviral vectors have proven efficient for transducing mouse HSCs, this finding has not been easily translatable to large animals or humans (*6*). This lack of success possibly reflects the quiescent nature of human HSCs and the requirement of cell division for retroviral integration. Although retroviral transduction is efficient for human hematopoietic progenitor cells that have been stimulated to divide by cytokines, exposure to cytokines can lead to differentiation of the HSCs, possible loss of homing abilities, and probable reductions in long-term repopulating capacity (*7*).

We and others have developed HIV vectors that can transduce nondividing cells (*8*, *9*). The HIV vector was pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV-G) to ensure a broad host range and facilitate concentration of virus to high titers. HIV vectors mediate efficient and stable transduction of postmitotic cells in brain, liver, muscle, and retina (*8*, *10*). Therefore, HIV vectors may facilitate the transduction of quiescent human HSCs. In this study, we evaluated whether HIV vectors could transfer genes into human $CD34^+$ cells that provide for long-term repopulation of NOD/SCID mice.

$CD34^+$ cells were isolated from human umbilical cord blood and maintained in serum-free medium before transduction (*11*). To minimize cycling and to maintain the in vivo repopulating capability, we transduced $CD34^+$ cells by means of a simple protocol in the absence of any exogenous cytokines. $CD34^+$ cells were transduced for 5 hours with VSV-G–pseudotyped HIV vector that contained the green fluorescent protein (GFP) gene under the control of the internal cytomegalovirus (CMV) promoter at a multiplicity of infection (MOI) of 60 or 300 (*12*). For comparison, VSV-G–pseudotyped MLV vector containing the same CMV-GFP expression cassette was used.

Transduction efficiencies were first assessed by in vitro assays. A portion of transduced $CD34^+$ cells was cultured for 5 days in serum-free medium containing recombinant human stem cell factor (SCF), interleukin-3 (IL-3), and IL-6 (*13*). Under these conditions, about 60% of the cells retained the $CD34^+$ phenotype while cells were expanded about 18-fold. $CD34^+$ cells transduced with either HIV or MLV vector showed comparable numbers of GFP^+ cells at an MOI of 60 and 300 [mean ± SE: 35 ± 5% and 54 ± 8% (HIV vector) and 33 ± 16% and 50 ± 22% (MLV vector) for MOI of 60 and 300, respectively], as determined by flow cytometry. A fivefold increase in MOI yielded a 1.5-fold increase in transduction efficiency for either HIV or MLV vector, suggesting that a higher MOI would provide little gain in transduction efficiency. To determine the transduction efficiency of colony-forming cell (CFC) progenitors, we plated transduced $CD34^+$ cells in methylcellulose with cytokines (*14*). GFP^+ CFC colonies, including burst-forming unit–erythroid (BFU-E), colony-forming unit–granulocyte, macrophage (CFU-GM), CFU–granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), and high proliferative potential–CFC (HPP-CFC) colonies, were scored by fluorescence microscopy at days 14 and 21. The number of GFP^+ CFC colonies transduced with the HIV vector was 12-fold higher at an MOI of 60 (12 ± 1%) and eightfold higher at an MOI of 300 (17 ± 3%) than that of GFP^+ CFC colonies transduced with the MLV vector. These results indicate that the HIV vector was more efficient than the MLV vector for transduction of $CD34^+$ progenitors that generate CFCs. No adverse effect of transduction on cell viability and proliferation was observed in these in vitro assays.

To assess the transduction efficiency of SRCs in the $CD34^+$ cell population, we transplanted transduced $CD34^+$ cells into sublethally irradiated NOD/SCID mice (*15*). Mice were serially bled from 7 to 22 weeks after transplantation to monitor GFP^+ human cells in the peripheral blood (PB). The percentage of GFP^+ human cells in the PB was

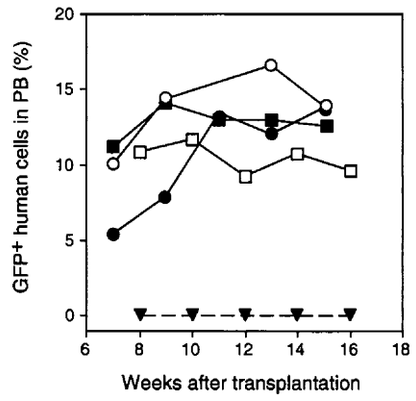

Fig. 1. Maintenance of GFP^+ human cells in the PB of NOD/SCID mice transplanted with HIV vector–transduced $CD34^+$ cells. Mononuclear cells were isolated from mice at indicated times after transplantation. The percentages of GFP^+ cells in human PB cells were assessed by two-color flow cytometry with an antibody to human CD45 (leukocyte common antigen). Representative results from four mice transplanted with HIV vector–transduced $CD34^+$ cells and all mice (n = 6) transplanted with MLV vector–transduced $CD34^+$ cells are shown. ■, mouse number 84, HIV (MOI 60); ●, mouse number 95, HIV (MOI 60); □, mouse number 10, HIV (MOI 300); ○, mouse number 92; ▼, all MLV (MOI 60 and 300)

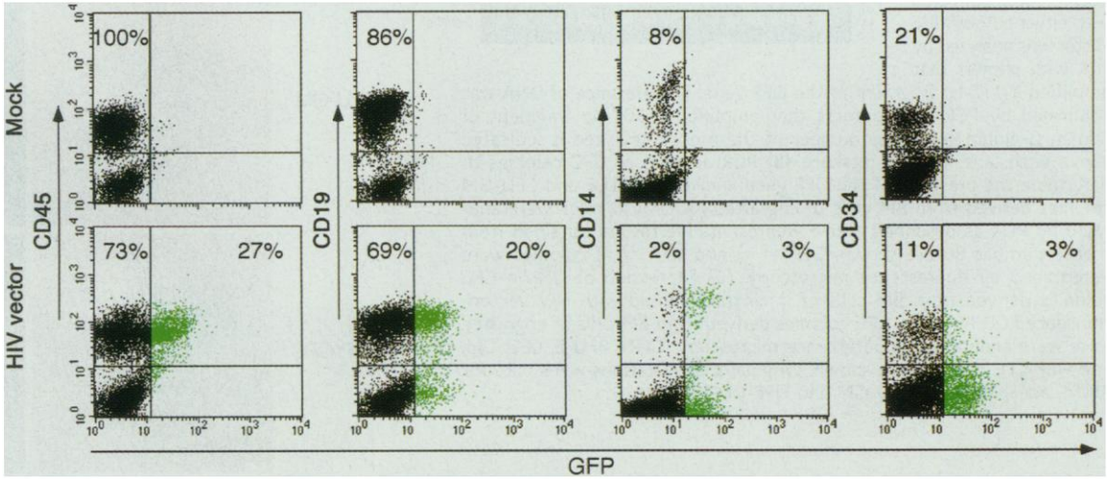

Fig. 2. GFP expression in human lymphoid and myeloid cells from the BM of NOD/SCID mice transplanted with HIV vector–transduced $CD34^+$ cells. Representative flow cytometric analyses of BM cells from mice transplanted with mock- or HIV vector–transduced $CD34^+$ cells (mouse number 95) are shown. Both mice had similar levels of human cell engraftment. Presented values are the percentages of total human cells.

Table 1. HIV, but not MLV, vectors can transduce human CD34$^+$ cells that give rise to lymphoid and myeloid lineages in engrafted NOD/SCID mice.

Vector	MOI*	Mouse number†	Weeks after transplantation	Human cell engraftment in BM (%)	GFP$^+$ human cells in (%):			GFP$^+$ CFC colonies‡	GFP gene in GFP$^-$ CFC colonies§ (%)	Transduced CFC colonies‖ (%)
					BM	Spleen	PB			
HIV	60	86	8	46	17	15	20	22	14	33
		924	9	41	12	10	13	19	5	23
		84	15	43	3	17	13	12	0	12
		95	15	33	27	16	15	67	20	74
		7	16	45	4	9	5	52	14	59
		93	17	5	6	14	12	36	7	40
		88	22	51	9	11	11	27	8	33
		5	22	28	2	4	2	20	7	26
				Mean ± SE¶: 37 ± 5	10 ± 3	12 ± 2	11 ± 2	32 ± 7	9 ± 2	38 ± 7
	300	87	9	46	1	17	11	3	14	17
		8	9	28	1	4	4	4	ND#	≥4
		92	15	30	15	22	14	77	57	90
		10	16	74	1	9	10	7	21	27
		85	17	46	1	9	9	14	0	14
		9	22	42	4	3	3	14	0	14
				Mean ± SE: 44 ± 6	4 ± 2	11 ± 3	9 ± 2	20 ± 12	18 ± 10	28 ± 13
MLV**	60	all (n = 3)	9, 16, 16	4, 11, 56	0	0	0	0	0	0
	300	all (n = 3)	9, 16, 16	42, 9, 34	0	0	0	0	0	0

*Multiplicity of infection for CD34$^+$ cells. †Results from three independent experiments are shown. ‡The percentage of human GFP$^+$ CFC colonies, obtained from BM cells, in total human CFC colonies observed. §The percentage of GFP gene positive CFC colonies in GFP$^-$ CFC colonies as determined by PCR. ‖The percentage of total transduced CFC colonies was calculated as follows: (% GFP$^+$ CFC colonies) + (% GFP gene positive CFC colonies in GFP$^-$ CFC colonies)(100 − % GFP$^+$ CFC colonies)/100. ¶Standard error of the mean. #ND, not done. **Because all humans cells were GFP$^-$ by flow cytometry and PCR analysis, all results are grouped for MOI.

determined by two-color flow cytometry (*16*). Most mice showed high levels of human cells in the PB, and the majority (≥95%) of these human cells were CD19$^+$ B cells, reflecting the dominance of B cell lymphopoiesis in this model (*17*). Representative results shown in Fig. 1 demonstrate that CD34$^+$ cells transduced with the HIV vector gave rise to GFP$^+$ human cells in the PB of engrafted mice and that the proportion of GFP$^+$ human cells remained roughly constant until the animals were killed. GFP$^+$ human cells were present in the PB for up to 22 weeks (see Table 1), the longest engraftment time period analyzed, demonstrating sustained production of human cells with integrated vector. In contrast, none of the mice (n = 6) transplanted with MLV vector–transduced CD34$^+$ cells had detectable GFP$^+$ human cells in the PB (Fig. 1), although these mice had numbers of human cells in the PB similar to those of mice transplanted with HIV vector–transduced CD34$^+$ cells.

GFP expression in human myeloid and lym-

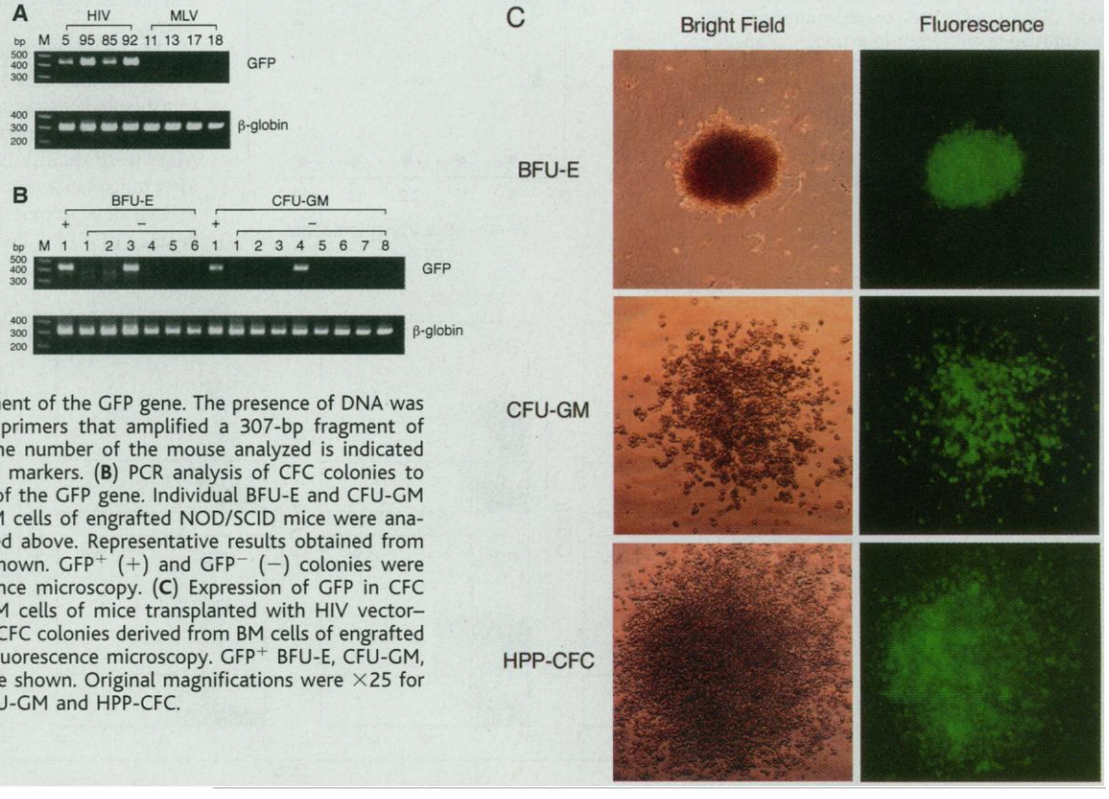

Fig. 3. GFP gene detection and expression in BM cells and CFC colonies derived from engrafted NOD/SCID mice. (**A**) PCR analysis of BM cells from engrafted mice to determine the presence of the GFP gene. Genomic DNA isolated from BM cells of NOD/SCID mice transplanted with CD34$^+$ cells infected with either HIV or MLV vector was analyzed by PCR with primers that amplified a 417-bp fragment of the GFP gene. The presence of DNA was confirmed by PCR with primers that amplified a 307-bp fragment of human β-globin gene. The number of the mouse analyzed is indicated above each lane. M, size markers. (**B**) PCR analysis of CFC colonies to determine the presence of the GFP gene. Individual BFU-E and CFU-GM colonies derived from BM cells of engrafted NOD/SCID mice were analyzed by PCR as described above. Representative results obtained from mouse number 86 are shown. GFP$^+$ (+) and GFP$^-$ (−) colonies were determined by fluorescence microscopy. (**C**) Expression of GFP in CFC colonies derived from BM cells of mice transplanted with HIV vector–transduced CD34$^+$ cells. CFC colonies derived from BM cells of engrafted mice were analyzed by fluorescence microscopy. GFP$^+$ BFU-E, CFU-GM, and HPP-CFC colonies are shown. Original magnifications were ×25 for BFU-E and ×12.5 for CFU-GM and HPP-CFC.

phoid lineages in the bone marrow (BM) and spleen of engrafted mice was evaluated at various time points after transplantation (*16*). High levels of human cell engraftment were evident in the BM of most of the mice (see Table 1). Representative flow cytometry results of BM cells from a mouse transplanted with HIV vector–transduced $CD34^+$ cells are shown in Fig. 2. About 27% of human ($CD45^+$) cells in the BM expressed the GFP gene. GFP expression was detected in $CD19^+$ B cells, the predominant population, and $CD14^+$ myeloid cells. In addition to differentiated human cells, GFP^+ $CD34^+$ cells were detected, suggesting that immature GFP^+ cells were maintained in the BM. The results from three separate experiments are summarized in Table 1. These results document the presence of GFP^+ human cells in the BM (range 1 to 27%), spleen (range 3 to 22%), and PB (range 2 to 20%) of all mice (n = 14) transplanted with HIV vector–transduced $CD34^+$ cells. There was no substantial difference between an MOI of 60 and 300. On the other hand, no GFP^+ human cells were detected in all mice (n = 6) transplanted with MLV vector–transduced $CD34^+$ cells. In addition, polymerase chain reaction (PCR) analysis of genomic DNA from BM cells revealed the presence of the GFP gene only in the BM cells from mice transplanted with HIV vector–transduced $CD34^+$ cells (Fig. 3A).

To determine the percentages of GFP^+ myeloid and erythroid progenitors in the BM of engrafted mice, we performed CFC assays in methylcellulose cultures that only support outgrowth of human progenitors (*14*). As shown in Fig. 3C, multiple lineages of human CFC colonies, including BFU-E, CFU-GM, and HPP-CFC colonies, derived from BM cells of mice transplanted with HIV vector–transduced $CD34^+$ cells expressed GFP (range 3 to 77%) (see Table 1). The presence of the GFP gene in the GFP^+ CFC colonies was verified by PCR analysis of randomly selected colonies (Fig. 3B). In contrast, no GFP^+ CFC colonies were detected from mice transplanted with MLV vector–transduced $CD34^+$ cells, further confirming the flow cytometric analysis of PB, BM, and spleen cells.

To address whether HIV vector integration correlated with GFP expression, we randomly isolated individual GFP^- CFC colonies and analyzed them by PCR for the presence of the GFP gene (*18*). Although there were some GFP^- colonies containing the GFP gene (Fig. 3B), the majority of GFP^- colonies did not contain the GFP gene (see Table 1). Thus, a large proportion of CFCs transduced with the HIV vector did express the GFP at a level detectable by fluorescence microscopy. On the basis of the results from fluorescence microscopy and PCR analysis of CFC colonies, the average percentage of transduction of CFC colonies from mice transplanted with HIV vector–transduced $CD34^+$ cells was 38 ± 7% (range 12 to 74%) at an MOI of 60 and 28 ± 13% (range 4 to 90%) at an MOI of 300 (Table 1). These results, together with data from PB longitudinal studies, demonstrated that the CMV promoter in the HIV vector can function properly in differentiated myeloid, erythroid, and lymphoid human cells with little silencing. The lack of expression of GFP in CFC colonies containing the GFP gene may have resulted from positional effects of the proviral integration site. Alternatively, these colonies expressed GFP at levels not detectable by fluorescence microscopy. HIV vector–mediated transduction had no adverse effect on human cell engraftment in NOD/SCID mice or colony-forming ability of progenitor cells as compared with mock transduction.

On the basis of human transplantation studies, human HSCs are known to be included in the $CD34^+$ cell population. A number of groups have provided strong evidence that the $Lin^-CD34^+CD38^-$ cell subpopulation contains SRCs, and it has been proposed that this subpopulation contains HSCs (*3*). It has also been shown that SRCs were transduced by retroviral vectors only when cytokine prestimulation was used (*19*). In contrast, our results demonstrate that HIV vectors can mediate efficient transduction of SRCs without cytokine prestimulation. Although several groups have recently shown, by in vitro assays, that $CD34^+$ cells can be transduced (*20*), we have established here the ability of HIV vectors to transduce human hematopoietic cells capable of long-term repopulation in vivo. Increases in MOI did not improve the transduction efficiency of SRCs, suggesting that there is a subpopulation of SRCs that is refractory for transduction with HIV vectors under the conditions we used. Recent studies have shown that the Lin^-CD34^- cell population also has long-term repopulating capacity and may be a precursor of Lin^-CD34^+ HSCs (*21*). Therefore, it would be of interest to determine whether HIV vectors can transduce these Lin^-CD34^- cells and possibly confirm the proposed role of these cells in the hierarchy of the hematopoietic system. Finally, a potential problem for the application of HIV vectors to human studies is safety. In this regard, recent improvements, including self-inactivating vectors, packaging constructs eliminating all accessory genes, and inducible packaging cell lines, could further minimize the risk (*22*). The use of HIV vectors provides a previously unexplored basis for the study of hematopoiesis and for human gene therapy with the use of HSCs.

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11. Cord blood was obtained at the Scripps Memorial Hospital from infants with uncompromised deliveries. Mononuclear cells were obtained from cord blood as previously described [B. Reinhardt *et al.*, *AIDS Res. Hum. Retroviruses* **10**, 131 (1994)]. $CD34^+$ cells were isolated from cord blood mononuclear cells with a VarioMACS device (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. After isolation, $CD34^+$ cells were resuspended at a concentration of 4.5×10^6 cells/ml in serum-free medium. Serum-free medium was composed of Iscove's modified Dulbecco's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% BIT 9500 serum substitute (Stem Cell Technologies, Vancouver, Canada). The purity of $CD34^+$ cells was >95% as determined by flow cytometry.
12. The HIV and MLV vectors used in this study and preparation of viral vector stocks have been described (*10*). Briefly, the VSV-G–pseudotyped HIV vector was generated by transient cotransfection of the vector construct pHR′-CMV-GFP (15 μg) with the VSV-G–expressing construct pMD.G (5 μg) and the packaging construct pCMVΔR8.2 (10 μg) into 293T cells. The VSV-G–pseudotyped MLV vector was generated by the same procedure as the HIV vector except with the vector construct pCLNCG and 293 gp/bsr cells that stably express MLV *gag* and *pol*. High-titer viral vector stocks were prepared by ultracentrifugation. The titers of viral vectors were determined by infection of 293T cells. 1.2×10^6 $CD34^+$ cells maintained in serum-free medium for 24 hours were transduced with each viral vector at an MOI of 60 or 300 in a total volume of 0.9 ml for 5 hours at 37°C in 5% CO_2. Transduced $CD34^+$ cells were washed with serum-free medium and then used for in vitro assays and reconstitution of NOD/SCID mice.
13. 2×10^4 transduced or mock-transduced $CD34^+$ cells/ml were incubated in serum-free medium containing the following recombinant human cytokines: SCF (100 ng/ml), IL-3 (50 ng/ml), and IL-6 (50 U/ml). Half of the medium was replaced every 2 days, and the transduction efficiency was determined by flow cytometry of GFP expression in the FL-1 channel at day 5.
14. For the CFC assay, 500 $CD34^+$ cells or total BM cells from engrafted mice containing 500 human $CD34^+$ cells, as determined by flow cytometry, were plated in triplicate 35-mm dishes with methylcellulose cultures containing the following recombinant human cytokines: SCF (50 ng/ml), IL-3 (10 ng/ml), granulocyte macrophage colony-stimulating factor (10 ng/ml), and erythropoietin (3 U/ml) (Stem Cell Technologies). These conditions support only the growth of human progenitor cells. Total and GFP^+ CFC colonies were counted under an inverted fluorescence microscope (Zeiss). BFU-E, CFU-GM, and CFU-GEMM colonies were counted at day 14, and HPP-CFC colonies were counted at day 21 on the basis of morphological criteria.
15. 2.0 to 3.5×10^5 transduced or mock-transduced $CD34^+$ cells were transplanted by tail-vein injection into sublethally irradiated (300 centigrays by ^{137}Cs γ-irradiation) 8- to 10-week-old NOD/SCID mice.
16. Mononuclear cells were isolated from PB, BM, and

Acetogenesis from H_2 Plus CO_2 by Spirochetes from Termite Guts

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Pure cultures of termite gut spirochetes were obtained and were shown to catalyze the synthesis of acetate from H_2 plus CO_2. The 16*S* ribosomal DNA sequences of two strains were 98 percent similar and were affiliated with those of the genus *Treponema*. However, neither was closely related to any known treponeme. These findings imply an important role for spirochetes in termite nutrition, help to reconcile the dominance of acetogenesis over methanogenesis as an H_2 sink in termite hindguts, suggest that the motility of termite gut protozoa by means of attached spirochetes may be based on interspecies H_2 transfer, and underscore the importance of termites as a rich reservoir of novel microbial diversity.

There are few, if any, habitats on Earth in which spirochetes are such major members of the microbial community as in the gut of termites (*1*). As many as half of the prokaryotes in termite guts are spirochetes (*2*), which range in size from small cells (0.1 to 0.2 μm by 3 μm) to much larger ones (1 by 100 μm). However, since they were first observed by Leidy over a century ago (*3*), none had ever been obtained in pure culture. Recent analyses of spirochetal 16S ribosomal RNA (rRNA)–encoding genes (16*S* rDNA) amplified by polymerase chain reaction (PCR) from termite guts revealed that they were affiliated with the treponemes, but none were closely related to any known species of *Treponema* (*2, 4, 5*).

We established enrichment cultures of spirochetes from hindgut contents of *Zootermopsis angusticollis* (Hagen) (Isoptera; Termopsidae) by using an anoxic medium under H_2 plus CO_2 (*6*). The medium contained rifamycin and phosphomycin (two drugs to which many spirochetes are resistant), as well as bromoethanesulfonate to inhibit the growth of H_2-consuming methanogens (*7*). During 10 to 12 weeks of incubation at 23°C, growth of a mixture of spirochetes (each 0.2 to 0.3 μm by 5 to 15 μm in size) was accompanied by consumption of H_2 and CO_2 and by formation of up to 30 mM acetate (*8*). Little or no spirochetal growth or acetate production occurred if the H_2 in the headspace was replaced by N_2. Two spirochete strains were isolated from an enrichment in which spirochetes outnumbered nonspirochetal bacteria by about 50 to 1.

Strains ZAS-1 and ZAS-2 were similar in morphology and size (0.2 μm by 3 to 7 μm) (Fig. 1A). Both had two periplasmic flagella (each inserted at opposite ends and overlapping for most of the length of the cells) interposed between the protoplasmic cylinder and the outer sheath (Fig. 1, B and C). The nucleotide sequences of the 16*S* rDNAs of ZAS-1 and ZAS-2 were 98% similar and were affiliated with those of the genus *Treponema* (Fig. 1D). Consistent with this assignment were the presence of phylum- and genus-level "signature" nucleotides in the inferred 16*S* rRNA sequences (*9*). However, neither strain was closely related [that is, bore >97% sequence similarity (*10*)] to any known species of *Treponema*. Phylogenetically, they grouped within a cluster of 16*S* rDNA clones from not-yet-cultured termite gut treponemes that ranged from 89% similar (clone NL1) to 97% similar (clones RFS3 and RFS25) and that included a clone (ZAS89; 95% similarity) from *Z. angusticollis* (*11*). The most similar sequences from cultivated relatives were from *Spirochaeta caldaria* and *S. stenostrepta* (92 to 93% similarity), two anaerobic spirochetes that are currently assigned to the genus *Spirochaeta* because they are free-living but that group within the treponemes on the basis of 16*S* rRNA sequence (*2, 4, 5, 8*). These results implied that ZAS-1 and ZAS-2 represented at least one new species of *Treponema*. However, we are postponing assignment of a species epithet or epithets until more is known about them.

ZAS-1 and ZAS-2 grew poorly in the medium used for enrichments (*6*). At 23°C their doubling time was ≥10 days and cell yields were $<10^8$ cells/ml. Growth was markedly improved in a medium containing yeast autolysate (YA) and a cofactor solution (*12*) and by increasing the incubation temperature. In 4YACo medium at 30°C, ZAS-1 grew with a doubling time of 23 to 24 hours to densities of 1.4×10^9 cells/ml, and ZAS-2 grew with a doubling time of 48 hours to 2.8×10^8 cells/ml. Little or no growth of either strain occurred if the cofactor solution

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spleens of engrafted mice. The human cell engraftment and GFP expression in each sample were analyzed by two-color flow cytometry with phycoerythrin (PE)–conjugated antibody to human CD45 with sample collection on a FACScan running Cell Quest software (Becton Dickinson). Specific subsets of human cells were detected by staining with PE-conjugated antibodies to human CD14 (monocytes), CD19 (B cells), and CD34 (progenitor cells). PE-conjugated mouse immunoglobulin G1 was used as an isotype control. All antibodies were purchased from Becton Dickinson. In each experiment, cells from mice transplanted with mock-transduced $CD34^+$ cells were analyzed as a negative control for GFP expression.

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18. The presence of the GFP gene was determined by PCR analysis. Genomic DNA was isolated from the BM cells of engrafted mice by the method as described [Q. Wu, M. Chen, M. Buchwald, R. A. Phillips, *Nucleic Acids Res.* **23**, 5087 (1995)]. Individual CFC colonies (BFU-E and CFU-GM) derived from BM cells of engrafted mice were plucked and incubated in lysis buffer [10 mM tris-HCl (pH 8.5), 50 mM KCl, 0.01% gelatine, 0.45% IGEPAL CA-630, 0.45% Tween 20, and proteinase K (100 μg/ml)] at 56°C for 12 hours, followed by heat inactivation of proteinase K at 95°C for 15 min. Typically, 14 to 22 colonies for each mouse were analyzed. PCR was performed with TaqPlus Precision PCR system (Stratagene) according to the manufacturer's instructions. Amplification conditions were as follows: 95°C for 3 min, then 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by extension at 72°C for 10 min. The primers used to amplify the GFP gene were 5′-ACCCCGACCACATGAAGCAGC-3′ and 5′-CGTTGGGGTCTTTGCTCAGGG-3′, giving a 417–base pair (bp) fragment. The presence of DNA was confirmed by PCR with primers specific for the human β-globin gene, 5′-GGGCAAGGTGAACGTGGATGA-3′ and 5′-CCATCACTAAAGGCACCGAGC-3′, giving a 307-bp fragment. BM cells or CFCs from mice transplanted with mock-transduced $CD34^+$ cells were used as a negative control. PCR products were electrophoresed on 2% agarose gel with ethidium bromide.
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