Initiation of Runaway Cell Death in an *Arabidopsis* Mutant by Extracellular Superoxide

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Reactive oxygen intermediates (ROIs) regulate apoptosis during normal development and disease in animals. ROIs are also implicated in hypersensitive resistance responses of plants against pathogens. *Arabidopsis lsd1* mutants exhibited impaired control of cell death in the absence of pathogen and could not control the spread of cell death once it was initiated. Superoxide was necessary and sufficient to initiate lesion formation; it accumulated before the onset of cell death and subsequently in live cells adjacent to spreading *lsd1* lesions. Thus, runaway cell death seen in *lsd1* plants reflected abnormal accumulation of superoxide and lack of responsiveness to signals derived from it.

The relation between developmentally programmed cell death (PCD) and pathogen-induced, localized, hypersensitive cell death in plants is poorly understood. Equally unclear is the relation between cell death in plants and various types of PCD in animals, including apoptosis (*1*). Certain plant mutations exhibit dead cell lesions in the absence of pathogen (*2*), which are often developmentally and environmentally regulated. Two classes of *Arabidopsis* mutants [termed "lesions simulating disease resistance" (*lsd*) (*3*) and "accelerated cell death" (*4*)] exhibit spontaneous lesions that are either determinate or propagate unchecked. Propagative *lsd1* lesions can be induced by pathogens or bioactive chemicals that induce systemic acquired resistance (SAR) (*5*) under short-day (SD) conditions that otherwise permit normal plant growth (*6*). These lesions spread beyond the site of infection or chemical application and engulf the affected leaf after 2 to 4 days. No pathogen is recovered from expanded lesions. Lesions do not propagate beyond the affected leaf. Lesion formation correlates with expression of biochemical and molecular markers of hypersensitive resistance response (HR) and SAR and is also induced by shifting *lsd1* plants to nonpermissive long-day (LD) growth conditions (*3*, *6*). In sum, the *lsd1* mutant phenotype is indicative of an HR lacking normal boundaries. Thus, *LSD1* is apparently required for limiting cell death caused during HR. We used the conditional nature of *lsd1* to investigate the role of ROIs in initiation and propagation of cell death in this mutant.

Local spot inoculation of an extracellular superoxide-generating system [xanthine and xanthine oxidase (X-XO)] into intercellular spaces of *lsd1* leaves triggered spreading lesion formation in a dose-dependent manner (Table 1) (*7*, *8*). Inoculation of X-XO caused no cell death on leaves of the parental *Arabidopsis* accession Ws-0. Co-inoculation of superoxide dismutase (SOD) greatly reduced lesion formation on *lsd1* but co-inoculation of catalase or peroxidase had no effect (*9*). Inoculation of a hydrogen peroxide (H_2O_2)–generating system, glucose plus glucose oxidase [which initiates PCD in a soybean cell suspension culture (*10*)], did not trigger lesions in *lsd1*. Inoculation of H_2O_2 above 30 mM rapidly killed all cells at the inoculation site in both wild-type and *lsd1* leaves but did not initiate the propagation of spreading *lsd1* lesions. Allopurinol, an inhibitor of intracellular xanthine oxidase and certain HR responses (*9*, *11*), did not influence initiation of *lsd1* lesions after shift to LD conditions. Other compounds influencing ROI metabolism in plants (*12*) had no effect on *lsd1* lesion formation. These data suggest that apoplastic superoxide generation is sufficient to initiate *lsd1* lesions.

Superoxide accumulation precedes initiation of *lsd1* cell death (Table 2 and Fig. 1). Superoxide accumulated in *lsd1* in the absence of cell death 16 hours after shift to LD conditions (Table 2). Lesions subsequently formed, and the purple formazan precipitate produced by reaction of nitro blue tetrazolium (NBT) (*7*, *8*) with superoxide was deposited at lesion boundaries in living tissue (Fig. 1, C and D). Inclusion of SOD in the staining mix abolished formazan formation (Fig. 1, E and F) but catalase did not (*9*), furthering the argument that extracellular superoxide is detected in this assay. Localized spot inoculation with X-XO caused immediate superoxide accumulation, which persisted in *lsd1* tissues with very little cell death until 16 hours (Table 2). At later time points, we observed superoxide production in living cells adjacent to spreading lesions (*9*). Superoxide was also generated in Ws-0 plants, but it disappeared slowly over 8 hours and never led to cell death (Table 2). When SD *lsd1* plants were treated with the SAR-inducing chemical 2,6-dichloroisonicotinic acid (INA) (*13*), superoxide accumulation began at 8 hours, well before the first visible cell death (Table 2). Subsequent spread of *lsd1* lesions was accompanied by superoxide production at lesion margins (Fig. 1, I and J). SAR-inducing chemicals did not cause either spreading cell death or superoxide accumulation in wild-type leaves (Fig. 1, G and H). Thus, superoxide production preceded spreading lesion formation in *lsd1* leaves after all three treatments. Once lesions were formed, more superoxide accumulated at their boundaries.

To assess whether superoxide is required for initiation and propagation of *lsd1* lesions, we inoculated leaf halves with the plasma membrane NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI) 3 hours before transfer from SD to LD conditions (*14*). Lesion formation in DPI-treated leaf halves was reduced 60 to 70% in a dose-dependent manner as compared with that in untreated halves of the same leaves (Fig. 2). This parallels inhibition of defense responses in cultured rose, soybean, and parsley cells treated with DPI (*10*, *15*). Half-maximal inhibition [approximate inhibition constant (K_i) of 1 to 3 mM] was within

Table 1. Generation of superoxide radicals is sufficient to induce *lsd1* lesions. *lsd1* leaves were treated (*8*) as shown, plants were kept in SD conditions, and spreading lesions were scored at 24 hours. Six independent experiments are pooled. When wild-type Ws-0 control leaves were treated with X-XO, 0 of 59 were lesioned.

Treatments			Lesioned leaves/total leaves (*n*)
XO	X	SOD	
–	–	–	0/70
–	+	–	1/74
–	+	+	0/14
–	–	+	2/23
0.02 U/ml	–	–	4/31
0.02 U/ml	+	–	84/104
0.02 U/ml	+	+	18/87
0.50 U/ml	–	–	3/17
0.50 U/ml	+	–	25/25
0.50 U/ml	+	+	6/28
Glucose oxidase		Glucose	
–		–	0/10
–		+	0/10
250 U/ml		+	0/17
H_2O_2			
0.001 to 10 mM			0/217

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ranges reported for DPI inhibition of the human neutrophil oxidative burst (5 mM (*16*) and the oxidative burst in plant cell cultures after elicitor stimulation (2 to 15 mM) (*10*, *15*). DPI pretreatment abolished subsequent NBT staining 48 hours after shift to LD conditions (9). Lesion formation was also inhibited in 40 ± 9.3% of *lsd1* leaves inoculated with SOD (0.5 mg/ml) 3 hours before shift to LD conditions (62 total leaves from three independent experiments). We conclude that superoxide formed by the action of a DPI-inhibitable plasma membrane NAD(P)H oxidase is necessary for most, if not all, *lsd1* lesion formation.

The expression of molecular markers extends these phenotypic observations. RNA isolated from *lsd1* and Ws-0 leaves after shift to LD conditions (*17*) showed accumulation of mRNA encoding the pathogenesis-related protein PR-1 (*3*), beginning just before visible lesion formation. Among expressed sequence tag (EST) probes encoding proteins with roles in oxygen metabolism (*17*), only peroxidase C (*PRXc*) and type III glutathione-S-transferase (*GST*) mRNAs accumulated (Fig. 3A). These two proteins are involved in maintenance of cellular redox balance (*18*). Thus, signaling events subsequent to superoxide generation in *lsd1* do not lead to general induction of ROI scavenging systems but have specific effects on parts of ROI metabolism and on genes associated with establishment of SAR. Among the probes tested were ESTs encoding all known isoforms of plant SOD (*18*). Although SOD isoforms are induced upon infection in some systems (*18*, *19*), neither SOD mRNA amounts nor activity levels differed between *lsd1* and Ws-0 plants (9). Thus, the *lsd1* mutation is unlikely to be an SOD mutation; if it were, it would be in an extracellular SOD gene (*19*) specifically involved in detoxification of superoxide induced during HR-like cell death.

Generation of superoxide in SD conditions though X-XO inoculation also drives mRNA accumulation of *PR-1*, *PRXc*, and *GST* in *lsd1* leaves (Fig. 3B). Only *GST* mRNA accumulates in Ws-0 leaves after this treatment, which is consistent with its normal role in ROI clearance in plant tissue (*7*, *18*). Accumulation of these marker mRNAs is diminished when SOD is coinoculated with X-XO (Fig. 3B). Leaves harvested after DPI inoculation and shift to LD conditions exhibited a dose-dependent diminution of marker gene expression (Fig. 3C) that correlated well with the observed inhibition of lesion formation in the same experiment (Fig. 2). We observed no effect of DPI treatment on expression of these three genes in Ws-0 leaves (Fig. 3C). Thus, phenotypic responses of *lsd1* to superoxide generation, or inhibition thereof, are mirrored by downstream responses exemplified by these marker genes.

To determine whether all responses we examined were a direct consequence of the *lsd1* mutation, we repeated each experiment using the *lsd1*/*phx21* suppressor line (*20*). These plants are wild type with respect to

Table 2. Evolution of superoxide precedes onset of cell death in *lsd1* leaves. Leaves (three to six per time point) were treated (*8*) and assayed for either onset of superoxide production measured through NBT staining (symbol at left in each column) or cell death measured by trypan blue uptake (symbol at right in each column) at the indicated time points. INA-treated (*17*) and X-XO–inoculated plants (*8*) were kept in SD conditions (*6*). Symbols for NBT staining are as follows: –, none on any leaf; +/–, light staining on some leaves; +, small, dark foci on all leaves with <50 cells; ++, larger foci on all leaves with >100 cells, surrounding lesion margin if present ; and +++, intense staining (spreading beyond inoculation site in X-XO experiments). Symbols for trypan blue staining (*3*) are as follows: –, none; +/–, single dead cells on some leaves; +, foci of 1 to 10 cells on each leaf; and ++, large foci of >50 cells on each leaf. ND, not done.

Treatment	Genotype	Measurement of NBT or trypan blue (hours)							
		0	4	8	16	24	32	40	48
Shift to LD	*lsd1*	–,–	–,–	–,–	++,–	++,+/–	++,+	++,++	++,++
	Ws-0	–,–	–,–	+/–,–	–,–	–,–	+/–,–	+–,–	–,–
X-XO	*lsd1*	++,–	++,+/–	++,+/–	+++,++	+++,++	ND	ND	ND
	Ws-0	++,–	+,–	+,–	+,–	+/–,–	ND	ND	ND
INA	*lsd1*	–,–	–,–	+,–	++,+	++,++	++,++	++,++	++,++
	Ws-0	–,–	–,–	–,–	+/–,–	+/–,–	–,–	–,–	–,–

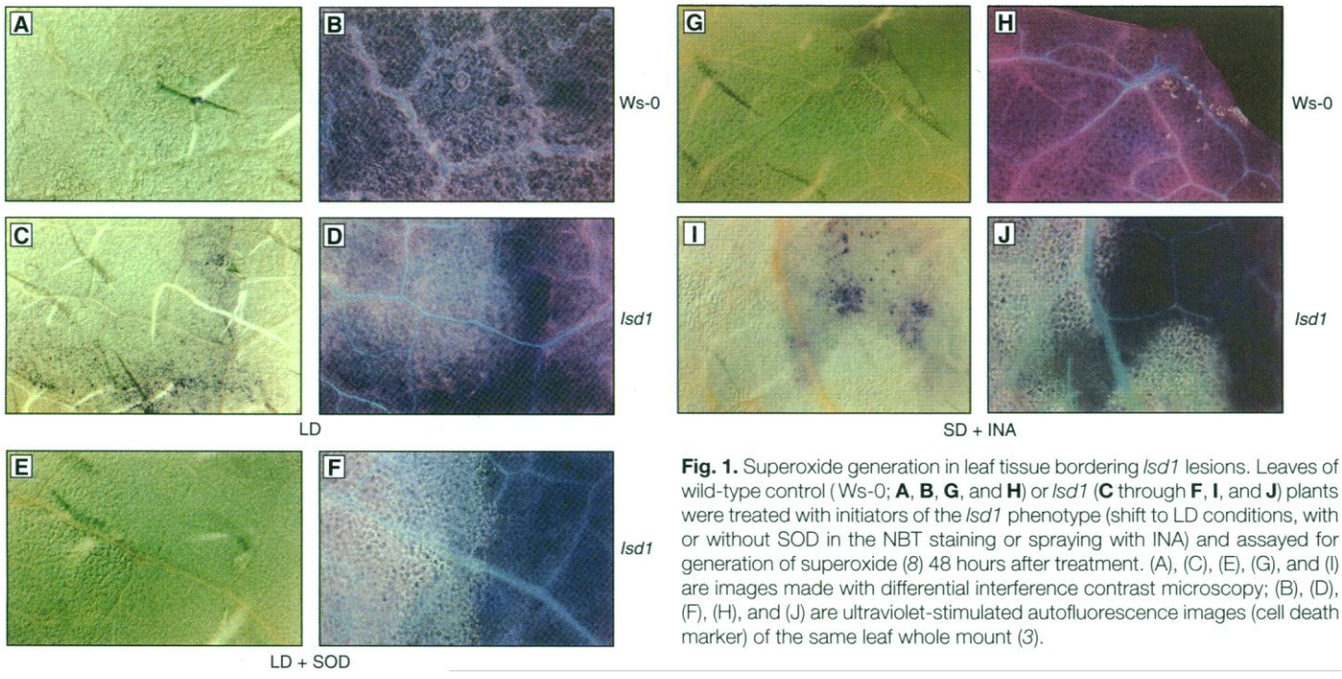

Fig. 1. Superoxide generation in leaf tissue bordering *lsd1* lesions. Leaves of wild-type control (Ws-0; **A**, **B**, **G**, and **H**) or *lsd1* (**C** through **F**, **I**, and **J**) plants were treated with initiators of the *lsd1* phenotype (shift to LD conditions, with or without SOD in the NBT staining or spraying with INA) and assayed for generation of superoxide (*8*) 48 hours after treatment. (A), (C), (E), (G), and (I) are images made with differential interference contrast microscopy; (B), (D), (F), (H), and (J) are ultraviolet-stimulated autofluorescence images (cell death marker) of the same leaf whole mount (*3*).

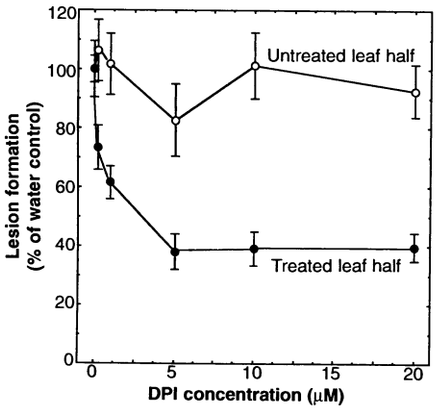

Fig. 2. Inhibition of *lsd1* lesion initiation through inhibition of plasma membrane NAD(P)H oxidase. Leaves in SD conditions were treated with DPI (*14*) and scored for spreading lesions 40 hours after shift to LD conditions. Values are means of at least 17 treated leaves per point from three independent experiments.

lesion induction by pathogens, shift to LD conditions, or treatment with SAR-inducing chemicals (*20*). No lesions formed after X-XO inoculation (*9*), and marker gene expression was unaffected by stimuli triggering both lesions and marker gene expression in *lsd1* (Fig. 3D). Thus, the effect of superoxide on the *lsd1* mutant is a specific and direct consequence of the *lsd1* mutation.

The *Arabidopsis lsd1* mutation apparently removes a critical negative regulatory control step, resulting in both a lowered threshhold for initiation of the cell death program and inability to limit the extent of subsequent cell death. Our data show that superoxide, which does not cross membranes and is not particularly toxic, is a key component in this control circuit. Other studies provide evidence for ROI involvement in the HR, and H_2O_2 derived from superoxide is often implicated as a critical component (*10*, *18*, *21*). Collectively, these results suggest that very high local doses of ROIs may participate in direct killing of plant cells and potentially also of pathogens. Lower doses of ROIs could also emanate as a signal to surrounding tissue to induce ROI-detoxification systems and to potentially act as a local trigger for defense gene activation (*10*).

The *lsd1* mutation allows accumulation of extracellular superoxide; *lsd1* cells are acutely sensitive to signals derived from extracellular superoxide and cell death is initiated. This initiates subsequent superoxide formation in live neighboring cells, auto-amplifying a cell nonautonomous signal leading to runaway cell death. Our data suggest that wild-type LSD1 monitors a feed-forward signal normally leading to cell death in plant cells. LSD1 may act as a rheostat, sensing signals that activate the HR cell death program. LSD1 could trigger initiation of cell death at high signal levels and down-regulate, or dampen, cell death as the signal level falls. This rheostat could respond to SA, found in a steep concentration gradient around infection sites (*22*). Demonstrations both of a requirement for SA in ROI-associated responses during HR and establishment of SAR, and for feedback amplification of SA-dependent lesion formation (*23*) are consistent with this model. Alternatively, LSD1 could inhibit constitutive low level signals feeding into the ROI-generating system acting between pathogen recognition and the specific oxidative burst associated with HR (*10*, *18*, *21*). The *lsd1* mutation would allow continuous signaling, leading to ROI generation and also resulting in runaway amplification of a positive signal for cell death (*24*).

Roles for ROIs, and in particular for superoxide, in animal diseases have been established (*25*). Our demonstration that superoxide is a critical signal in a process monitored via LSD1 both to initiate localized cell death and to inhibit the spread of this cell death provides a novel perspective for dissection of similarities and differences between control of PCD in plants and animals.

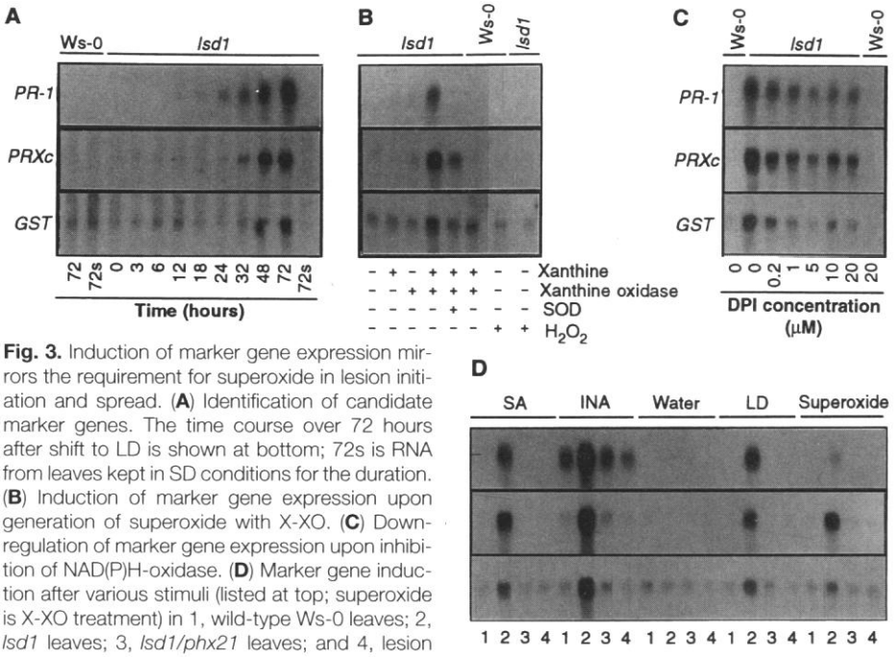

Fig. 3. Induction of marker gene expression mirrors the requirement for superoxide in lesion initiation and spread. (**A**) Identification of candidate marker genes. The time course over 72 hours after shift to LD is shown at bottom; 72s is RNA from leaves kept in SD conditions for the duration. (**B**) Induction of marker gene expression upon generation of superoxide with X-XO. (**C**) Down-regulation of marker gene expression upon inhibition of NAD(P)H-oxidase. (**D**) Marker gene induction after various stimuli (listed at top; superoxide is X-XO treatment) in 1, wild-type Ws-0 leaves; 2, *lsd1* leaves; 3, *lsd1/phx21* leaves; and 4, lesion minus *lsd3* leaves (*3*) as a control for nonspecific gene induction. Samples in (B) through (D) were harvested 48 hours after treatment.

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17. RNA (5 μg per lane) was isolated (10 leaves from three plants) and blots were prepared, probed, and washed as described [S. Kiedrowski, P. Kawalleck, K. Hahlbrock, I. E. Somssich, J. L. Dangl, *EMBO J.* **11**, 4677 (1992)]. The probes used were *PR-1* cDNA [S. Uknes *et al.*, *Plant Cell* **5**, 159 (1993)] and ESTs [T. Newman *et al.*, *Plant Physiol.* **106**, 1241 (1994)]. The ESTs were from the Arabidopsis Biological Resource Center, Ohio State University. EST identification numbers are as follows: B25XP, homologous to *PRXcb* (GenBank accession number X71794); 90B13T7, *GST* (GenBank accession number T20657), and a type III GST, similar to an auxin-regulated protein and *GST* clones [F. N. J. Droog, P. J. J. Hooykaas, B. J. van der Zaal, *Plant Physiol.* **107**, 11139 (1995)]. Other *Arabidopsis GSTs* described are class I (*GST2* and PMA239x14). ESTs showing no difference in expression between *lsd1* and the wild type putatively encode Fe-SOD (34D9T7); Cu/Zn-SOD (2G11T7P and 92L6T7); Mn-SOD (105G4T7); Catalase I (35F2T7, 38C1T7, and 40A1T7); anionic lignin-forming peroxide (ATTS0592); cationic peroxidase (40H1T7); P7 peroxidase (11B11T7); glutathione peroxidase (139F9T7); and lipoxygenase I (92H8T7). The apparent lack of SA-induced *PR-1* mRNA accumulation in wild-type leaves (Fig. 2D) is due to deliberate underexposure of the autoradiogram in order to visualize the stronger signals. Normal exposure clearly shows *PR-1* mRNA accumulation.
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Genetic Restriction of HIV-1 Infection and Progression to AIDS by a Deletion Allele of the *CKR5* Structural Gene

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The chemokine receptor 5 (CKR5) protein serves as a secondary receptor on $CD4^+$ T lymphocytes for certain strains of human immunodeficiency virus–type 1 (HIV-1). The *CKR5* structural gene was mapped to human chromosome 3p21, and a 32–base pair deletion allele (*CKR5Δ32*) was identified that is present at a frequency of ~0.10 in the Caucasian population of the United States. An examination of 1955 patients included among six well-characterized acquired immunodeficiency syndrome (AIDS) cohort studies revealed that 17 deletion homozygotes occurred exclusively among 612 exposed HIV-1 antibody–negative individuals (2.8 percent) and not at all in 1343 HIV-1–infected individuals. The frequency of *CKR5* deletion heterozygotes was significantly elevated in groups of individuals that had survived HIV-1 infection for more than 10 years, and, in some risk groups, twice as frequent as their occurrence in rapid progressors to AIDS. Survival analysis clearly shows that disease progression is slower in *CKR5* deletion heterozygotes than in individuals homozygous for the normal *CKR5* gene. The *CKR5Δ32* deletion may act as a recessive restriction gene against HIV-1 infection and may exert a dominant phenotype of delaying progression to AIDS among infected individuals.

In all well-characterized epidemics there are individuals in the population that respond differently to the infectious agent (*1*, *2*). Although resistance to infection is the most common variable phenotype, variation in disease outcomes has also been observed. Epidemiologic studies have shown that inherited factors are involved in the risk of mortality from infectious agents (*3*, *4*). The HIV-1 epidemic presents a critical challenge for the application of current genetic techniques to the study of host genetic variation for infection and susceptibility to infection. This problem is confounded in the studies of HIV-1 by the rapid rate of evolution of the virus (*5*–*7*). However, a number of groups have shown that specific alleles of the human lymphocyte antigen (HLA) locus are associated with different rates of progression from infection to an AIDS diagnosis (*8*). Yet little evidence for non-HLA loci regulating HIV-1 infection or AIDS progression has been reported, although it does seem likely that other host genetic factors would play a role in AIDS epidemiology (*8*, *9*).

The recent demonstration that the chemokines RANTES, MIP-1α, and MIP-1β act as natural suppressors of HIV-1 infection (*10*) has focused attention on the role of these chemokines during HIV-1 infection and clinical pathogenesis. Feng *et al.*