M. Locksley, *J. Exp. Med.* **179**, 447 (1994).
10. T. Yanagida, T. Kato, O. Igarashi, T. Inoue, H. Nariuchi, *J. Immunol.* **152**, 4919 (1994).
11. C.-S. Hsieh *et al.*, *Science* **260**, 547 (1993).
12. J. D. Gorham, M. Güler, A. Mackey, R. G. Steen, W. F. Dietrich, K. M. Murphy, in preparation.
13. M. Roberts, B. A. Mock, J. M. Blackwell, *Eur. J. Immunogenet.* **20**, 349 (1993).
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Susceptibility to *Leishmania major* Infection in Interleukin-4–Deficient Mice

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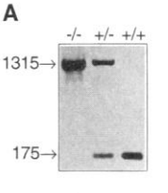
Interleukin-4 (IL-4), a pleiotropic cytokine, is a major regulator of the immune system and is considered crucial for the development of T helper cell type 2 (T_H2) responses. The susceptibility of BALB/c mice to infection with *Leishmania major* has been associated with a polarized T_H2 response and an inability to down-modulate IL-4 production. The role of IL-4 in vivo was examined directly by disrupting the IL-4 gene in BALB/c embryonic stem cells. Despite the absence of IL-4, the genetically pure BALB/c mutant mice remained susceptible to *L. major* infection, showed no signs of lesion healing or parasite clearance, and did not switch to a T_H1 phenotype.

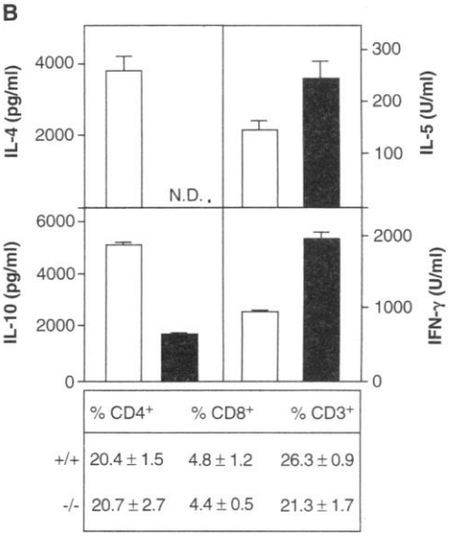
Infection of mice with *Leishmania major* is widely used as a model to study the differential development of $CD4^+$ T helper cell subsets (T_H1 and T_H2) in vivo. Polarized T_H1 or T_H2 responses mediate healing or progressive disease in genetically resistant or susceptible strains of mice (*1*). Susceptibility to infection is correlated with the expansion of IL-4–producing T_H2 cells, whereas the expansion of interferon γ (IFN-γ)–producing T_H1 cells in resistant strains of mice results in control of the infection (*2*, *3*). Cytokines released by one T helper cell subset cross-regulate the development of the other subset: T_H2 cells suppress T_H1 cells by secreting IL-4, whereas IFN-γ inhibits T_H2 cell expansion (*4*). BALB/c mice are highly susceptible to *L. major* infection: They develop progressive cutaneous leishmaniasis and fail to control the replication of the intracellular parasites. On the basis of ample published evidence (*3*, *5*), IL-4 is considered to be a crucial cytokine for the development of T_H2 responses and the susceptibility of BALB/c mice to infection with *L. major*; thus, it is thought that the inability of BALB/c mice to control *L. major* infection is due to their failure to down-regulate IL-4 production during infection (*3*). IL-4–deficient BALB/c mice were therefore used to study the function of IL-4 during the course of *L. major* infection.

The IL-4 mutant mice used in the present study were generated from a recently derived BALB/c embryonic stem (ES) cell line (*6*). Other IL-4–deficient strains (*7*, *8*) contain genetic contributions from the 129 inbred strain, which is resistant to *L. major* infection (*9*). The use of IL-4–deficient mice of mixed susceptible and resistant genetic backgrounds has led to conflicting interpretations of the role of IL-4 in murine acquired immunodeficiency syndrome (AIDS) infection (*10*). The use of the BALB/c ES cell line to generate genetically pure BALB/c mice was required because the loci for susceptibility to leishmaniasis (*Scl1*), for IL-12p40 (*Il12b*), and for inducible nitric oxide synthase (*Nos2*) are linked to the IL-4 structural gene (*Il4*) on chromosome 11 (*11*).

BALB/c mice defective in IL-4 expression were generated as described (*6*). IL-4 homozygous mutant (−/−), heterozygous (+/−), and wild-type (+/+) mice were detected by polymerase chain reaction (PCR) amplification of tail DNA (Fig. 1A). The naïve IL-4 mutant BALB/c mice did not display an overtly abnormal phenotype, and lymphocyte composition and function were comparable with wild-type littermates. Spleens from naïve $IL\text{-}4^{+/+}$ and $IL\text{-}4^{-/-}$ mice contained comparable numbers of $CD3^+$, $CD4^+$, and $CD8^+$ cells, and lymphocytes from $IL\text{-}4^{-/-}$ mice secreted similar amounts of the T_H2 cytokine IL-5, but reduced amounts of IL-10 and no IL-4 after stimulation with monoclonal antibody (mAb) to CD3 (anti-CD3) in vitro (Fig. 1B). In contrast, a defective T_H2 cytokine response in other IL-4–targeted mice has been reported (*8*). These discrepancies may be due to differences in stimulation protocols or the mouse strains used.

The susceptibility of BALB/c mice to *L. major* infection has been correlated with a strong IL-4–dominated T_H2 response that concomitantly down-regulates the potentially protective IFN-γ–dependent T_H1 response, resulting in a poor cell-mediated immune response and an inability to activate macrophages to kill the intracellular parasites. Therefore, we expected the IL-4–deficient BALB/c mutant mice to control infection; however, infection with *L. major* resulted in the development of pronounced cutaneous lesions in all groups (−/−, +/−, and +/+) (Fig. 2A). Lesions began to open and ulcerate in the fifth week after infection in all groups. At the same time, the lesions of control-infected, genetically re-





	% CD4+	% CD8+	% CD3+
+/+	20.4 ± 1.5	4.8 ± 1.2	26.3 ± 0.9
-/-	20.7 ± 2.7	4.4 ± 0.5	21.3 ± 1.7

Fig. 1. Phenotypic analysis of naïve IL-4–deficient BALB/c mice. (**A**) PCR screening of IL-4–targeted mice. Offspring from IL-4 heterozygous matings were screened for the presence of the targeted allele by PCR amplification of tail genomic DNA (*26*). Numbers at left indicate base pairs. (**B**) Lymphocyte composition in wild-type (+/+) and IL-4–targeted BALB/c mice (−/−) and cytokine secretion of naïve cells after polyclonal stimulation. Naïve spleen cells from both groups of mice were analyzed for CD3, CD4, and CD8 expression by flow cytometry (FACScan, Becton Dickinson) and stimulated with anti-CD3 for the determination of the cytokine pattern (*27*). $IL\text{-}4^{+/+}$ open bars; $IL\text{-}4^{-/-}$ black bars; N.D., not detectable.

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sistant C57BL/6 mice started to decline (*12*). The development of nonhealing lesions in all groups of IL-4–deficient mutant mice indicates that IL-4 is not the exclusive determining factor in the nonhealing disease in BALB/c mice. Furthermore, the absence of IL-4 did not result in a more efficient killing of the obligate intracellular parasites because uncontrolled replication of *L. major* occurred in all BALB/c groups (Fig. 2B). Thus, the data obtained in mice with a genetic deletion of IL-4 fail to support a simple causal relation between IL-4 and susceptibility to *L. major* infection in BALB/c mice. This contrasts with findings showing that depletion of IL-4 with neutralizing mAb to IL-4 (11B11) induces healing of the lesions and the development of a T_H1 response in BALB/c mice. Comparison of *L. major* infection in the two types of IL-4–deficient BALB/c mice confirmed that IL-4 depletion by anti–IL-4 strongly reduced the lesion size and the parasite burden (11×10^6 compared with 7×10^3 *L. major* parasites per lesion) (Fig. 2C), whereas injection of the same dose of neutralizing anti–IL-4 into IL-$4^{-/-}$ mice neither reduced the lesion size nor the parasite multiplication (2.5×10^6 compared with 4.6×10^6 parasites per lesion) (Fig. 2C).

Cytokines control the class of immunoglobulin (Ig) expressed by B cells, and the role of IL-4 in the induction of Ig class switch recombination is generally accepted. This raises the question of whether Ig class switching to IgG1 and IgE is impaired in vivo in −/− mice. The absence of IL-4 in BALB/c mice clearly resulted in reduced serum concentrations of IgG1 and IgE in infected mutant mice (Fig. 3A). These data confirm the importance of IL-4 for these Ig class switches in vivo (*7*, *8*, *13*). Alternative pathways for IgE class switching can be triggered in IL-4–deficient mice (*14*); however, the strong reduction of IgG1 and IgE in *L. major*–infected −/− mice indicates that these activities of IL-4 are not completely compensated for by other cytokines in vivo.

The analysis of the *L. major*–specific cytokine production revealed neither a significant increase in antigen-induced IFN-γ secretion nor a consistent reduction of T_H2 cytokines during the course of infection of +/+ and −/− mice. Lymph node (LN) cells from *L. major*–infected IL-$4^{+/-}$ BALB/c mice consistently produced 58 to 82% less IL-4 than those from infected IL-$4^{+/+}$ mice. A similar tendency was observed in the spleen cells (*12*). LN cells from anti–IL-4–treated, infected wild-type mice displayed 59 days after infection an IFN-γ response 3.1 times that in −/− mice and drastically reduced IL-4 concentrations (Fig. 3B). The unaltered concentrations of IL-10 (2500 pg/ml in −/− mice and 2150 pg/ml in +/+ mice treated with anti–IL-4) at 59 days after infection indicate that antigen-specific T_H2 cytokine secretion is not impaired in the absence of IL-4. IL-10 and transforming growth factor–β (TGF-β) can down-regulate T_H1 effector functions and cell-mediated immunity (*5*, *15*), thereby interfering with the efficient activation of the nitric oxide pathway, which is necessary for parasite elimination (*16*).

Direct analysis of cytokine transcripts by reverse transcriptase PCR of LN RNA from mice 4 and 8 weeks after infection confirmed the lack of IL-4 mRNA and showed no compensatory increases in IL-13 transcripts in the mutant mice (Fig. 4A). Small amounts of IL-5 transcripts were detected only in the day 27 IL-$4^{+/+}$ LN cells after longer exposure of the autoradiogram. Anti–IL-4–treated BALB/c mice did show a significant reduction in IL-4 transcripts relative to the untreated mouse at 8 weeks after infection. Amounts of IL-10, IFN-γ, IL-12p40, TNF-α, and TGF-β1 mRNA were

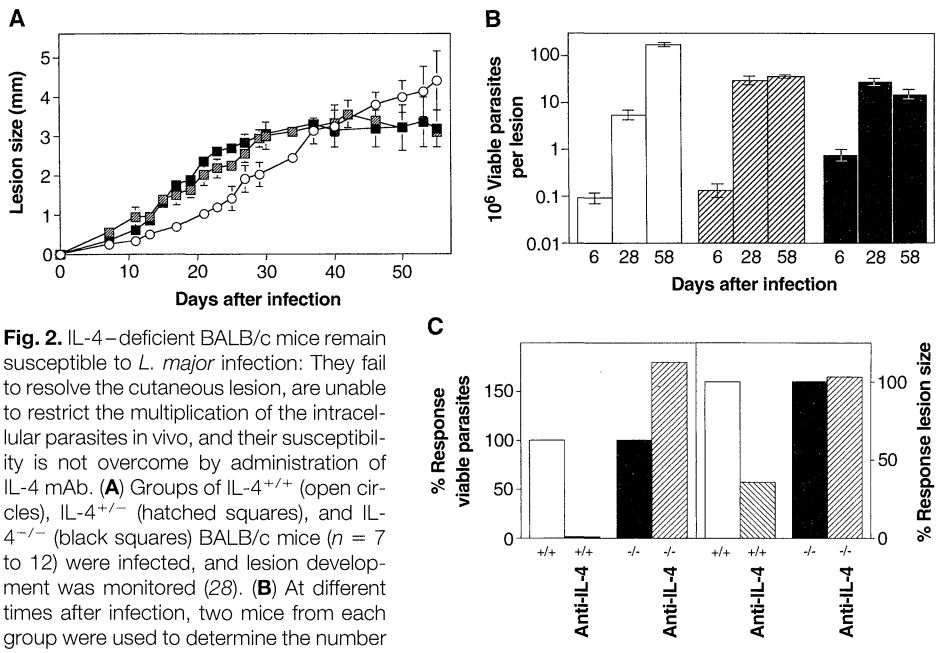

Fig. 2. IL-4–deficient BALB/c mice remain susceptible to *L. major* infection: They fail to resolve the cutaneous lesion, are unable to restrict the multiplication of the intracellular parasites in vivo, and their susceptibility is not overcome by administration of IL-4 mAb. (**A**) Groups of IL-$4^{+/+}$ (open circles), IL-$4^{+/-}$ (hatched squares), and IL-$4^{-/-}$ (black squares) BALB/c mice (n = 7 to 12) were infected, and lesion development was monitored (*28*). (**B**) At different times after infection, two mice from each group were used to determine the number of living *L. major* in the infected footpads by a parasite limiting-dilution assay (*28*). There was no significant difference in the parasite load between the three groups (P = 0.5591, analysis of variance). The development of lesions and the parasite load were determined in three experiments, data from one course of infection are shown. IL-$4^{+/+}$ open bars; IL-$4^{+/-}$ hatched bars; IL-$4^{-/-}$ black bars. (**C**) A total of 10 mg of IL-4 mAb (11B11) was injected intraperitoneally on day 2 and day 1 before infection of BALB/c wild-type and IL-$4^{-/-}$ mice with *L. major* promastigotes, and the effect of this treatment on the parasite load (left panel) and on the lesion size (right panel) in the four different groups of mice was determined 59 days after infection.

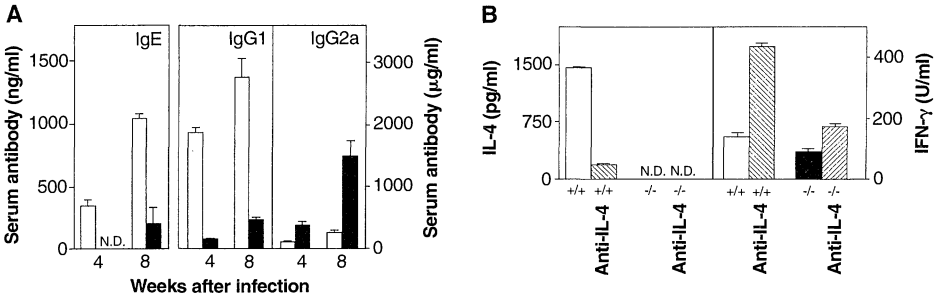

Fig. 3. Serum antibody concentrations and the cytokine profile in *L. major*–infected wild-type and IL-4–deficient BALB/c mice during the course of cutaneous leishmaniasis, and direct comparison of the effects of anti–IL-4 on cytokine secretion. (**A**) Serum antibody concentrations were determined in *L. major*–infected wild-type (open bars) and IL-4–deficient BALB/c (black bars) mice (*29*). (**B**) Mice were treated as described in Fig. 2C, and the effects of anti–IL-4 administration on cytokine secretion by popliteal lymph node (LN) cells from *L. major*–infected BALB/c wild-type and IL-$4^{-/-}$ mice were determined 59 days after infection (*29*). T_H2 cytokine release by LN and spleen cells from anti–IL-4–treated and untreated mice was due to $CD4^+$ T cells, because treatment with CD4 mAb and complement abrogated the response (*12*).

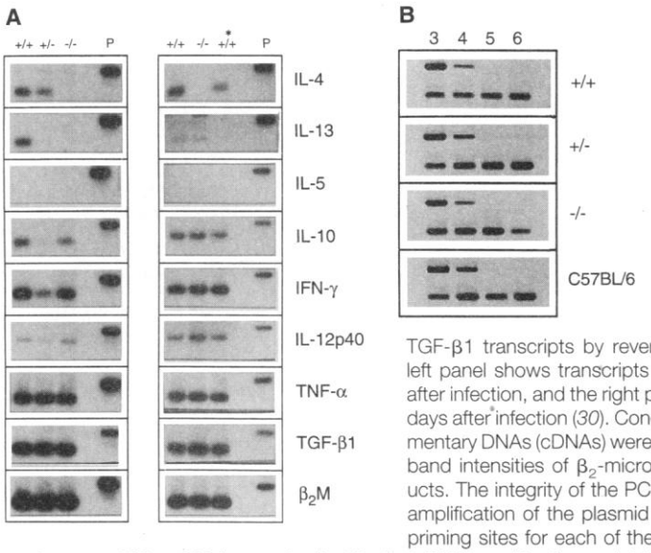

Fig. 4. (**A**) Cytokine mRNA profile in *L. major*–infected wild-type, IL-4–deficient, and anti–IL-4–treated BALB/c mice. LN draining the site of infection from individual IL-4$^{+/+}$, IL-4$^{+/-}$, IL-4$^{-/-}$, and anti–IL-4–treated mice (indicated by asterisk) (see Fig. 2C) were analyzed for IL-4, IL-13, IL-5, IL-10, IFN-γ, IL-12p40, TNF-α, and TGF-β1 transcripts by reverse-transcriptase PCR. The left panel shows transcripts from LN removed 27 days after infection, and the right panel is from LN removed 59 days after infection (*30*). Concentrations of input complementary DNAs (cDNAs) were equalized by comparing the band intensities of β_2-microglobulin amplification products. The integrity of the PCR was controlled by parallel amplification of the plasmid pMus3 (P), which contains priming sites for each of the target cytokines. Products are between 200 to 300 base pairs (bp) for the cDNA amplification and 440 bp for pMus3. (**B**) TGF-β1 transcripts from spleens of day 27–infected IL-4$^{+/+}$, IL-4$^{+/-}$, and IL-4$^{-/-}$ BALB/c and wild-type C57BL/6 mice were compared by quantitative PCR by titrating amounts of plasmid pMus3 in competition with a constant input of cDNA. The upper band is pMus3 in fourfold dilutions (3 to 6) ranging from 240 pg (dilution 3) to 3.75 pg (dilution 6), in competition with a constant input of 60 ng of cDNA. At equivalence, pMus3 and cDNA products reveal bands of equal intensities.

comparable among control, IL-4 mutant, and anti–IL-4–treated mice. With a sensitive quantitative PCR technique, amounts of TGF-β1 transcripts were also comparable among the IL-4 mutants and the genetically resistant C57BL/6 mice (Fig. 4B). Neither the pattern of *Leishmania*-induced cytokine secretion nor the direct analysis of cytokine transcripts showed a shift to a T_H1 cytokine profile in IL-4 mutant mice.

Despite significant correlations between T_H2 responses and disease progression (*3*, *17*), the exact role of IL-4 in vivo is unclear; indeed, both disease-promoting as well as protective functions have been reported. Expression of an IL-4 transgene in resistant mice rendered them susceptible (*9*). Injection of anti–IL-4 in the beginning of infection allowed healing and development of protective immunity in susceptible BALB/c mice (*18*). Even established T_H2 responses in infected BALB/c mice could be switched to a T_H1 response by a combination of IL-4 neutralization and reduction of the parasite burden (*19*). Injection of IL-4 around the lesion surprisingly inhibited the development of established *L. major* infections and rendered BALB/c mice resistant to reinfection (*20*), and administration of IL-4 in ongoing lesions starting at 5 weeks after infection decreased lesion size and parasite numbers (*21*). Infusion of exogenous IL-4 did not, however, establish T_H2 responses in genetically resistant mice (*22*). Furthermore, chimeric mouse studies recently showed that neither IL-4 production nor the onset of a T_H2 cytokine profile is sufficient to promote disease (*23*). In both genetically susceptible BALB/c and resistant C57BL/6 mice the cytokine mRNA amounts are very similar early in infection (*3*). The genetic background of the inbred strains of mice can determine differences in T cell signaling pathways (*24*), and T cells transgenic for the D011.10 T cell receptor derived from a BALB/c background secreted less IFN-γ and more IL-4 than those derived from a B10.D2 background (*25*).

The initial cytokine milieu is thought to be important for the differentiation of T helper precursors. The biological effects of cytokines are transmitted by signaling through membrane-bound receptors, but the precise signaling mechanisms underlying the development of different T helper phenotypes in resistant and susceptible mice after *L. major* infection are not fully understood.

The pleiotropic nature of IL-4 and the redundancy of the cytokines require caution in interpreting results obtained with cytokine-deficient mice. Regardless of the mechanism involved, it is clear that IL-4 alone is not responsible for the susceptibility of BALB/c mice to infection with *L. major*. The hypothesis that susceptibility to disease is due to a preferential expansion of IL-4–producing T_H2 cells must be revised on the basis of the data presented here.

REFERENCES AND NOTES

1. J. G. Howard, *Int. Rev. Exp. Pathol.* **28**, 79 (1986); F. Y. Liew, *Immunol. Today* **10**, 40 (1989).
2. F. P. Heinzel, M. D. Sadick, B. J. Holaday, R. L. Coffman, R. M. Locksley, *J. Exp. Med.* **169**, 59 (1989); P. Scott, *Exp. Parasitol.* **68**, 369 (1989); F. P. Heinzel, M. D. Sadick, S. S. Mutha, R. M. Locksley, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7011 (1991); Z.-E. Wang, S. L. Reiner, S. Zheng, D. K. Dalton, R. M. Locksley, *J. Exp. Med.* **179**, 1367 (1994).
3. S. L. Reiner and R. M. Locksley, *Annu. Rev. Immunol.* **13**, 151 (1995).
4. T. F. Gajewski and F. W. Fitch, *J. Immunol.* **140**, 4245 (1988); T. R. Mosmann and R. L. Coffman, *Annu. Rev. Immunol.* **7**, 145 (1989); G. G. LeGros, S. S. Ben-Sasson, R. Seder, F. D. Finkelman, W. E. Paul, *J. Exp. Med.* **172**, 921 (1990); S. L. Swain, A. D. Weinberg, M. English, G. Huston, *J. Immunol.* **145**, 3796 (1990).
5. F. Y. Liew and C. A. O'Donnel, *Adv. Parasitol.* **32**, 162 (1993).
6. N. Noben-Trauth, G. Köhler, K. Bürki, B. Ledermann, *Transgenic Res.*, in press.
7. R. Kühn, K. Rajewsky, W. Müller, *Science* **254**, 707 (1991).
8. M. Kopf *et al.*, *Nature* **362**, 245 (1993).
9. L. M. Leal, D. W. Moss, R. Kühn, W. Müller, F. Y. Liew, *Eur. J. Immunol.* **23**, 566 (1993).
10. O. Kanagawa, B. A. Vaupel, S. Gayama, G. Köhler, M. Kopf, *Science* **262**, 240 (1993); R. A. Morawetz *et al.*, *ibid.* **265**, 264 (1994).
11. B. Mock, J. Blackwell, J. Hilgers, M. Potter, C. Nacy, *Eur. J. Immunogenet.* **20**, 335 (1993); N. Noben-Trauth *et al.*, in preparation; I. C. Gerling *et al.*, *Mamm. Genome* **5**, 318 (1994).
12. P. Kropf, data not shown.
13. R. L. Coffman, D. A. Lebman, P. Rothman, *Adv. Immunol.* **54**, 229 (1993).
14. T. von der Weid, M. Kopf, G. Köhler, J. Langhorne, *Eur. J. Immunol.* **24**, 2285 (1994); S. Hjulström, A. Landlin, L. Jansson, R. Holmdahl, B. Heyman, *ibid.* **25**, 1469 (1995).
15. R. Powrie, S. Menon, R. L. Coffman, *ibid.* **23**, 2223 (1993); T. R. Mosmann and K. W. Moore, *Immunoparasitol. Today* **12/7**, A49 (1991); A. Ding *et al.*, *J. Immunol.* **145**, 940 (1990); C. A. Nacy, B. J. Nelson, M. S. Meltzer, S. J. Green, *Res. Immunol.* **142**, 573 (1991); I. P. Oswald, R. T. Gazzinelli, A. Sher, S. L. James, *J. Immunol.* **148**, 3578 (1992).
16. X. Wei *et al.*, *Nature* **375**, 408 (1995).
17. R. M. Locksley and P. Scott, *Immunoparasitol. Today* **12/7**, A58 (1991).
18. M. D. Sadick *et al.*, *J. Exp. Med.* **171**, 115 (1990); I. Müller, T. Pedrazzini, P. Kropf, J. Louis, G. Milon, *Int. Immunol.* **3**, 587 (1991).
19. G. S. Nabors and J. P. Farrell, *Infect. Immun.* **62**, 5498 (1994).
20. K. C. Carter, G. Gallagher, A. J. Baillie, J. Alexander, *Eur. J. Immunol.* **19**, 779 (1989).
21. C. M. Lezama-Davila, D. M. Williams, G. Gallagher, J. Alexander, *Parasite Immunol.* **14**, 37 (1992).
22. M. D. Sadick, N. Street, T. Mosmann, R. M. Locksley, *Infect. Immun.* **59**, 4710 (1991); R. Chatelain, K. Varkila, R. L. Coffman, *J. Immunol.* **148**, 1182 (1992).
23. A. H. Shankar and R. G. Titus, *J. Exp. Med.* **181**, 845 (1995).
24. S. J. Szabo, N. G. Jacobson, A. S. Dighe, U. Gubler, K. M. Murphy, *Immunity* **2**, 665 (1995).
25. C.-S. Hsieh, S. E. Macatonia, A. O'Garra, K. M. Murphy, *J. Exp. Med.* **181**, 713 (1995).
26. IL-4 primer sequences obtained from exon 3 amplify a 1315–base pair (bp) product for the disrupted allele and a 175-bp fragment for the wild-type allele. The primer sequences were IL-4for: 5′-GTGAGCAGATGACATGGGGC-3′ and IL-4rev: 5′-CTTCAAGCATGGAGTTTTCCC-3′. The cycling conditions were 20 s at 94°C, 20 s at 59°C, and 3 min at 72°C for five cycles followed by 28 cycles of 20 s at 94°C, 20 s at 59°C, and 2.5 min at 72°C.
27. Naïve spleen cells (1×10^7 per milliliter) were stimulated with immobilized mAb to CD3 (1 μg/ml) [O. Leo, M. Foo, D. H. Sachs, L. E. Samelson, J. A. Bluestone, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1374 (1987)] in 24-well tissue culture plates. Culture supernatants were collected 24 and 72 hours later and assayed for the presence of T_H1 or T_H2 cytokines by enzyme-linked immunosorbent assay (ELISA) with paired mAbs {IFN-γ [S. S. Slade and J. Langhorne, *Immunobiology* **179**, 353 (1989)], IL-10 (PharMingen), and IL-4, IL-5 (Endogen)}. The detection limits were 1 U/ml for IFN-γ, 3 U/ml for IL-5, 7 pg/ml for

IL-4, and 40 pg/ml for IL-10.

28. Mice were infected subcutaneously in one hind footpad with 2×10^6 *L. major* LV 39 promastigotes (MRHO/SU/59/P strain). Lesion development was monitored with a dial gauge caliper (Schnelltaster, Kröplin, Germany) and expressed as lesion size = thickness of the infected footpad minus the uninfected contralateral footpad. For the determination of living parasites [R. G. Titus, M. Marchand, T. Boon, J. A. Louis, *Parasite Immunol.* **7**, 545 (1985)], footpad tissues from infected mice were homogenized, and serial 10-fold dilutions were distributed in wells of microtiter plates containing rabbit blood agar slants. After 10 to 14 days of incubation at 26°C, the wells containing growing promastigotes were identified by microscopic examination. The frequency of living parasites recovered from infected footpads was determined by minimum χ^2 analysis applied to a Poisson distribution.
29. Mice were infected as described in Fig. 2. Sera were collected at different times after infection, and the serum concentration of the indicated Ig isotypes were determined by ELISA according to standard protocols. The detection limit for IgG1 and IgG2a were 0.5 ng/ml (Southern Biotechnology), and the detection limit for IgE was 30 ng/ml (PharMingen). Popliteal LN cells (1×10^7 per milliliter) were stimulated with 4×10^6 live *L. major* promastigotes as antigen in 24-well plates for the determination of antigen-specific cytokine production. Nonstimulated and anti-CD3–stimulated (1 μg/ml) cells were used as controls (*12*).
30. Popliteal LN from individual IL-4 control, mutant, and anti–IL-4 (11B11)–treated mice were removed on days 27 and 59 of infection, flash frozen in liquid nitrogen, and homogenized, and total RNA was extracted in Ultraspec (Biotecx). The RNA was treated with DNase I (Gibco, BRL), and 5 μg was reverse-transcribed with murine Moloney leukemia virus reverse transcriptase and oligo$(dT)_{15}$ primers (Superscript II amplification system, Gibco, BRL). For β_2-microglobulin amplification, 5 ng of cDNA per reaction was used and 60 ng was used to amplify cytokine transcripts. The cycling conditions were 20 s at 94°C, 30 s at 56°C, and 30 s at 72°C for 35 cycles. 1 μCi of [α-^{32}P]deoxycytidine triphosphate was included per reaction. Half of the PCR products were run through a 2% agarose gel. Band intensities were quantitated with a Fujix Bio-Imaging Analyzer BAS100 equipped with MacBase software.
31. We thank F. Fitch, J. Augers, and J. Bluestone for their help, advice, and generosity in letting us use their FACScan; W. E. Paul for the gift of IL-4 mAb 11B11; D. Shire, Sanofi Recherche, France, for primer sequences and plasmid pMus3; K. Geiger for help with the statistics; B. Ledermann for helpful discussions; and R. Etges for his comments on the manuscript. Partially supported by funds from the University of Notre Dame (I.M. and P.K.), NIH grant NIH-1R29 AI37636-01 (I.M.), and the American Cancer Society and United States Public Health Service (B. Knowles). N.N.-T. was supported by a postdoctoral fellowship from the National Cancer Institute.

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Role of a Peptide Tagging System in Degradation of Proteins Synthesized from Damaged Messenger RNA

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Variants of λ repressor and cytochrome b_{562} translated from messenger RNAs without stop codons were modified by carboxyl terminal addition of an *ssrA*-encoded peptide tag and subsequently degraded by carboxyl terminal–specific proteases present in both the cytoplasm and periplasm of *Escherichia coli*. The tag appears to be added to the carboxyl terminus of the nascent polypeptide chain by cotranslational switching of the ribosome from the damaged messenger RNA to *ssrA* RNA.

Although many intracellular proteases have been identified (*1*), the determinants that render certain proteins the targets of particular proteases are generally not known. Studies of recombinant interleukin-6 (IL-6) purified from inclusion bodies of *Escherichia coli* revealed that some IL-6 molecules are modified by COOH-terminal truncation at different positions in the sequence and addition of an 11-residue peptide tag (AANDENYALAA) (*2*) at the truncation point (*3*). The last 10 residues of this peptide tag are encoded by the *ssrA* gene of *E. coli*, and tagging of IL-6 does not occur in *ssrA*$^-$ cells. The *ssrA* transcript is a stable 362-nucleotide RNA molecule that exhibits some tRNA-like properties and can be charged with alanine (*4*). The COOH-terminal residues of the *ssrA*-encoded peptide tag (YALAA) are similar to a COOH-terminal tail sequence (WVAAA) recognized by Tsp (*5*), a periplasmic endoprotease, and by a cytoplasmic protease that also degrades proteins in a tail-dependent manner (*6*). Thus, we reasoned that tagging with the *ssrA*-encoded peptide might target proteins for degradation by COOH-terminal–specific proteases.

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To test this hypothesis, we constructed genes encoding variants of the NH_2-terminal domain (residues 1 to 93) of λ repressor (a cytoplasmic protein) and cytochrome b_{562} (a periplasmic protein) in which the *ssrA* peptide tag sequence (AANDENYALAA) was encoded at the DNA level as a COOH-terminal tail (*7*). Variants of each protein were also constructed with a control tag (AANDENYALDD), because both Tsp and its cytoplasmic counterpart do not cleave proteins with charged residues at the COOH-terminus (*5*, *6*). In pulse-chase experiments (Fig. 1, A and B), the λ repressor and cytochrome b_{562} proteins with the *ssrA* peptide tag were degraded with half-lives of <5 min, whereas proteins with the control tag had half-lives of >1 hour. When the *ssrA* peptide–tagged cytochrome b_{562} protein was expressed in an *E. coli* strain deleted for the *tsp* gene, its half-life increased to >1 hour (Fig. 1C). As expected, degradation of the *ssrA* peptide–tagged λ repressor protein in the cytoplasm was not affected in the *tsp*$^-$ strain (Table 1). Thus, the presence of the *ssrA* peptide tag at the COOH-termini of these proteins resulted in rapid intracellular degradation in both the cytoplasmic and periplasmic compartments of the cell. In the periplasm, this rapid degradation required the presence of Tsp. Incubation of the purified variant cytochrome proteins with Tsp in vitro (*8*) resulted in cleavage of the *ssrA* peptide–tagged variant but not of the control peptide–tagged variant (Fig. 1D), thus supporting a direct role for Tsp in the periplasmic degradation of *ssrA* peptide–tagged substrates in vivo (*9*).

Figure 1E shows a model that addresses the mechanisms by which the *ssrA*-encoded tag sequence may be added to the COOH-terminus of a protein and by which specific proteins in the cell may be chosen for modification by peptide tagging, as well as the

Table 1. Half-lives of the λ repressor and cytochrome b_{562} constructs in *tsp*$^+$ *ssrA*$^+$ (X90), *tsp*$^-$ *ssrA*$^+$ (KS1000), and *tsp*$^+$ *ssrA*$^-$ (X90 *ssrA1::cat*) strains. ND, not determined.

Protein construct	Half-life (min)		
	tsp$^+$ *ssrA*$^+$	*tsp*$^-$ *ssrA*$^+$	*tsp*$^+$ *ssrA*$^-$
λ repressor(1–93)–AANDENYALAA	4	4	ND
λ repressor(1–93)–AANDENYALDD	>60	>60	ND
λ repressor(1–93)–M2-H_6-*trpAt*	2	ND	>60
Cyt b_{562}–AANDENYALAA	4	>60	ND
Cyt b_{562}–AANDENYALDD	>60	>60	ND
Cyt b_{562}–*trpAt*	<0.5	~30	ND
Cyt b_{562}–M2-H_6-*trpAt*	<0.5*	~40	~60

*The half-life is that of the full-length protein; a metastable proteolytic intermediate is produced that is degraded with a half-life of ~15 min.