5. A. W. Simpson and P. H. Hodkinson, *Nature* **237**, 320 (1972).
6. S. T. Schober, J. Friedrich, A. Altmann, *J. Appl. Phys.* **71**, 2206 (1992).
7. C. A. Murray and D. G. Grier, *Annu. Rev. Phys. Chem.* **47**, 421 (1996).
8. A. van Blaaderen, R. Ruel, P. Wiltzius, *Nature* **385**, 321 (1997).
9. P. W. Green, R. R. A. Syms, E. M. Yeatman, *J. Microelectromech. Syst.* **4**, 170 (1995).
10. We estimated the interfacial free energy of the alloy/aqueous KBr interface using data from F. H. Howie and E. D. Hondros, *J. Mater. Science* **17**, 1434 (1982); M. A. Carroll and M. E. Warwick, *Mater. Sci. Technol.* **3**, 1040 (1987); and *Handbook of Chemistry and Physics*, D. R. Lide, Ed. (CRC Press, Boston, ed. 71, 1990), pp. 4-137 to 4-143. A liquid with a lower surface tension, such as the hydrophobic lubricant used in (*2*) and (*4*), was unable to generate capillary forces that were strong enough to hold objects together.
11. The designs selected for the components were used to fabricate machined aluminum masters; these masters were used to fabricate an elastomeric mold. We placed the masters in a petri dish, covered them with a liquid polydimethylsiloxane prepolymer (Sylgard 184, DuPont), and cured the prepolymer with heat (60°C, 30 min). Subsequent removal of the aluminum masters left an elastomeric mold consisting of wells in the shape of the aluminum masters. We filled the wells with a liquid PU prepolymer (NOA 73, Norland), cured the polymer by exposure to ultraviolet light (45 min), and removed the solid PU pieces. We patterned selected faces of these objects using adhesive copper foil (Scotch Brand Electrical Tape, 3M Corp., St. Paul, MN). This tape was cut to size and applied to the selected planes (or portions of these planes) of the polyhedra. This process was labor-intensive and limited the number of objects we were willing to examine in any experiment; it also limited these objects to sizes greater than 5 mm. The pattern of copper foil was coated with molten alloy (Bismuth Alloy 117, melting point: 47°C, Small Parts, Miami Lakes, FL) by dipping the pieces into a beaker containing molten alloy (under an aqueous acetic acid solution at pH 4 to dissolve oxidized alloy). Cooling left the faces of the copper foil coated with a film (~0.5 mm thick) of solid alloy. Self-assembly was allowed to take place in a 500-ml Morton flask filled with an aqueous solution of KBr. Acetic acid (3 ml) was added to dissolve oxide from the surface of the alloy. The flask and its contents were rotated horizontally with a rotary evaporator motor at 5 to 10 rpm and heated to 60°C in an oil bath. A typical assembly was complete after ~1 hour of agitation. We reduced the intensity of agitation about halfway through the experiment to obtain complete assemblies of the component pieces by increasing the concentration and thus the density (ranging from 1.1 to 1.3 g/ml) of the aqueous KBr solution.
12. Fragility resulted from delamination of tape from polymer, not fracture of alloy connections.
13. We assembled each array twice (with the exception of the dodecahedra, which were assembled three times). With the exception of the dodecahedra, the arrays incorporated all the starting pieces; for the dodecahedra, an excess of pieces was required. If we broke apart an array during assembly with a brief increase in rotation speed, the resulting fragments reassembled into a crystalline, monolithic array in ~1 hour.
14. N. Bowden, I. S. Choi, B. Grzybowski, G. M. Whitesides, *J. Am. Chem. Soc.*, in press.
15. G. M. Whitesides, *Sci. Am.* **273**, 146 (September 1995).
16. We thank S. Brittain for photography and K. Paul for discussions. Supported by the DARPA and the NSF. T.L.B. and S.R.J.O. thank the NSERC of Canada for postdoctoral fellowships; J.T. acknowledges the NSF for a predoctoral fellowship. T.H. was supported by the MRSEC program of the NSF under award number DMR-9400396.

9 February 1999; accepted 29 March 1999



# Requirement for Type 2 NO Synthase for IL-12 Signaling in Innate Immunity

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Interleukin-12 (IL-12) and type 2 NO synthase (NOS2) are crucial for defense against bacterial and parasitic pathogens, but their relationship in innate immunity is unknown. In the absence of NOS2 activity, IL-12 was unable to prevent spreading of *Leishmania* parasites, did not stimulate natural killer (NK) cells for cytotoxicity or interferon-γ (IFN-γ) release, and failed to activate Tyk2 kinase and to tyrosine phosphorylate Stat4 (the central signal transducer of IL-12) in NK cells. Activation of Tyk2 in NK cells by IFN-α/β also required NOS2. Thus, NOS2-derived NO is a prerequisite for cytokine signaling and function in innate immunity.

The innate immune response to bacteria and protozoan parasites is characterized by the rapid recognition of microbial antigens, after which activated inflammatory cells release soluble mediators and antimicrobial effector molecules (*1*). The early production of IL-12 by granulocytes, macrophages, or dendritic cells stimulates the cytotoxic activity of NK cells and enhances their release of IFN-γ. Along with IL-12, IFN-γ then facilitates the later development of type 1 T helper cells ($T_H1$ cells) that ultimately activate macrophages for the destruction of intracellular pathogens through cognate interactions and further secretion of IFN-γ (*2*). A key antimicrobial agent implicated in this killing process is nitric oxide (NO) generated from the amino acid L-arginine by the inducible isoform of NO synthase (called iNOS or NOS2) (*3*). NOS2-derived NO can also regulate T cell proliferation, cytokine production, apoptosis, and signaling activity in vitro (*4–6*) and in vivo (*7–9*). In genetically resistant mice cutaneously infected with the protozoan parasite *Leishmania major*, the inhibition of early parasite spreading, the up-regulation of IFN-γ, and the induction of NK cell cytotoxicity at day 1 of infection were abolished after genetic deletion or functional inactivation of NOS2 (*9*), a response that is similar to that of mice after neutralization of IL-12 (*10*). This suggests that NOS2 deficiency might affect the availability of IL-12. Although the baseline expression of IL-12 subunit p40 mRNA in naïve $NOS2^{-/-}$ mice was lower as compared to that of $NOS2^{+/+}$ mice, the production of active IL-12 p70 heterodimers by inflammatory macrophages was not reduced in the absence of NOS2 (*9*). Thus, both NO/NOS2 and IL-12 regulate the innate response to *L. major*, but whether and how they might interact in vivo remained unknown. We show here that NO/NOS2 is an integral part of the IL-12 signaling cascade in NK cells and therefore constitutes a prerequisite for the function of IL-12 in innate immunity.

We initially analyzed whether responsiveness to exogenous IL-12 in vivo required NOS2 activity. Wild-type $NOS2^{+/+}$ mice (129/SvEv × C57BL/6) were cutaneously infected with *L. major* promastigotes (*11*). At 24 hours, the parasites were found at the site of inoculation and in the draining popliteal lymph node (pLN). In NOS2-deficient mice (*9*, *12*), in contrast, the parasites were disseminated to visceral organs, even after treatment with IL-12 (*13*) (Fig. 1A). In genetically susceptible BALB/c mice, which lack functional IL-12 early during infection (*10*, *14*), treatment with IL-12 prevented parasite spreading at day 1 of infection (Fig. 1A). However, simultaneous application of L-$N^6$-iminoethyl-lysine (L-NIL) (*13*), a potent competitive inhibitor of NOS2 (*9*), reversed the protective effect of IL-12 (Fig. 1A). Treatment of $NOS2^{+/+}$ mice with IL-12 increased expression of IFN-γ mRNA and NK cell cytotoxic activity in the pLN after infection, whereas no such effect was observed in $NOS2^{-/-}$ mice or in $NOS2^{+/+}$ mice injected with L-NIL (Fig. 1, B and C). Similar amounts of IL-12 receptor β1 and β2 mRNA (*15*) were detected in the pLN of $NOS2^{+/+}$ and $NOS2^{-/-}$ mice at day 1 of infection (Fig. 1D), indicating that the impaired parasite control, IFN-γ expression, and NK cell activity in $NOS2^{-/-}$ mice at day 1 of infection (*9*) resulted from an inability to respond to IL-12 but not from a lack of IL-12

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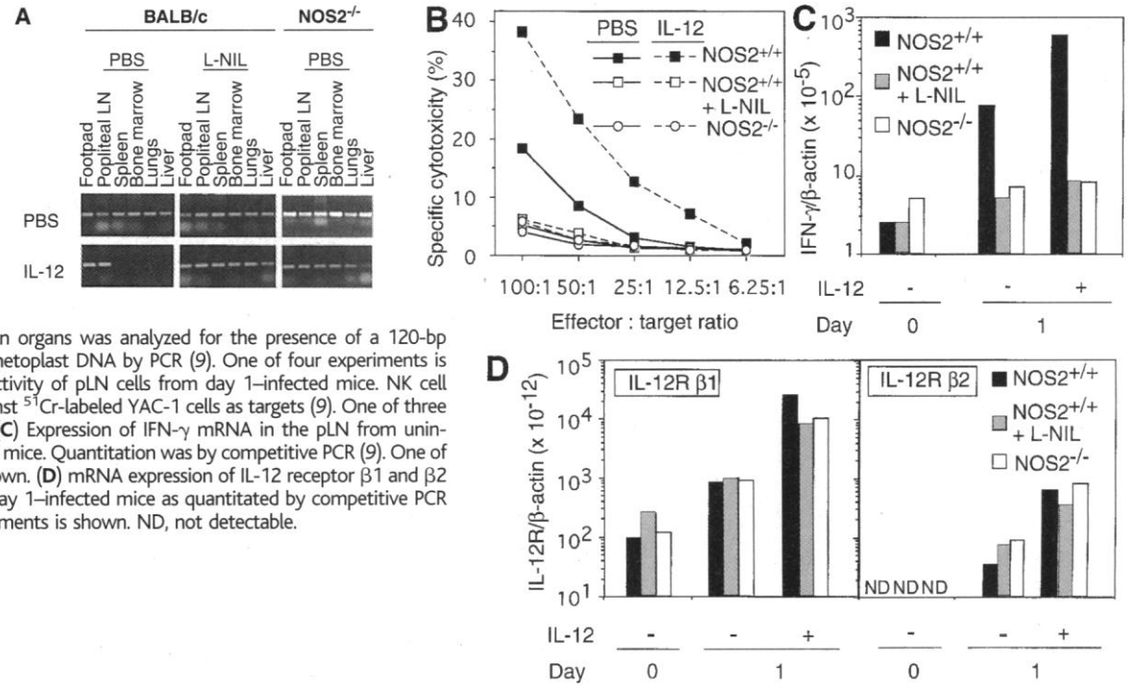


**Fig. 1.** NOS2 is required for IL-12 responsiveness in vivo. NOS2$^{+/+}$ or NOS2$^{-/-}$ mice (129/SvEv × C57BL/6) (*9*, *12*) or BALB/c mice were infected with *L. major* (*11*) with or without application of PBS, rmIL-12, or L-NIL (*13*). (**A**) Parasite spreading in *L. major*–infected mice at 24 hours of infection. DNA from given organs was analyzed for the presence of a 120-bp fragment of *L. major* kinetoplast DNA by PCR (*9*). One of four experiments is shown. (**B**) Cytotoxic activity of pLN cells from day 1–infected mice. NK cell activity was tested against $^{51}$Cr-labeled YAC-1 cells as targets (*9*). One of three experiments is shown. (**C**) Expression of IFN-γ mRNA in the pLN from uninfected or day 1–infected mice. Quantitation was by competitive PCR (*9*). One of three experiments is shown. (**D**) mRNA expression of IL-12 receptor β1 and β2 chain in uninfected or day 1–infected mice as quantitated by competitive PCR (*15*). One of two experiments is shown. ND, not detectable.

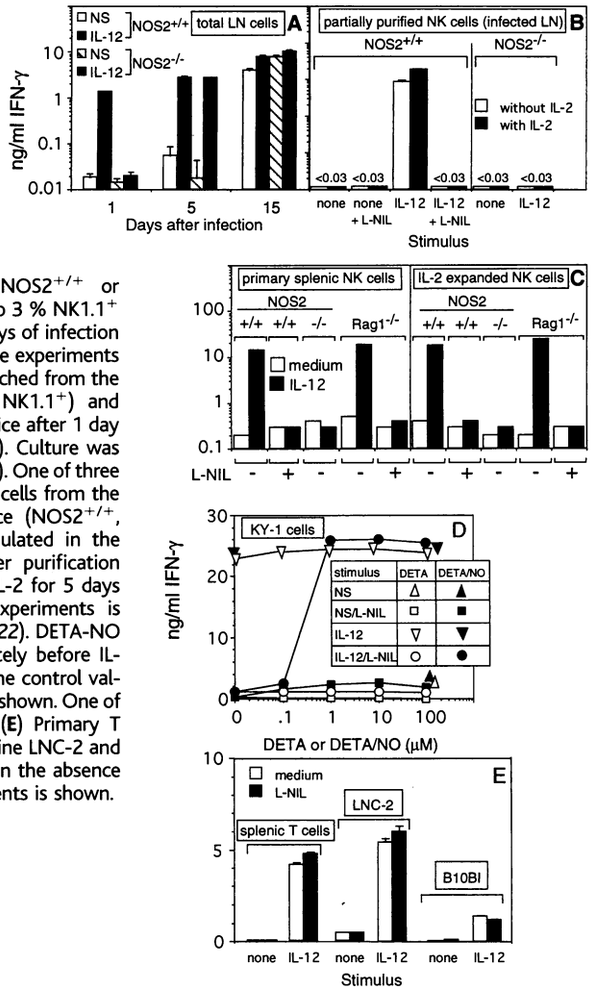


**Fig. 2.** NOS2 is required for IL-12–induced IFN-γ production by NK cells. pLN cells, NK cells, or T$_H$1 cells were stimulated with IL-12 (100 pg/ml). The NOS2 inhibitor L-NIL (1 mM) was added ≥2 hours before stimulation. After 24 hours, IFN-γ was determined in the culture supernatants by ELISA (sensitivity 19.5 to 39 pg/ml) (*9*). (**A**) Total pLN cells from NOS2$^{+/+}$ or NOS2$^{-/-}$ mice (each with 1 to 3 % NK1.1$^+$ CD3$^-$ cells) after 1, 5, or 15 days of infection with *L. major* (*11*). One of three experiments is shown. (**B**) NK cells (*18*) enriched from the pLN of NOS2$^{+/+}$ (10.7 % NK1.1$^+$) and NOS2$^{-/-}$ (10.4 % NK1.1$^+$) mice after 1 day of infection with *L.* major (*11*). Culture was with or without IL-2 (100 U/ml). One of three experiments is shown. (**C**) NK cells from the spleen of naïve C57BL/6 mice (NOS2$^{+/+}$, NOS2$^{-/-}$, or Rag1$^{-/-}$), stimulated in the presence of IL-2 directly after purification (*19*) or after expansion with IL-2 for 5 days (*20*). One of two to five experiments is shown. (**D**) KY-1 NK cells (*21*, *22*). DETA-NO or DETA was added immediately before IL-12. For clarity, only some of the control values in the absence of L-NIL are shown. One of three experiments is shown. (**E**) Primary T cells (*24*) or CD4+ T$_H$1 cells [line LNC-2 and clone B10BI (*25*)], stimulated in the absence of IL-2. One of three experiments is shown.

receptor expression.

The primary source of IFN-γ during the innate response to *Leishmania* is the NK cell (*16*). We therefore tested the production of IFN-γ by various NK cell populations for its dependency on NOS2-derived NO upon stimulation with IL-12. By flow cytometry analysis, total pLN cells from day 1–infected NOS2$^{+/+}$ and NOS2$^{-/-}$ mice always contained a similar percentage (1 to 3%) of NK1.1$^+$ CD3$^-$ cells (*17*). NOS2$^{+/+}$ pLN cells produced up to 2 to 5 ng of IFN-γ per milliliter after stimulation with 0.1 to 10 ng of IL-12 per milliliter, whereas less than 200 pg/ml or no IFN-γ was detectable in cultures from NOS2$^{-/-}$ mice or in L-NIL–treated cultures from NOS2$^{+/+}$ mice (Fig. 2A). The unresponsiveness of NOS2$^{-/-}$ cells was restricted to day 1 of infection and was not due to an up-regulation of endogenous transforming growth factor–β (*17*). At later time points, during the expansion of IFN-γ–producing T$_H$1 cells, wild-type and NOS2-deficient pLN cells responded equally well to IL-12 (Fig. 2A). Also, when the pLN cells were stimulated with a T cell mitogen (concanavalin A) instead of IL-12, the production of IFN-γ by NOS2$^{+/+}$ at day 1 to 21 of infection was indistinguishable from that of NOS2$^{-/-}$ cells (*17*). These data suggest NOS2-dependent IFN-γ production by early responding NK cells rather than T cells.

Next, we partially purified NK cells from pLN cell suspensions of day 1–infected mice (*18*). The NK cell–enriched population from NOS2$^{+/+}$ mice showed high production of IFN-γ after stimulation with IL-12 (in the presence or absence of IL-2) that was completely

blocked by L-NIL. The corresponding cell population from NOS2$^{-/-}$ mice was comparably composed but did not respond to IL-12 (Fig. 2B). Similar results were obtained (i) with NK1.1$^{+}$ CD3$^{-}$ cells purified to near homogeneity from the spleen of noninfected C57BL/6 (NOS2$^{+/+}$, NOS2$^{-/-}$, or T and B cell–free Rag1$^{-/-}$) mice (*19*) (Fig. 2C); (ii) with T cell–depleted and IL-2–expanded adherent splenic NK1.1$^{+}$ CD3$^{-}$ NK cells derived from naïve NOS2$^{+/+}$ or NOS2$^{-/-}$ mice (*20*) (Fig. 2C); and (iii) with the mouse NK cell clone KY-1 (*21*, *22*). IL-12 (≥0.1 pg/ml) strongly upregulated the IFN-γ release by KY-1 cells, which was blocked by L-NIL [Fig. 2D and Web Fig. 1 (*23*)] but not by L-nitroarginine-methylester (L-NAME), which primarily inhibits constitutive NO synthases, or by the inactive analog D-NAME (*17*). The addition of small amounts (≥1 μM) of NO donors (DETA/NO or *S*-nitrosoglutathione) completely reversed the inhibitory effect of L-NIL, indicating that exogenous NO can facilitate the activation of NK cells. The control compounds diethylene-triamine (DETA) and glutathione were inactive [(Fig. 2D) and (*17*)]. Thus, IL-12–stimulated NK cell production of IFN-γ is NOS2-dependent.

IL-2–propagated KY-1 cells expressed small amounts of NOS2 protein, which was partially dependent on endogenous IFN-γ (*17*) and was increased upon stimulation with IL-12 (Fig. 3A). Treatment of KY-1 cells with IL-12 led to the accumulation of 8.8 ± 0.9 μM $NO_2^-$ within 24 hours, as compared to 2.6 ± 0.7 μM in the absence of IL-12 and 0.8 ± 0.1 μM in cultures without IL-2 and IL-12 (mean ± SEM of five experiments). L-NIL, which antagonized the IL-12–induced production of IFN-γ, did not prevent the stimulatory effect of IL-12 on NOS2 protein expression, which indicates that some functions of IL-12 are preserved in the absence of NOS2 activity (Fig. 3A). NOS2 protein was also found in wild-type (but not in NOS2$^{-/-}$) splenic NK cells expanded in IL-2 and stimulated by IL-12 (Fig. 3A). The average production of $NO_2^-$ by these cells was 12.2 ± 2.9 μM $NO_2^-$ within 48 hours, as compared to 4.9 ± 2.1 μM $NO_2^-$ in the absence of IL-12 (mean ± SEM of five experiments). In contrast to NK cells, the IL-12–induced production of IFN-γ by purified splenic T cells (*24*) and by two $T_H1$ cell clones (B10BI and LNC-2) was refractory to inhibition by L-NIL (Fig. 2E). This is in line with the lack of NOS2 in these cells (*25*). Thus, NOS2-derived NO is required for the activation of NK cells, but not T cells, by IL-12.

Because IL-12 signaling in NK cells is strictly dependent on the signal transducer and activator of transcription 4 (Stat4) (*26*), we investigated whether NOS2 activity was critical for the activation of Stat4 (*27*). Stimulation of KY-1 cells with IL-12 caused tyrosine phosphorylation of Stat4 at 15 to 90 min, which was abolished when L-NIL was added to KY-1 cells at least 2 hours before IL-12 [(Fig. 3B) (*17*)]. The same result was obtained with IL-2–expanded splenic NK cells from NOS2$^{+/+}$ versus NOS2$^{-/-}$ mice, whereas tyrosine phosphorylation of Stat4 in purified primary T cells was NOS2-independent (Fig. 3B). Tyrosine phosphorylation of Stat4 in NK cells was completely restored when L-NIL–treated cells were exposed to small amounts of DETA/NO (Fig. 3C). As in human NK cells after culture in IL-2 (*28*), KY-1 cells tyrosine phosphorylated Stat1α, but unlike Stat4, the tyrosine phosphorylation was neither induced nor modulated by IL-12 and was also not affected by inhibition of NOS2 activity (*17*). Thus, NOS2 activity is

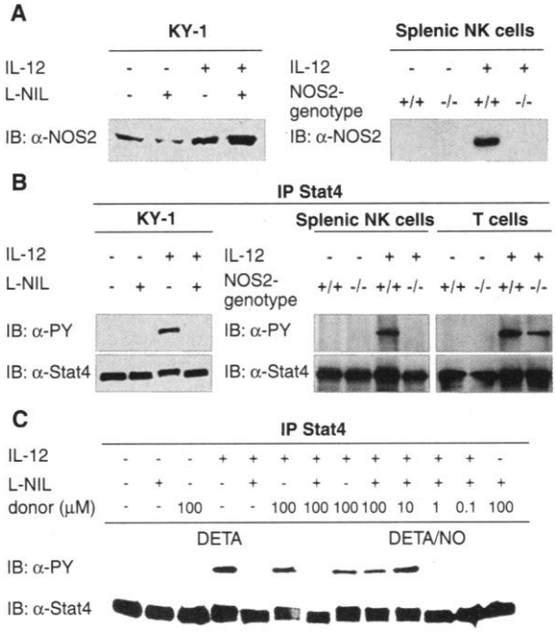


**Fig. 3.** NK cells express NOS2 activity, which is required for tyrosine phosphorylation of Stat4. KY-1 cells (*21*, *22*), IL-2–expanded splenic NK cells (*20*), or splenic T-cells (*24*) from C57BL/6 mice (NOS2$^{+/+}$ or NOS2$^{-/-}$) were stimulated with IL-12. L-NIL (1 mM) was added 24 hours before stimulation and DETA/NO or DETA was added 2 hours before stimulation. One of two to four experiments is shown. (**A**) Anti-NOS2 immunoblot (IB) after 24 hours of stimulation with IL-12 (*38*). (**B** and **C**) Detection of tyrosine-phosphorylated Stat4 and total Stat4 protein in NK or T cells after 60 min of stimulation by immunoprecipitation (IP) and subsequent immunoblotting (*27*). α, anti.

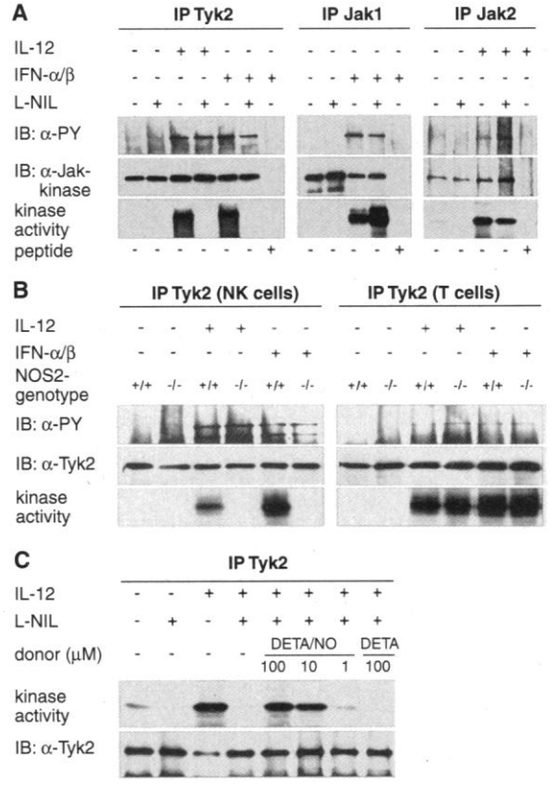


**Fig. 4.** NOS2-derived NO is required for the activation of Tyk2 kinase but not of Jak1 or Jak2 kinase. (**A** through **C**) KY-1 cells (*21*, *22*) or splenic NK (*20*) or T cells (*24*) from C57BL/6 (NOS2$^{+/+}$ or NOS2$^{-/-}$) mice were stimulated with IL-12 or IFN-α/β (±1 mM L-NIL) for 15 min, lysed, and subjected to immunoprecipitation with antibody to Jak1 (α-Jak1), α-Jak2, or α-Tyk2 (in the absence or presence of blocking peptide), followed by anti-phosphotyrosine immunoblotting (*32*), or were immunoprecipitated for in vitro autokinase assays (*33*). To control for equal loading of the lanes, blots were reprobed with α-Jak1, α-Jak2, or α-Tyk2. One of three experiments is shown. (C) NO donor (DETA/NO) or DETA was added to L-NIL–treated KY-1 cells 1 hour before IL-12.

specifically required for IL-12–stimulated Stat4 activation in NK cells.

NO is known to activate soluble guanylyl cyclase, which results in elevated concentrations of cyclic guanosine monophosphate (cGMP) and activation of cGMP-dependent kinases and ion channels (*29*). We added a potent and highly specific activator of cGMP-dependent kinases and of cGMP-gated ion channels [8-(4-chlorophenylthio)-guanosine-3′,5′-cyclic monophosphate (8-pCPT-cGMP) (*29*)] to KY-1 cells that were stimulated with IL-12 in the presence of L-NIL. 8-pCPT-cGMP did not restore tyrosine phosphorylation of Stat4 [Web Fig. 2 (*30*)]. Thus, NOS2-derived NO did not activate Stat4 via cGMP-dependent pathways.

The Janus kinases Jak2 and Tyk2 are tyrosine phosphorylated and activated in response to IL-12 and are thought to phosphorylate the Stat4 transcription factor in hematopoietic cells, including NK cells (*28*, *31*). We therefore analyzed the effect of NOS2/NO on the activation of Jak2 and Tyk2 (*32*). Stimulation of KY-1 cells with IL-12 caused tyrosine phosphorylation of Jak2 and Tyk2, which remained unaltered by L-NIL (Fig. 4A). Thus, IL-12–induced tyrosine phosphorylation of both kinases (and hence the expression of a functional IL-12 receptor) was not dependent on NOS2 activity. However, when both kinases were immunoprecipitated from KY-1 cells after stimulation with IL-12 and subsequently tested for their catalytic activity (*33*), inhibition of NOS2 by L-NIL blocked the autokinase activity of Tyk2 but not of Jak2 (Fig. 4A). These results were confirmed with IL-2–expanded splenic NK cells from $NOS2^{+/+}$ and $NOS2^{-/-}$ mice [(Fig. 4B) (*17*)]. In contrast, the induction of Tyk2 kinase activity by IL-12 in purified splenic T cells or in the $T_H1$ cell line B10BI was independent of NOS2 [(Fig. 4B) (*17*)]. Tyk2 kinase activity of KY-1 cells was restored when the intact cells (stimulated with IL-12 plus L-NIL) (Fig. 4C) or the respective anti-Tyk2 immunoprecipitates were treated with DETA/NO [Web Fig. 3 (*34*)]. DETA/NO did not alter the tyrosine phosphorylation of Tyk2 (*17*). These findings suggest that stimulation of NK cells with IL-12 first causes tyrosine (auto)phosphorylation and activation of Jak2, which then transphosphorylates Tyk2. For the activation of Tyk2 function, NOS2-derived NO is required as an independent second signal.

Finally, we tested whether Tyk2 kinase also requires NOS2/NO for activity when type I interferon (IFN-α/β) is used as an alternative stimulus (*35*). In KY-1 cells or primary splenic NK cells (from $NOS2^{+/+}$, $NOS2^{-/-}$, or $Rag1^{-/-}$ C57BL/6 mice) activated with IFN-α/β, the activity of Tyk2 kinase as well as the production of IFN-γ were dependent on endogenous NOS2 activity, whereas the activity of Jak1 remained unaffected in the absence of NOS2 [(Fig. 4, A and B) (Web Fig. 4) (*17*, *36*)]. In contrast, when NK cells were activated with IL-18, which does not signal via Tyk2 and Stat4 (*37*), the production of IFN-γ remained unaltered in the absence of NOS2 (*36*). In primary T cells or $T_H1$ cell lines (B10BI and LNC-2) IFN-α/β–mediated activation of Tyk2 kinase and IFN-γ production were NOS2-independent [(Fig. 4B) (Web Fig. 4) (*17*, *36*)]. These results are in line with the strict requirement for endogenous NOS2 for a protective function of IFN-α/β at day 1 of *L. major* infection (*9*).

Several interactions between NOS2 and IL-12 have been reported in the past, including the inhibition of macrophage IL-12 production by NO, the possible induction of the IL-12 antagonist $IL\text{-}12(p40)_2$ by NO, and the NOS2-dependent suppression of T cell responses by IL-12 (*6*, *7*). The latter might result from an inhibition of Jak2 and Jak3 kinase or a disruption of the Jak3/Stat5 signaling pathway (*5*). These negative regulatory functions of NO contrast with our study, which identifies NOS2-derived NO as an indispensable and positive regulatory element in the IL-12 signaling pathway of NK cells during the innate response to a protozoan parasite. The effect of NO, whose molecular nature remains to be determined, is selective because (i) it pertains to NK cells (expressing NOS2) but not to T cells (lacking NOS2), and (ii) it is a prerequisite for the activation of Tyk2 but not of two other Janus kinases (Jak1 and Jak2). At day 1 of infection with *L. major*, NOS2 was focally expressed by dermal macrophages, most likely as a consequence of the induction of IFN-α/β by the parasites (*9*). We hypothesize that the small quantities of NO generated very rapidly after infection by macrophages (or endogenously by NK cells after exposure to IL-2) capacitate NK cells to respond to IL-12 and IFN-α/β, which leads them to become cytotoxic and to release IFN-γ. We propose that this signaling function of NO is critical for the T cell–independent containment of bacterial and parasitic infections at a time when direct NO-mediated control of the microbes does not yet occur.

### References and Notes


1. D. T. Fearon and R. M. Locksley, *Science* **272**, 50 (1996); R. Medzhitov and C. A. Janeway, *Curr. Opin. Immunol.* **9**, 4 (1997).
2. G. Trinchieri and F. Gerosa, *J. Leukocyte Biol.* **59**, 505 (1996); C. Reis e Sousa *et al.*, *J. Exp. Med.* **11**, 1819 (1997); E. Y. Denkers and A. J. Marshall, *Immunologist* **6**, 116 (1998); R. D. Stout, *Curr. Opin. Immunol.* **5**, 398 (1993).
3. C. Bogdan, *Behring Inst. Res. Commun.* **99**, 58 (1997); J. MacMicking, Q.-w. Xie, C. Nathan, *Annu. Rev. Immunol.* **15**, 323 (1997).
4. C. Bogdan, *J. Exp. Med.* **9**, 1361 (1998); H. M. Lander, *FASEB J.* **11**, 118 (1997).
5. R. J. Duhé *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 126 (1998); R. M. Bingisser, P. A. Tilbrook, P. G. Holt, U. R. Kees, *J. Immunol.* **160**, 5729 (1998).
6. H. Rothe, B. Hartmann, P. Geerlings, H. Kolb, *Biochem. Biophys. Res. Commun.* **224**, 159 (1996); F.-P. Huang *et al.*, *Eur. J. Immunol.* **28**, 4062 (1998).
7. T. K. Tarrant *et al.*, *J. Exp. Med.* **189**, 219 (1999).
8. C. Hierholzer *et al.*, *ibid.* **187**, 917 (1998).
9. A. Diefenbach *et al.*, *Immunity* **8**, 77 (1998).
10. T. Scharton-Kersten, L. C. C. Afonso, M. Wysocka, G. Trinchieri, P. Scott, *J. Immunol.* **154**, 5320 (1995).
11. Mice were infected in the right hind footpad with $2 \times 10^6$ to $3 \times 10^6$ *L. major* promastigotes (strain MHOM/IL/81/FE/BNI) as described (*9*).
12. J. D. MacMicking *et al.*, *Cell* **81**, 641 (1995).
13. Recombinant mouse IL-12 (250 to 300 ng per injection; Genetics Institute, Cambridge, MA) or phosphate-buffered saline (PBS) was injected intraperitoneally (i.p.) at 24 and 4 hours before infection. L-NIL (2.5 mg per injection; Searle/Monsanto, St. Louis, MO) or PBS was injected i.p. twice daily, starting 48 to 72 hours before infection.
14. T. Laskay, A. Diefenbach, M. Röllinghoff, W. Solbach, *Eur. J. Immunol.* **25**, 2220 (1995); P. Launois, K. G. Swihart, G. Milon, J. A. Louis, *J. Immunol.* **158**, 3317 (1997).
15. IL-12Rβ1 and the IL-12Rβ2 competitors were constructed by the linker primer technique from the original cDNAs and were used with the following primers: IL-12Rβ1 sense, 5′-CGG GGG TCC TGA CGC AAT ACG-3′[base pair (bp) position 1545 → 1565 of the mouse IL-12Rβ1 sequence]; IL-12Rβ1 antisense, 5′-CTC CGG GCA TCT CGA CCA CCA G-3′ (bp position 1976 → 1997); IL-12Rβ2 sense, 5′-CTG CGA GAT CTG AGA CCG TTT ACA-3′ (bp position 1027 → 1050 of the mouse IL-12Rβ2 sequence); IL-12Rβ2 antisense, 5′-GGG GGA TCC GCA GCC AGT G-3′ (bp position 1597 → 1615). The sequences of the linker primers were 5′-CTC GAC CAC CAG GCC CTG TTT AAG CCA ATG T-3′ (IL-12Rβ1) and 5′-CCG CAG CCA GTG TCC GAC TTT GCA GAG ACC T-3′ (IL-12Rβ2). The annealing temperatures were 58°C and the number of polymerase chain reaction (PCR) cycles was 35.
16. T. M. Scharton and P. Scott, *J. Exp. Med.* **178**, 567 (1993); S. L. Reiner, S. Zheng, Z.-E. Wang, L. Stowring, R. M. Locksley, *ibid.* **179**, 447 (1994); T. Laskay, M. Röllinghoff, W. Solbach, *Eur. J. Immunol.* **23**, 2237 (1993).
17. A. Diefenbach, H. Schindler, M. Röllinghoff, C. Bogdan, data not shown.
18. pLN cells from infected mice were depleted of $CD4^+$, $CD8^+$, and $CD3^+$ cells by complement-mediated cell lysis with rat antibodies to CD4 and CD8 (gift of T. Winkler, Erlangen, Germany) and by incubation with rat antibody to CD3 (clone KT3, Dianova, Hamburg, Germany), followed by anti-rat immunoglobulin (Ig) Dynabeads (Dynal, Hamburg, Germany).
19. NK cells (NK1.1 bright and CD3-negative cells) were purified from splenocytes of naïve C57BL/6 mice ($NOS2^{+/+}$, $NOS2^{-/-}$, or $Rag1^{-/-}$) by fluorescence-activated cell sorting using phycoerythrin-conjugated rat antibody to mouse NK1.1 (clone PK136) and fluorescein isothyocyanate–conjugated hamster antibody to mouse CD3 (clone 500A2) (Pharmingen, San Diego, CA). They were then analyzed by flow cytometry (>98% $NK1.1^+$ and <1% each $CD3^+$, $CD4^+$, $CD8^+$, or $CD19^+$ cells) .
20. Total splenocytes were cultured in RPMI 1640 with 10% fetal bovine serum and rhuIL-2 (1 μg/ml) (Chiron Therapeutics, Ratingen, Germany). After 3 days, adherent and nonadherent cells were harvested; depleted of $CD3^+$, $CD4^+$, and $CD8^+$ T cells by antibody- and complement-mediated lysis; and further cultured in IL-2–containing medium. At day 5 to 9, nonadherent cells were discarded, and the adherent cells were harvested with PBS plus 1mM EDTA and then analyzed by flow cytometry (≥96% $NK1.1^+$ and <1% each of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, or $F4/80^+$ cells) before stimulation with rmIL-12 (0.1 to 1 ng/ml) or mouse IFN-α/β (100 to 200 U/ml).
21. F. M. Karlhofer, M. M. Orihuela, W. M. Yokoyama, *J. Exp. Med.* **181**, 1785 (1995).
22. The NK cell clone KY-1 (derived from p53-deficient C57BL/6 mice) shows stable proliferation in the presence of IL-2. Cells were expanded in IL-2 (50 to 400 U/ml), washed, and treated as indicated. Stimulation with IL-12 (0.1 to 1 ng/ml) or mouse IFN-α/β (200 U/ml; gift of I. Gresser, CNRS, Villejuif, France) for ≤24 hours did not require the routine addition of fresh IL-2.
23. See Web Fig. 1 at www.sciencemag.org/feature/data/986543.shl.
24. T cells were purified by Thy 1.2 magnetic cell sorting (Miltenyi Biotec, Bergisch-Gladbach, Germany) or by

nylon wool passage of total splenocytes from C57BL/6 mice (NOS2$^{+/+}$ or NOS2$^{-/-}$), analyzed by flow cytometry (CD3$^+$ >93%, NK1.1$^+$ <3%, CD19$^+$ <1%, and F4/80$^+$ <1%), and stimulated with rmIL-12 (0.1 to 1 ng/ml) or mouse IFN-α/β (100 to 500 U/ml).
25. H. Thüring, S. Stenger, D. Gmehling, M. Röllinghoff, C. Bogdan, *Eur. J. Immunol.* **25**, 3229 (1995).
26. W. E. Thierfelder *et al.*, *Nature* **382**, 171 (1996); M. H. Kaplan, Y.-L. Sun, T. Hoey, M. J. Grusby, *ibid.*, p. 174.
27. After stimulation, KY-1 cells or IL-2–expanded splenic NK cells were washed twice with ice-cold PBS (plus 100 μM sodium orthovanadate) and lysed in 20 mM tris buffer (pH 8.0) containing 150 mM NaCl; 1% Triton X-100; 0.5% NP-40; 1 mM each of EDTA, EGTA, sodium orthovanadate, sodium pyrophosphate, sodium fluoride, and PMSF; 0.1 mM sodium molybdate; and pepstatin A, aprotinin, chymostatin, and leupeptin (5 μg/ml each). Protein lysates were immunoprecipitated with 1 μg of affinity-purified rabbit anti-mouse Stat4 IgG (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) in the absence or presence of the respective Stat4 blocking peptide (5 μg/ml) using protein A/G-Plus-agarose (Santa Cruz Biotechnology); separated by 7.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE); and transferred to nitrocellulose. Immunoblotting (IB) of tyrosine phosphorylated Stat4 was performed with anti-phosphotyrosine mouse IgG [PY99, Santa Cruz Biotechnology; 1 μg/ml in dilution buffer (tris-buffered saline with 5% nonfat dry milk and 0.05% Tween 20)], followed by incubation with peroxidase-conjugated goat anti-mouse IgG (Dianova; 160 ng/ml in dilution buffer) and detection with ECL Plus (Amersham Pharmacia Biotech). Blots were reprobed with anti-Stat4 IgG (200 ng/ml in dilution buffer).
28. C.-R. Yu *et al.*, *J. Immunol.* **157**, 126 (1996).
29. H. H. H. W. Schmidt and U. Walter, *Cell* **78**, 919 (1994); J. Geiger, C. Nolte, E. Butt, S. O. Sage, U. Walter, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1031 (1992).
30. See Web Fig. 2 at www.sciencemag.org/feature/data/986543.shl.
31. C. M. Bacon *et al.*, *J. Exp. Med.* **181**, 399 (1995); S. J. Szabo, N. G. Jacobson, A. S. Dighe, U. Gubler, K. M. Murphy, *Immunity* **2**, 665 (1995); J. J. O'Shea, *ibid.* **7**, 1 (1997).
32. KY-1 cells, primary NK cells, or T cells were lysed (27) and immunoprecipitated with rabbit anti-Jak1 IgG (HR-785, Santa Cruz Biotechnology; 2 μg/ml), rabbit anti-Jak2 IgG (HR-758, Santa Cruz Biotechnology; 2 μg/ml), or rabbit anti-Tyk2 {C-15, specific for amino acids 1173 through 1187 [O. Colamonici *et al.*, *Mol. Cell. Biol.* **14**, 8133 (1994)]; 1:200} in the absence or presence of the respective blocking peptides (10 μg/ml) using protein A–Sepharose. They were then separated by 7.5% SDS-PAGE and transferred to nitrocellulose. Immunoblotting of tyrosine-phosphorylated Jak1, Jak2, or Tyk2 was performed with anti-phosphotyrosine mouse IgG (PY99, 1 μg/ml; Santa Cruz Biotechnology) as described (27). After stripping, blots were reprobed with antibody to Jak1 (800 ng/ml), Jak2 (600 ng/ml), or Tyk2 [C-15 combined with C-20 (Santa Cruz Biotechnology), 600 ng/ml].
33. Jak1, Jak2, or Tyk2 immunoprecipitates were washed with lysis buffer and with kinase buffer [20 mM Hepes (pH 7.4), 50 mM NaCl, 2 mM EGTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 5 mM $MnCl_2$, and 5 mM $MgCl_2$]. The protein bead complexes were resuspended in 50 μl kinase buffer containing 10 μCi [γ-$^{32}$P]ATP (>3000 Ci/mmol) and incubated for 30 min at room temperature. After several washes in tris buffer (pH 7.5) containing 0.1% Triton X-100 and 100 μM orthovanadate and a final wash in Triton-free tris buffer (pH 6.8), the immunoprecipitates were eluted in Laemmli-loading buffer, fractionated on 7.5% SDS-PAGE, and transferred to reinforced nitrocellulose (Schleicher & Schuell, Dassel, Germany) for autoradiography. In some cases, the protein bead immunocomplexes were treated with DETA/NO or DETA (1 to 100 μM in kinase buffer) for 60 min before the in vitro kinase reaction.
34. See Web Fig. 3 at www.sciencemag.org/feature/data/986543.shl.
35. G. Barbieri, L. Velazquez, M. Scrobogna, M. Fellous, S. Pellegrini, *Eur. J. Biochem.* **223**, 427 (1994); O. R. Colamonici, H. Uyttendaele, P. Domanski, H. Yan, J. J. Krolewski, *J. Biol. Chem.* **269**, 3518 (1994).
36. See Web Fig. 4 at www.sciencemag.org/feature/data/986543.shl.
37. D. Robinson *et al.*, *Immunity* **7**, 571 (1997); O. Adachi *et al.*, *ibid.* **9**, 143 (1998).
38. Cell lysates were prepared in 40 mM tris buffer (pH 8.0) containing 200 μM phenylmethylsulfonyl fluoride and aprotinin, chymostatin, pepstatin A, and leupeptin (5 μg/ml each), then separated on a 7.5% SDS-PAGE followed by protein immunoblotting with a rabbit anti-mouse NOS2 IgG (9) using the ECL Plus detection system (Amersham Pharmacia Biotech, Freiburg, Germany).

39. Supported by grants from the Deutsche Forschungsgemeinschaft (SFB263, project A5 to C.B. and Di764/1-1 to A.D.) and from NIH (W.M.Y.). We thank H. Bang, Chiron Therapeutics, O. Colamonici, I. Gresser, M. Lohoff, P. Manning, E. Schmitt, T. Winkler, S. Wolff, and Q.-w. Xie for their gifts of reagents or cell lines; J. Mudgett, J. MacMicking, and C. Nathan for breeding pairs of NOS2$^{-/-}$ mice; C. Brooks, A. Ding, M. Müller, and E. Parganas for advice; D. Raulet and R. E. Vance for discussions; K. Schröppel for help with the electronic data files; and E. Lorenz and N. Donhauser for their outstanding technical assistance.

17 November 1998; accepted 30 March 1999

# Interaction of Diphtheria Toxin T Domain with Molten Globule-Like Proteins and Its Implications for Translocation

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The transmembrane (T) domain of diphtheria toxin has a critical role in the low pH–induced translocation of the catalytic domain (A chain) of the toxin across membranes. Here it is shown that at low pH, addition of proteins in a partly unfolded, molten globule-like conformation converted the T domain from a shallow membrane-inserted form to its transmembrane form. Fluorescence energy transfer demonstrated that molten globule-like proteins bound to the T domain. Thus, the T domain recognizes proteins that are partly unfolded and may function in translocation of the A chain as a transmembrane chaperone.

Diphtheria toxin, a protein secreted by *Corynebacterium diphtheriae*, consists of an A chain (21 kD) and a B chain (37 kD). The A chain is the catalytic domain, and the B chain contains the transmembrane (T) and receptor-binding domains (*1*). After binding to mammalian cells and undergoing endocytosis, the toxin partially unfolds within the low pH of the endosomal lumen. This exposes hydrophobic sites, induces membrane insertion, and results in translocation of the A chain into the cytoplasm (*2–4*). Translocation is believed to involve the interaction of a transmembrane structure formed largely by the T domain with the partly unfolded A chain (*2–6*). Recent studies have shown the T domain can exist in both partially membrane-penetrating (P) and transmembrane (TM) conformations (*7, 8*). Conversion of the P to the TM conformation can be detected by the blue shift of the emission of fluorescent groups attached to single Cys residues introduced into TH8 or TH9, such as residue $C^{356}$ (*7*). TH8 and TH9 are hydrophobic helices that become buried in the TM conformation (*7–9*).

At low pH, the addition of bovine serum albumin (BSA), human serum albumin (HSA), α-lactalbumin, apomyoglobin, or diphtheria toxin A chain to T domain bound in the P conformation to dioleoylphosphatidylglycerol (DOPG)/dioleoylphosphatidylcholine (DOPC) model membranes induced a blue shift in the fluorescence of bimane attached to $C^{356}$ (Fig. 1A). Below pH 5 these added proteins have molten globule (MG)-like conformations, which exhibit some degree of partial unfolding and increased hydrophobicity (*4, 10–12*). In contrast, no effect on bimane fluorescence was observed when proteins that do not form an MG-like state [egg white lysozyme, ovalbumin, and an anti-dansyl immunoglobulin G (IgG)] were added (*13*) (Fig. 1B).

The ability of HSA to blue shift bimane fluorescence was not diminished by predialysis in tubing with a 10,000-kD cutoff and could not be induced by an equivalent volume of an ultrafiltrate of an HSA solution. Thus, HSA itself induced the blue shift.

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