bud sites (*11*), no growth defect has been reported. The results reveal a functional redundancy of Ras and Rsr1 in spite of their known individual roles.

When the *ras1*Δ *ras2*Δ *rsr1*Δ *cyr1*Δ (YEpT-TPK1, YCpLeGAL1-RAS2-1) cells were shifted to a galactose-free medium at 30°C, they ceased to proliferate as large budded cells with chromosomal DNA evenly distributed in mother cells and buds (Fig. 2, A to C), indicating that the cells were arrested at or near the end of the M phase. This arrest was accompanied by an accumulation of cells with DNA content characteristic of the $G_2$ and M phases (Fig. 2F) and by sustained Cdc28 histone H1 kinase activity (Fig. 2H), consistent with an M phase arrest. Cells carrying an intact *RAS* gene did not accumulate such cells (*10*). This terminal phenotype was different from the $G_1$ arrest of *ras1*Δ *ras2*Δ cells when the cells were starved of exogenous cAMP. In this case, the cells were arrested as unbudded cells (Fig. 2D) with a single nucleus (Fig. 2E), Cdc28 kinase activity decreased (Fig. 2G), and cells with $G_1$ DNA content accumulated (*10*). These results indicate that the cells with the *RAS1 RAS2 RSR1* triple disruption are defective in the completion of the M phase.

We examined genetic interactions between *RAS* and other genes that participate in M phase completion, including *DBF2*, *CDC5* (*18*), *CDC15* (*19*), *SPO12*, and *TEM1*. Both *CDC5* and *CDC15* encode protein kinases. We introduced multicopy plasmids carrying each of these genes into the *ras1*Δ *ras2*Δ *rsr1*Δ *cyr1*Δ (YEpT-TPK1, YCpLeGAL1-RAS2-1) cells, and the resulting transformants were examined for growth on a galactose-free plate. Each of the multicopy plasmids suppressed the growth defects of the cells (Fig. 3A). None of these genes suppressed the cAMP requirement of the *ras1*Δ *ras2*Δ cells (*10*). Mammalian c-Ha-*RAS*, or its activated form ($Val^{12}$, $Thr^{59}$), also suppressed the lethality, indicating that mammalian Ras can substitute for yeast Ras in this other function (Fig. 3B).

We have shown here that *S. cerevisiae* Ras functions in the completion of the M phase. The genetic interactions between *RAS* and other genes involved in M phase completion suggest a network of signal transduction pathways in which low molecular weight GTP binding proteins and various protein kinases are involved. Activation of Ras and downstream protein kinase cascade by growth factors in mammalian cells is necessary for cell cycle progression through the $G_1$ phase to the S phase (*2*). Several reports contain arguments for another function of Ras in the $G_2$-M boundary in vertebrate cells (*7*, *20*). Yeast and mammalian Ras proteins could share the same effector molecule in this signal transduction pathway.

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26. We thank K. Tanaka, Y. Ohya, and A. Nakano for valuable suggestions; H. Sekiguchi and E. Nishida for critical reading of the manuscript; and J. B. Gibbs, M. S. Marshall, and A. Sugino for materials. Supported by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture of Japan. T.M. is supported by the Research Development Corporation of Japan.

10 February 1995; accepted 24 August 1995


# Ligand-Induced Autoregulation of IFN-γ Receptor β Chain Expression in T Helper Cell Subsets


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Interferon γ (IFN-γ) responsiveness in certain cells depends on the state of cellular differentiation or activation. Here an in vitro developmental system was used to show that IFN-γ produced during generation of the $CD4^+$ T helper cell type 1 ($T_H1$) subset extinguishes expression of the IFN-γ receptor β subunit, resulting in $T_H1$ cells that are unresponsive to IFN-γ. This β chain loss also occurred in IFN-γ–treated $T_H2$ cells and thus represents a specific response of $CD4^+$ T cells to IFN-γ rather than a $T_H1$-specific differentiation event. These results define a mechanism of cellular desensitization where a cytokine down-regulates expression of a receptor subunit required primarily for signaling and not ligand binding.


Recently it was reported that $T_H1$ and $T_H2$ cells develop opposing patterns of responsiveness to the cytokines interleukin-12 (IL-12) and IFN-γ, with $T_H1$ retaining only IL-12 responsiveness and $T_H2$ retaining only IFN-γ responsiveness (*1*). Because this differential responsiveness significantly affects $T_H1$ and $T_H2$ subset stability in vivo, the developmental mechanism controlling the loss of cytokine receptor signaling becomes central to the understanding of pathogen susceptibility or resistance (*1*, *2*). Using fully differentiated long-term T cell clones, Pernis *et al.* observed that expression of the IFN-γ receptor β chain, the receptor subunit required primarily for signaling and not ligand binding, is limited to $T_H2$ cells, and it was implied that this was important to phenotype differentiation (*3*, *4*). To characterize the mechanism controlling developmental expression of the two IFN-γ receptor subunits and to critically test their


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roles in directing T cell differentiation, we used an αβ T cell receptor (TCR) transgenic system. This allowed us to examine and manipulate the expression of the two IFN-γ receptor subunits during differentiation of naïve T cells. Our results show that IFN-γ receptor β chain loss is not intrinsic to $T_H1$ development but rather represents an unusual mechanism of ligand-dependent cellular desensitization.

Naïve splenic $CD4^+$ T cells from DO11.10 TCR transgenic mice were differentiated in vitro into $T_H1$ or $T_H2$ subsets, exposed to IFN-γ, and then analyzed for major histocompatibility complex (MHC) class I expression (Fig. 1A) (*5*, *6*). Treatment with IFN-γ did not enhance MHC class I expression on $T_H1$ cells but upregulated expression on $T_H2$ cells. In contrast, both cell types responded to IFN-α, indicating that the class I response pathway was intact. IFN-γ also selectively activated the Jak-STAT (Janus kinase–signal transducers and activators of transcription) pathway only in early developing $T_H2$ cells and not in $T_H1$ cells, although both cell types expressed comparable amounts of the Jak-STAT proteins required for IFN-γ signaling (*7*, *8*). As was shown for long-term $T_H$ clones, early developing $T_H1$ cells expressed substantially reduced amounts of IFN-γ receptor β chain message compared with $T_H2$ cells (Fig. 1B) (*3*). This observation was substantiated by protein immunoblot analysis in which a hamster monoclonal antibody (mAb) (MOB-47) was used that was specific for the murine IFN-γ receptor β chain (*9*). Whereas $T_H2$ cells contained β chain protein, $T_H1$ cells did not (Fig. 1C). Both subsets expressed comparable amounts of IFN-γ receptor α chain as detected with a mAb (GR-20) specific for the IFN-γ receptor α chain (Fig. 1C) (*10*). These experiments confirm the previous observations of Pernis *et al.* (*3*) and demonstrate that the receptor β chain protein as well as message is extinguished in $T_H1$ cells as early as 14 days after differentiation.

To define the mechanism underlying this process, we monitored surface expression of the two IFN-γ receptor subunits on $CD4^+$ T cells during the process of $T_H$ subset differentiation. Because the receptor epitopes recognized by the mAbs GR-20 and MOB-47 are masked by bound IFN-γ, cells were treated with dilute acid to remove bound ligand before they were stained (*11*). Naïve $CD4^+$

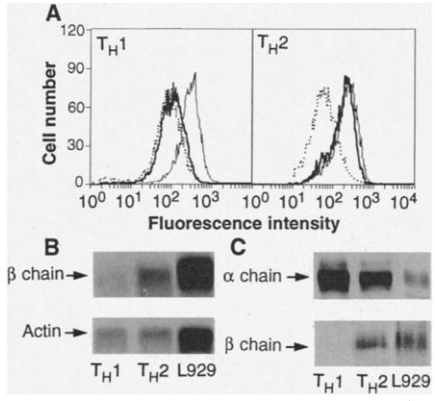


**Fig. 1.** Lack of IFN-γ receptor β chain expression in early developing $T_H1$ cells. $T_H1$ and $T_H2$ cells were differentiated for 14 days as described (*13*). (**A**) Cells were treated for 72 hours with medium (dotted line), murine IFN-γ (MuIFN-γ) [1000 international reference units (IRU) per milliliter, thick solid line], or MuIFN-α (1000 IRU/ml, thin solid line) and analyzed by fluorescence-activated cell sorting (FACS) for MHC class I expression with a biotinylated H-$2^d$–specific antibody (Pharmingen, San Diego, California) as described (*11*). (**B**) Northern (RNA) blot analysis of total RNA from $T_H1$, $T_H2$, or L929 cells was performed with $^{32}P$-labeled murine β chain complementary DNA (cDNA) (*4*) as described (*11*). (**C**) $T_H1$ cells ($6.7 \times 10^7$) or $T_H2$ cells ($9 \times 10^7$) were lysed as described (*11*), and receptor subunits were immunoprecipitated with either 1 μg of GR-20 (*10*) or 0.5 μg of MOB-47 (*9*), mAbs specific for the MuIFN-γ receptor α and β chains, respectively. Precipitates were separated on 7 or 9% SDS-polyacrylamide gels for analysis of α or β chains, respectively. Protein immunoblot analysis was performed with MuIFN-γ receptor α chain–specific mAbs (1G5, 1F1, and 2E2) (*9*) or biotinylated MOB-47 (*9*) as described (*14*).

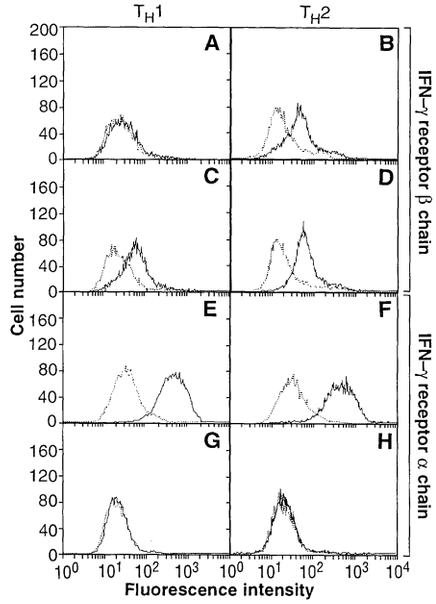


**Fig. 3.** Maintenance of the IFN-γ receptor β chain on $T_H1$ cells derived from mice deficient for IFN-γ receptor α chain. IFN-γ–unresponsive mice expressing the DO11.10 TCR transgene were generated by breeding the transgenic TCR (*5*) into mice carrying a null mutation for the IFN-γ receptor α chain (IFN-$\gamma R^{o/o}$) (*12*). $T_H1$ and $T_H2$ cells were differentiated as described (*13*). Fourteen-day-differentiated $T_H1$ and $T_H2$ cells from DO11.10–IFN-$\gamma R^{o/o}$ (**C**, **D**, **G**, and **H**) or DO11.10 control (**A**, **B**, **E**, and **F**) mice were analyzed as described in Fig. 2 for expression of the IFN-γ receptor β chain [(A) to (D)] or α chain [(E) to (H)].

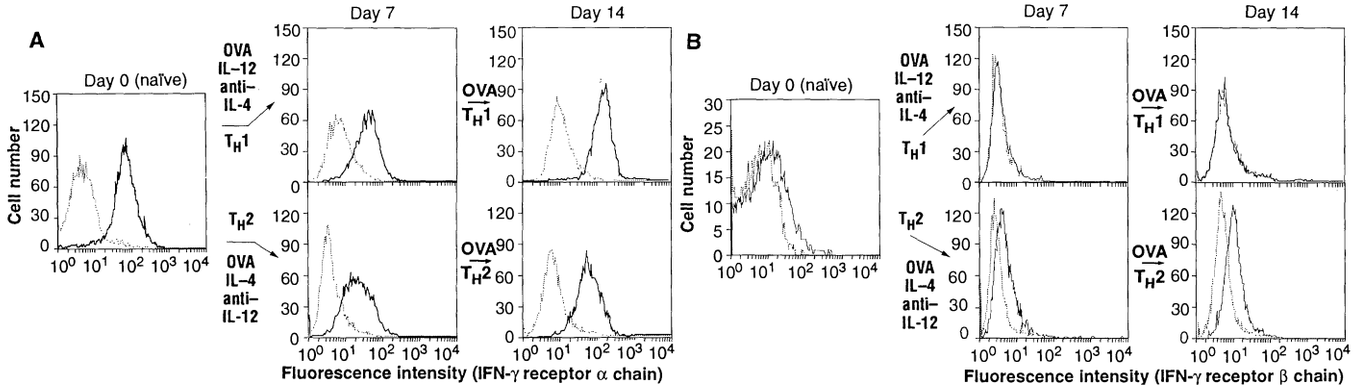


**Fig. 2.** Loss of IFN-γ receptor β chain during $T_H1$ development. Naïve $CD4^+$ T cells or $T_H1$ and $T_H2$ cells treated with dilute acetic acid as described (*11*) were analyzed by FACS for the presence of IFN-γ receptor α and β chains after 7 or 14 days of differentiation (*13*). (**A**) Cells were left untreated (solid line) or were treated with 10 μg of MuIFN-γ for 45 min (dotted line), and then stained with 1 μg of biotinylated GR-20 and streptavidin-phycoerythrin (PE) (*11*). (**B**) Cells were stained with 0.3 μg of MOB-47 (solid line) or 0.3 μg of species-matched control mAb (dotted line) and developed with 1 μg of biotinylated polyclonal goat antibody to hamster immunoglobulin and streptavidin-PE. OVA, ovalbumin.

T cells and acid-stripped $T_H1$ and $T_H2$ cells expressed similar amounts of the IFN-γ receptor α chain (Fig. 2A). In contrast, whereas naïve $CD4^+$ T cells and early developing $T_H2$ cells expressed IFN-γ receptor β chain, $T_H1$ cells did not (Fig. 2B). Loss of the receptor β chain on $T_H1$ cells occurred within 5 days after the initiation of primary culture (*8*). Thus, $CD4^+$ T cells lose cell surface expression of the IFN-γ receptor β chain as they differentiate to the $T_H1$ subset.

These observations suggested that the loss of the IFN-γ receptor β chain on $T_H1$ cells was either a process intrinsic to $T_H1$ differentiation or a consequence of it. We distinguished between these possibilities by breeding the DO11.10 TCR into IFN-γ–unresponsive mice lacking the IFN-γ receptor α chain (*12*). T cells from these mice differentiated normally into $T_H1$ and $T_H2$ subsets, as evidenced by polarized production of subset-specific cytokines (*8*). However, unlike $T_H1$ cells derived from IFN-γ–responsive transgenic mice, β chain expression was retained on $T_H1$ cells derived from TCR transgenic mice lacking the IFN-γ receptor α chain (Fig. 3C). These results demonstrate that loss of the IFN-γ receptor β chain requires the presence of a functionally active IFN-γ receptor and represents a response of the cells to ligand. Moreover, the results show that the process is not intrinsically linked to $T_H1$ differentiation.

To directly test whether the loss of the IFN-γ receptor β chain represented a general biologic response of $CD4^+$ T cells to IFN-γ, $T_H2$ cells were treated with three different doses of IFN-γ (100, 500, or 2000 international reference units per milliliter), quantities typically produced by $T_H1$ cultures, and analyzed for surface expression of the receptor β chain (Fig. 4) (*8*). At all doses tested, IFN-γ induced a loss of receptor β chain expression on $T_H2$ cells (Fig. 4A) (*8*). The kinetics of receptor β chain loss on IFN-γ–treated $T_H2$ cells was similar to that displayed on $T_H1$ cells (*8*). In contrast, IFN-γ did not down-regulate expression of the β chain on L929 fibroblasts and actually increased expression in some experiments (Fig. 4D). Protein immunoblot analysis demonstrated that all T cells expressed the IFN-γ receptor α chain and untreated $T_H2$ cells expressed the receptor β chain (Fig. 4B). In contrast, no β chain was detected in either $T_H1$ or IFN-γ–treated $T_H2$ cells. Analysis of cytokine production by the T cell cultures showed that IFN-γ treatment of $T_H2$ cells did not alter the $T_H2$ phenotype (*8*). IFN-γ did not enhance MHC class I expression on $T_H2$ cells pretreated with IFN-γ (Fig. 4C). In contrast, these cells remained fully responsive to IFN-α. Thus, IFN-γ–treated $T_H2$ cells become unresponsive to IFN-γ in a manner similar to that of $T_H1$ cells.

With a TCR transgenic system, we have been able to distinguish between cellular responses intrinsic to $T_H$ subset differentiation and those that arise as a result of this process. Whereas our observations confirm and extend those of Pernis *et al.* (*3*), our study draws different conclusions about the mechanism of this process. Specifically, we show that the $T_H1$ subset develops normally from IFN-γ–unresponsive naïve T cells and maintains expression of the IFN-γ receptor β chain. This result demonstrates that IFN-γ receptor β chain loss is not intrinsic to the $T_H$ differentiation process. We also show that β chain loss and inactivation of IFN-γ signaling occurs in $T_H2$ cells when they are exposed to IFN-γ. Thus, the loss of IFN-γ receptor β chain expression on T cells is a consequence of exposure to IFN-γ rather than a true marker of phenotype. Our observations indicate that inactivation of IFN-γ signaling in $CD4^+$ T cells is a dynamic regulatory process that represents a cellular response to cytokines present in the local environment.

Equally significant is our observation that on T cells, IFN-γ down-regulates expression of a component of its own receptor involved predominantly in signaling rather than ligand binding (*4*). Previous work with several cell types has demonstrated that after receptor ligation, the IFN-γ receptor α chain is internalized and, on most cells, dissociates from ligand and recycles back to the cell surface (*7*). However, on a limited number of cell types, IFN-γ induces down-regulation of the receptor α chain by affecting receptor α chain internalization (*7*). Internalization of a ligand-binding receptor subunit is a common mechanism that leads to a transient insensitivity of cells to a variety of hormone and cytokine ligands. However, our observation that ligand induces the down-regulation of biosynthesis of a signaling component of a receptor without affecting expression of the ligand-binding chain represents a heretofore unrecognized mechanism of ligand-induced cellular desensitization. That β chain down-regulation occurs on T cells but not on certain other cells suggests that this process can modulate the ability of specific cells to respond to subsequent reexposure to ligand. In this manner, IFN-γ–induced cellular responses may be differentially regulated in distinct cell types. It will be important in the future to explore which cells are desensitized to IFN-γ in this manner and to determine whether down-regulation of a receptor signaling chain represents a paradigm controlling the activity of other cytokine receptors as well.

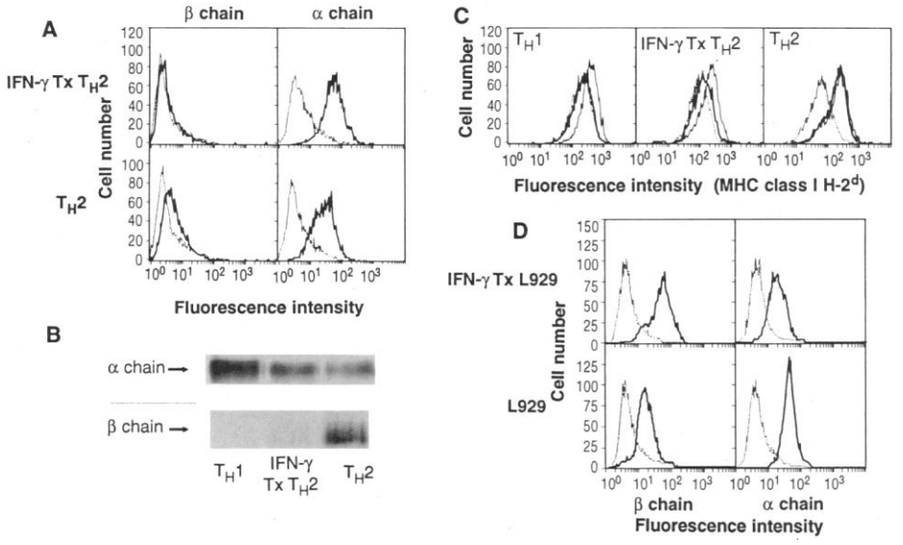


**Fig. 4.** Loss of IFN-γ receptor β chain expression and IFN-γ responsiveness in IFN-γ–treated $T_H2$ cells. $T_H2$ cells were treated with MuIFN-γ (2000 IRU/ml) on days 0 to 5 of both primary and secondary stimulation (IFN-γ Tx $T_H2$) (*13*). $T_H1$, $T_H2$, and IFN-γ Tx $T_H2$ cells were differentiated for 14 days as described (*13*). (**A**) FACS analysis for expression of IFN-γ receptor α and β chains on $T_H2$ and IFN-γ Tx $T_H2$ was performed as described in Fig. 2. (**B**) Protein immunoblot analysis for IFN-γ receptor α and β chains from $6.7 \times 10^7$ $T_H1$, $9 \times 10^7$ $T_H2$, and $9 \times 10^7$ IFN-γ Tx $T_H2$ cells was performed as described in Fig. 1. (**C**) MHC class I expression on $T_H1$, IFN-γ Tx $T_H2$, and $T_H2$ cells after treatment with medium (dotted line), MuIFN-γ (thick solid line), or MuIFN-α (thin solid line) was assessed by FACS as described in Fig. 1. (**D**) L929 fibroblasts were treated with MuIFN-γ (1000 IRU/ml) and analyzed by FACS 72 hours later for expression of the IFN-γ receptor polypeptides as described in Fig. 2 with mAbs 1G5 and MOB-55 to stain IFN-γ receptor α and β chains, respectively (*9*).

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13. Naïve $CD4^+$ $Mel14^{hi}$ T cells from the ovalbumin (OVA)–reactive DO11.10 αβ TCR transgenic mouse (*5*) were purified by FACS as described (*6*) and differentiated in vitro into $T_H1$ or $T_H2$ cells in the presence of 0.3 μM OVA peptide and irradiated BALB/c splenocytes by culture with MuIL-12 (10 U/ml Hoffmann–La Roche, Nutley, NJ) and MuIL-4 mAb (11B11) (10 μg/ml, DNAX, Palo Alto, CA), or MuIL-4 (200 U/ml, Genzyme, Cambridge, MA) and MuIL-12 mAb (3 μg/ml) (TOSH), respectively. Seven days after primary stimulation, cells were restimulated with antigenic peptide in the absence of exogenous cytokine reagents and incubated for an additional 7 days.
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18 July 1995; accepted 16 October 1995

## TECHNICAL COMMENTS

# Attenuated Retrovirus Vaccines and AIDS

In their recent report, Timothy W. Baba *et al.* state that a deletion mutant of simian immunodeficiency virus (SIVΔ3), which does not cause disease in adult macaques and has been successfully used as a vaccine against challenge with pathogenic virus (*1*), causes acquired immunodeficiency syndrome (AIDS) in newborn macaques. They ascribe the differential outcome of SIVΔ3 infection of neonatal and adult macaques to several possibilities including the amount of virus replication early after inoculation, the route of virus inoculation, and the developing neonatal immune system. However, their study does not allow separation of these important variables.

We found that high-dose intravenous inoculation of newborn rhesus macaques with molecularly cloned SIVmac239 (the parental virus from which SIVΔ3 was derived) resulted in persistently high amounts of virus in peripheral blood mononuclear cells (PBMC) and plasma (higher than those reported by Baba *et al.* for SIVΔ3). Rhesus newborns infected with SIVmac239 did not experience rapid $CD4^+$ T lymphocyte depletion, and the time course before fatal immunodeficiency developed was consistent with that previously reported for SIVmac239-infected adult macaques (that is, 6 to 24 months) (*2, 3*). Thus, an age-related difference does not explain why rhesus infants inoculated with an attenuated triple-deletion mutant of SIVmac239 appear to experience a more rapid $CD4^+$ T cell depletion and $CD4^+/CD8^+$ T cell ratio inversion than rhesus infants inoculated with the pathogenic parental virus, SIVmac239. We also found that absolute $CD4^+$ T lymphocyte numbers were not a reliable marker of disease progression in infant rhesus macaques because of extreme variability of absolute lymphocyte counts in response to stress (for example, handling). Only absolute $CD4^+$ T cell numbers that are persistently below 500 per microliter reliably suggested $CD4^+$ T lymphocyte depletion in neonatal macaques (*2–4*).

Baba *et al.* hypothesize that the oral route of inoculation may be responsible for increased virulence of SIVΔ3 in newborns. Our observations with five orally and six intravenously inoculated newborn macaques did not demonstrate a more severe course of infection with uncloned pathogenic SIVmac251 for the oral route (*2–4*). With regard to the postulated age-dependence of SIV virulence, we have also compared the time course of infection of the nonpathogenic molecular clone, SIVmac1A11, and SIV/human immunodeficiency virus–1 (HIV-1) envelope chimeric viruses in macaques of different ages: We have no evidence that an SIV strain that is attenuated in older macaques becomes pathogenic when inoculated intravenously or orally into newborn macaques (*2, 5*). Instead, inoculation of fetal and newborn macaques with attenuated SIVmac1A11 proved to be a safe and effective vaccine against challenge with pathogenic uncloned SIVmac later in life (*3*). Finally, our studies indicate that the neonatal immune system was not overwhelmed by attenuated SIV isolates or by a pathogenic SIV clone (*2, 3*).

Caution must be used when assigning the underlying cause of death in SIV-infected macaques to immunodeficiency. For the one SIVΔ3-inoculated macaque that died in their study, the classical hallmarks of simian AIDS (such as the presence of opportunistic infections, encephalopathy, and so on) apparently were not demonstrated by Baba *et al.* Instead, this animal had severe anemia and thrombocytopenia, reportedly a result of peripheral autoimmune destruction of red blood cells and platelets. It is not clear whether this diagnosis of hemolytic anemia was mainly based on a positive direct Coombs test. Many healthy macaques will react positively if human Coombs test reagents are used (*6*). Clinical hemolytic anemia must be confirmed by additional evidence, such as hemoglobinuria, poikilocytosis, the presence of spherocytes, hemolytic or icteric plasma, and increased serum bilirubin and lactate dehydrogenase. The erythroid hyperplasia of the bone marrow, reported by Baba *et al.*, is a finding that we do not see in anemic SIV-infected animals; rather, their bone marrow aspirates reveal a myeloid hyperplasia with the erythroid series being normal or only slightly increased (*7*). Findings in addition to an abundance of megakaryocytes in the bone marrow are needed to support the hypothesis of peripheral platelet destruction. SIV-infected animals often have a megakaryocyte hyperplasia of the bone marrow, but these megakaryocytes have increased cytoplasmic vacuolization, which suggests that the thrombocytopenia is a result of decreased platelet production rather than peripheral platelet destruction (*6*).

Extra care needs to be taken to exclude all other pathogens that can adversely affect the immune system and the health of macaques. Although the animals in the study by Baba *et al.* were polymerase chain reaction–negative (by an assay able to detect approximately one infected cell in 8000 PBMC) and seronegative for simian type D retroviruses, virus isolation is more reliable for diagnosis of this viral infection, but was not reported by Baba *et al.*

Until a more thorough analysis is completed and results of Baba *et al.* are confirmed, it would be premature to dismiss the potential of SIV *nef*-deletion mutants as live-attenuated vaccines.

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