Although a majority of the BiP in the enriched PB fraction was released by detergent and salt, about 25% remained associated with the protein granules (Table 1). The ATP (but not ATP-γ-S) released almost all of this tightly bound BiP (Fig. 3A). Two major polypeptides with electrophoretic mobilities identical to BiP and prolamine (*16*) were evident in ATP-released products (Fig. 3B). The ATP-dependent dissociation of BiP from the PBs suggests that BiP has an essential role in the assembly of prolamines onto the PB and would account for the localization of rice BiP on the surface of, but not within, the PBs (*8*).

The tight binding of BiP-prolamine complexes is likely due to the preference of BiP for aliphatic amino acids (*17*), residues enriched in prolamines (*18*). The higher affinity of BiP for prolamines than for glutelins may explain the localization of BiP to the prolamine PBs but not to the cisternal ER where glutelins are synthesized (*2*). However, BiP may help the folding and assembly of glutelins in the ER before they are transported to the Golgi complexes. The association of BiP with glutelins is probably transient.

We propose that prolamine PB formation is a sequential process mediated by BiP. BiP binds to the nascent prolamine peptide as it emerges through the ER membrane. This initial complex, which resembles the interaction between cytosolic Hsp70 and nascent polypeptides (*19*), maintains the polypeptide chain in a competent state for subsequent assembly onto the PBs. The completion of protein synthesis results in the release of the prolamine-BiP complexes into the ER lumen. These protein complexes then interact with the PB surface, and prolamine molecules are dissociated from BiP and assembled onto the PB surface at the expense of ATP. The dissociated BiP is then recycled. The PB formation would be initiated when a critical concentration of BiP-prolamine complexes is accumulated in the ER lumen and BiP-mediated aggregation of the prolamine polypeptides begins. This involvement of BiP in prolamine PB formation is also supported by its elevated amounts in the PBs of maize mutants defective in zein accumulation (*20*).

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9. Rice seeds (12 to 14 days after flowering) were homogenized in a buffer (10 mM Hepes, 5 mM MgCl, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.2 M sucrose) modified from (*10*). The homogenate was filtered and then centrifuged at 100*g* for 5 min, then at 5000*g* for 10 min. The PB pellet was washed for 30 min at 4°C twice with 0.5% Triton X-100 (detergent extract), then with 200 mM NaCl (salt extract). Cell-free PBs were removed from the salt extract by centrifugation at 5000*g* for 10 min.
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13. The salt extract (4 absorbance units at 260 nm) was loaded onto a 4.5-ml sucrose density gradient (15 to 60%) in PB buffer (*17*) and centrifuged at 240,000*g* for 3 hours followed by fractionation. Polyribosome profiles were monitored at 254 nm.
14. Twenty micrograms of antiserum was incubated with 50 μl of agarose–protein A overnight at 4°C in 50 mM tris-HCl (pH 7.2), 500 mM NaCl, and 0.1% Tween 20 (TBST). The resulting agarose beads were washed thoroughly with TBST, then with PB buffer containing 1% Tween and 1% bovine serum albumin (buffer Q), followed by suspension in 0.5 ml of buffer Q. After addition of purified polyribosomes (0.1 absorbance unit at 260 nm), the mixture was incubated overnight at 4°C and the beads washed thoroughly with buffer Q. The bound polyribosomes were released by an incubation at 25°C for 10 min in 200 mM acetate.
15. Northern blot analyses showed that prolamine transcripts predominated in the salt extract (*8*), which indicates that these transcripts represented the principal mRNA species on the prolamine PB membrane (*2*).
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Formation and Hydrolysis of Cyclic ADP–Ribose Catalyzed by Lymphocyte Antigen CD38

Maureen Howard,* J. Christopher Grimaldi, J. Fernando Bazan, Frances E. Lund, Leopoldo Santos-Argumedo, R. M. E. Parkhouse, Timothy F. Walseth, Hon Cheung Lee*

CD38 is a 42-kilodalton glycoprotein expressed extensively on B and T lymphocytes. CD38 exhibits a structural homology to *Aplysia* adenosine diphosphate (ADP)–ribosyl cyclase. This enzyme catalyzes the synthesis of cyclic ADP–ribose (cADPR), a metabolite of nicotinamide adenine dinucleotide (NAD^+) with calcium-mobilizing activity. A complementary DNA encoding the extracellular domain of murine CD38 was constructed and expressed, and the resultant recombinant soluble CD38 was purified to homogeneity. Soluble CD38 catalyzed the formation and hydrolysis of cADPR when added to NAD^+. Purified cADPR augmented the proliferative response of activated murine B cells, potentially implicating the enzymatic activity of CD38 in lymphocyte function.

CD38 has been used predominantly as a phenotypic marker of different subpopulations of T and B lymphocytes (*1*, *2*). Antibodies to CD38 (anti-CD38) can stimulate the growth of lymphocytes in the presence of other stimuli such as interleukin-4 (IL-4) (*3*, *4*) or IL-2 and accessory cells (*2*). Anti-CD38–induced proliferation of murine B lymphocytes is preceded by a rapid Ca^{2+} influx that can be distinguished from the inositol triphosphate (IP_3)–dependent Ca^{2+} influx induced by stimulation of the same cells with antibody to immunoglobulin M (anti-IgM) (*3*). Sequence analysis of the complementary DNAs (cDNAs) encoding murine CD38 (*4*), human CD38

M. Howard, J. C. Grimaldi, J. F. Bazan, F. E. Lund, L. Santos-Argumedo, DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304.
R. M. E. Parkhouse, Institute for Animal Health, Surrey GU24 ONF, United Kingdom.
T. F. Walseth and H. C. Lee, Departments of Pharmacology and Physiology, University of Minnesota, Minneapolis, MN 55455.

*To whom correspondence should be addressed.

(5), and ADP–ribosyl cyclase, an enzyme originally isolated from the sea mollusk *Aplysia californica* (6–8) revealed a 24% sequence identity between the latter two proteins (9) and conservation of 10 out of 12 cysteine residues in all three proteins (10). The latter suggested that the tertiary structures of these polypeptides were similar and that they thus possibly shared functional properties. The ADP–ribosyl cyclase converts NAD^+ into cADPR, a Ca^{2+}-mobilizing agent that acts independently of IP_3, probably by activating ryanodine receptors (11–15). The cADPR causes release of intracellular Ca^{2+} in sea urchin eggs (16), rat pituitary cells (17), dorsal root ganglion cells (18), pancreatic islet β cells (13), brain microsomes (13, 14, 19), and cardiac sarcroplasmic reticulum (14). In light of the observed structural similarity between CD38 and the *Aplysia* enzyme, we investigated whether CD38 exhibited ADP–ribosyl cyclase activity.

Murine CD38 is a typical type II membrane glycoprotein that consists of a short cytoplasmic tail of 23 amino acids, a 22–amino acid transmembrane region, and a 259–amino acid extracellular domain (4). The extracellular domain of murine CD38 was expressed (20) and purified (21) by standard procedures. This material effectively antagonized proliferation of murine B cells induced by anti-CD38 but did not affect the proliferative response of murine B cells to anti-IgM (10). The ADP–ribosyl cyclase activity was evaluated with sea urchin egg homogenates to monitor cADPR production (15). Purified CD38 (1.35 μg/ml) was incubated with 3.4 mM NAD^+ for 22 min at 37°C, and then 1 μl of the mixture was added to the homogenates. Release of Ca^{2+} was readily observed (Fig. 1A). The homogenates became transiently desensitized to subsequent stimulation with cADPR but remained fully responsive to stimulation with IP_3 (Fig. 1A). Similarly, homogenates initially stimulated with cADPR became transiently desensitized to the CD38-NAD^+ reaction mixture but remained responsive to stimulation with IP_3 (Fig. 1B). Addition of an 8-NH_2 derivative of cADPR (8-NH_2-cADPR), an antagonist of cADPR in sea urchin egg homogenates and intact eggs (22), blocked the effect of cADPR on Ca^{2+} release from egg homogenates without altering the effect of IP_3 (Fig. 1C). The 8-NH_2-cADPR also blocked the Ca^{2+} release induced by the CD38-NAD^+ reaction mixture (Fig. 1D). Thus, the CD38-NAD^+ reaction mixture contained a Ca^{2+}-mobilizing agent that was indistinguishable from cADPR but readily distinguishable from IP_3.

The presumed production of cADPR in the CD38-NAD^+ reaction mixture was verified by high-performance liquid chromotography (HPLC) analysis, with an AG MP-1 column (Bio-Rad, Richmond, California) (23). When individual fractions from an HPLC chromatograph of the CD38-NAD^+ reaction mixture were assayed for Ca^{2+}-releasing activity in the egg homogenate assay, the activity had an elution time identical to that of a control preparation of cADPR (10). Importantly, native CD38 immunoprecipitated from the membranes of murine B cells also catalyzed the metabolism of NAD^+ (10). Although the latter experiments have not yet distinguished cADPR versus ADPR formation, cADPR formation can be readily demonstrated with whole lysates from CD38-expressing lymphocytes (10).

The time course of production of cADPR by CD38 was measured (Fig. 2B) and compared to a calibration curve of Ca^{2+} release produced by different concentrations of purified cADPR (Fig. 2A). When these kinetic studies were extended over a longer time course, they revealed that the production of cADPR reached a maximum concentration of 8 μM in about 10 min and then stayed constant for the next 30 min (Fig. 2, inset). This plateau suggested that CD38 not only made cADPR, but also degraded it. The latter was demonstrated directly by incubation of CD38 with cADPR, with subsequent HPLC analysis showing the conversion of this substrate to ADPR after a 60-min incubation (Fig. 3). No ADPR was detected in control incubations that contained cADPR but lacked CD38 (10). The initial rate of CD38-induced cADPR hydrolysis was measured to be 27 μmol mg^{-1} min^{-1} at 37°C. Assuming the CD38 hydrolase is saturated with 1.5 mM cADPR, this corresponds to a turnover rate of 18 s^{-1}. The kinetic data shown in Fig. 2 (inset) suggest that the apparent initial rate of cADPR production is approximately 1.2 μmol mg^{-1} min^{-1}. Assuming the CD38 cyclase is saturated with 3.4 mM NAD, this corresponds to approximately 0.8 s^{-1}. This latter rate is likely to be an underestimate because some of the cADPR produced will presumably be hydrolyzed.

The results shown in Figs. 2 and 3 indicate that CD38 not only can catalyze the production of cADPR, but also can hydrolyze cADPR. In this context, CD38 represents a bifunctional enzyme exhibiting both cyclase and hydrolase activity. Numerous other examples of bifunctional enzymes exist in the literature. Particularly pertinent to the current study are the several bacterial toxins involving single polypeptides that exhibit both nicotinamide adenine dinucleotidase and ADP–ribosyl transferase activities (24). This enzymatic bifunctionality is in contrast to that of the *Aplysia* cyclase, which has been shown definitively to possess only the cyclase activity

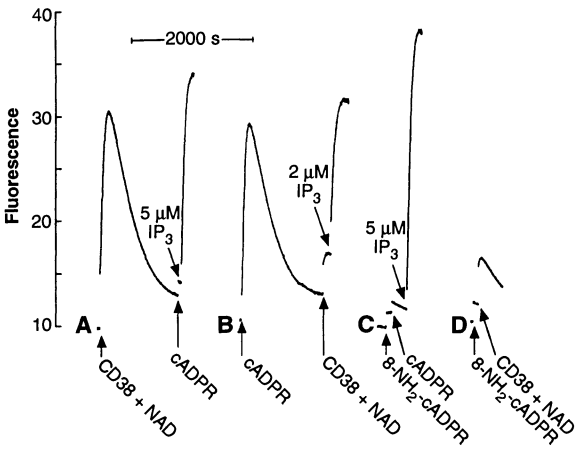

Fig. 1. Calcium-mobilizing activity of the reaction product obtained after incubation of NAD^+ with CD38. (**A**) CD38 + NAD stimulation with subsequent stimulation by cADPR and IP_3. (**B**) cADPR stimulation with subsequent CD38 + NAD and IP_3 stimulation. Addition of 8-NH_2-cADPR blocked the effect of cADPR (**C**) and CD38 + NAD (**D**), but not IP_3 (C). Where indicated, a portion (1 μl) of the CD38 + NAD mixture was added to 0.2 ml of 1.25% egg homogenate at a constant temperature of 17°C. Alternatively, cADPR, 8-NH_2-cADPR, and IP_3 were added at final concentrations of 90 nM, 0.88 μM, and either 5 μM or 2 μM, respectively.

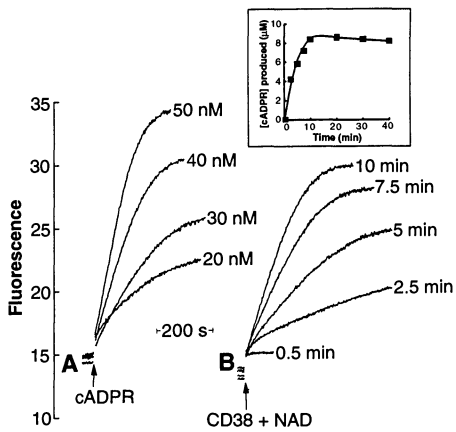

Fig. 2. Time course of production of cADPR catalyzed by CD38. (**A**) Calibration of the Ca^{2+} release activity of egg homogenates with various concentrations of standard cADPR. (**B**) Kinetics of production of Ca^{2+} release activity in a CD38-NAD^+ mixture. HPLC-purified CD38 (1.35 μg/ml) was incubated with 3.4 mM NAD^+ (pH 7.0) at 37°C. One microliter of this mixture was added to egg homogenates after the various times shown. (**Inset**) Time course of production of cADPR in the CD38-NAD^+ mixture.

with no detectable hydrolase activity (*7*). This major difference may presumably explain why the specific activity of the CD38 cyclase (that is, 0.8 s^{-1}) is so much lower than that of the *Aplysia* cyclase (that is, 450 s^{-1}) (*7*). Alternatively, native CD38 may be intimately associated with critical stimulatory subunits or cofactors also expressed in lymphocytes, and these accessory molecules may increase the specific activity of CD38 or the ratio of cyclase to hydrolase activity. Importantly, although the specific activity of recombinant CD38 is lower than that of the *Aplysia* cyclase, it is nevertheless comparable to the turnover rates of numerous other physiologically significant enzymes, such as lysozyme (0.5 s^{-1}) and DNA polymerase I (15 s^{-1}) (*25*). Thus, the rate of CD38 turnover is sufficiently high to render this activity of potential physiological significance.

Our data above suggest that the extracellular domain of the lymphocyte antigen CD38 may metabolize cADPR, an IP_3-independent Ca^{2+}-mobilizing agent. The possibility that this enzymatic activity contributes to the function of CD38 on lymphocytes is consistent with the known biological properties of this antigen. In particular, antibodies to CD38 induce a Ca^{2+} influx in murine B lymphocytes that can be distinguished mechanistically from the IP_3-dependent Ca^{2+} influx induced by the same cells with anti-IgM (*3*). Furthermore, although IP_3 is the only Ca^{2+}-mobilizing agent that has been identified in B lymphocytes to date (*26*), B cell activation involving Ca^{2+} mobilization has been shown to occur independently of phosphoinositide hydrolysis after some modes of immunoglobulin receptor stimulation (*27*).

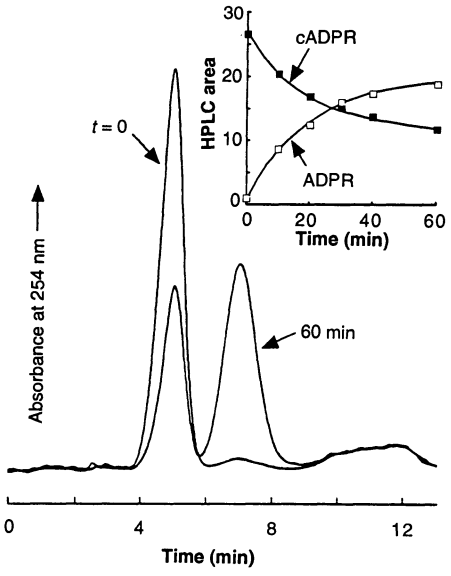

Fig. 3. Hydrolysis of cADPR catalyzed by CD38. CD38 (1.35 μg/ml) was incubated with 1.5 mM cADPR at 37°C. Portions of the mixture were removed at various times during incubation and analyzed by HPLC on an AG MP-1 column (*23*). The data shown represent 0- and 60-min incubation. (**Inset**) The areas of the peaks in the chromatographs that corresponded to cADPR and ADPR were obtained by integration and plotted against the time of incubation.

To explore the potential contribution of ADP–ribosyl cyclase to B cell activation, we added varying dilutions of cADPR to purified splenic lymphocytes in the presence or absence of growth costimulants. Although cADPR by itself had little effect on lymphocytes in terms of Ca^{2+} influx or membrane antigen modulation either in the presence or absence of other lymphocyte costimulants (*10*), it did significantly enhance the proliferative response of lymphocytes costimulated with suboptimal concentrations of a mixture of other growth costimulants (Fig. 4). In contrast, a wide concentration range of ADPR produced no growth-enhancing effects when it was added to the same induced B cells (Fig. 4). The inability of similar concentrations of cADPR to induce Ca^{2+} influx in B lymphocytes may well be explained by a recent article of Yamada *et al.* (*27*). These investigators have identified a weak, IP_3-independent Ca^{2+} influx in activated B lymphocytes that cannot be detected by conventional technologies and is evident only with the use of the highly sensitive procedure of digital image analysis of individual cells.

The concept of outer membrane expression by a large proportion of lymphocytes of an enzyme that metabolizes NAD^+ presents certain intriguing, unresolved issues. In particular, what is the physiological mechanism for regulating this extracellular enzyme and how does its activation lead to intracellular signaling? One may envision either an extracellular enzymatic role for CD38 or, alternatively, that CD38 becomes internalized in response to some external stimulant and then acts intracellularly. In terms of the former model and its obligate requirement of an extracellular source of NAD^+, low molecular weight "intracellular molecules" are sometimes found outside cells—for example, adenosine triphosphate (ATP) has recently been identified as an extracellular neurotransmitter (*28, 29*). In this context, one could imagine a mechanism whereby apoptosing lymphocytes, such as mature germinal center B cells or immature thymocytes, may release NAD^+, creating a transiently elevated local extracellular concentration of this substrate available to neighboring CD38-expressing lymphocytes. The alternate proposal that CD38 may become internalized after activation seems equally plausible. Indeed, recent experiments by Malavasi *et al.* (*2*) have shown that CD38 co-caps (that is, it localizes to one pole of the cell) with the B cell antigen receptor IgM after B cell activation, which suggests that CD38 may become internalized with IgM after antigen binding.

In either model, the binding of CD38-induced cADPR to as-yet-unidentified intracellular cADPR receptors would presumably require a transport mechanism for mobilizing cADPR from either its extracellular or intracellular vesicular site of production to the intracellular site of cADPR receptors. Alternatively, cADPR may produce its growth-augmenting effects on B cells (Fig. 4) through a receptor on the cell surface by means of a mechanism that is unrelated to its intracellular Ca^{2+}-mobiliz-

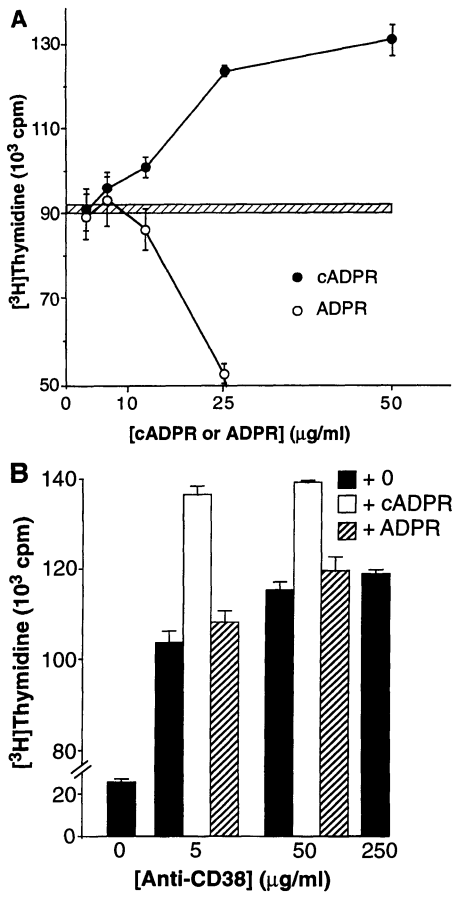

Fig. 4. Augmentation by cADPR of the proliferative response of splenic lymphocytes stimulated with CD38 antibodies, lipopolysaccharide (LPS), and IL-4. Lymphocytes were purified and cultured as described (*3*). Incorporation of [^{3}H]thymidine was used to evaluate proliferation after 3 days in culture. (**A**) Lymphocytes were cultured with suboptimal concentrations of anti-CD38 (5 μg/ml) and LPS (0.5 μg/ml), plus IL-4 (100 U/ml) either in the presence or absence (hatched region) of varying concentrations of either cADPR or ADPR. (**B**) Lymphocytes were cultured with IL-4 (100 U/ml) and suboptimal LPS (0.5 μg/ml) together with different concentrations of anti-CD38. Some cultures were additionally supplemented with the maximum nontoxic concentration of cADPR (50 μg/ml) or ADPR (12 μg/ml).

ing activity. This would be analogous to the extracellular functions of ATP or cyclic adenosine monophosphate (*28–30*), which are very much different from their intracellular roles. Further experimentation will be required to clarify these various possibilities and to fully elucidate the contribution of this lymphocyte-associated ADP–ribosyl cyclase activity in the immune system.

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20. Soluble CD38-FLAG was constructed with the polymerase chain reaction (PCR) to amplify the murine CD38 extracellular domain commencing with amino acid residue 46. The accuracy of the PCR-derived recombinant CD38 construct was confirmed by sequencing. The CD38 PCR fragment was ligated into a pMeV18S derivative containing a CD8 leader sequence adjacent to an eight–amino acid FLAG sequence [T. P. Hopp *et al.*, *BioTechnology* **6**, 1204 (1988)]. Expression of this construct in mammalian cells yielded a polypeptide that contained the entire extracellular domain of murine CD38, together with the FLAG octapeptide (NH_2-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-COOH) attached at the NH_2-terminus. The expressed material was detected by enzyme-linked immunosorbent assay with plates (96 well) coated with mouse monoclonal antibody to FLAG (International Biotechnologies, New Haven, CT). Bound CD38 was detected with anti-CD38.
21. Supernatants were concentrated and affinity-purified with a FLAG antibody column (International Biotechnologies, New Haven, CT). The protein was eluted with 0.1 M glycine-HCl (pH 3.0), then further purified on an HPLC Mono-Q column (Pharmacia) with a salt gradient of 10 mM tris-HCl (pH 7.5) and 2 M NaCl in 10 mM tris-HCl (pH 7.5). The resultant material was composed of a single protein species of approximately 40 kD after SDS–polyacrylamide gel electrophoresis.
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Immune Responses in Interleukin-2–Deficient Mice

Thomas M. Kündig,* Hubert Schorle, Martin F. Bachmann, Hans Hengartner, Rolf M. Zinkernagel, Ivan Horak

The role of costimulatory signals in T cell induction was evaluated in mice lacking the interleukin-2 (IL-2) gene. In vitro secondary antiviral T cell responses were absent unless IL-2 was added, confirming the crucial role of IL-2 in vitro. In vivo, primary and secondary cytotoxic T cell responses against vaccinia and lymphocytic choriomeningitis virus were within normal ranges. B cell reactivity to vesicular stomatitis virus was not impaired. T helper cell responses were delayed but biologically functional. Natural killer cell activity was markedly reduced but inducible. These normal in vivo immune responses in IL-2–deficient mice question the importance of IL-2 as defined by in vitro studies.

Stimulation of the T cell receptor (TCR) alone is insufficient for T cell induction; a second, costimulatory signal must be provided (*1*). The interaction between B7 and BB1 (*2*) on the antigen-presenting cell with CD28 (*3*) and CTLA-4 (*4*) on the T cell can provide this second signal. This costimulation drastically increases the production of IL-2 (*5*), which is crucial for in vitro proliferation of T cells (6) and is assumed to be the central growth hormone of the immune system. IL-2 is produced mainly by activated T helper (T_H) cells (*7*) and stimulates their proliferation in an autocrine fashion. IL-2 is also required for in vitro growth of cytotoxic T cell (CTL) lines and has been shown to enhance natural killer (NK) cell (*8*) and B cell (*9*) responses. In a study to assess the importance of IL-2 in immune responsiveness in vivo, mice made deficient for the IL-2 gene by homologous recombination (*10*) were evaluated for immune responses against three different viruses. CTL function was studied with the use of vaccinia virus and lymphocytic choriomeningitis virus (LCMV), since cytotoxic responses are crucial in resolving these two viral infections (*11*). Humoral immune responsiveness was assessed with vesicular stomatitis virus (VSV) as a model antigen where B cells and T_H cells play the major role in the immune response (*12*).

T. M. Kündig, M. F. Bachmann, H. Hengartner, R. M. Zinkernagel, Institute of Experimental Immunology, Schmelzbergstr. 12, University of Zürich, Zürich, Switzerland.

H. Schorle and I. Horak, Institute for Virology and Immunobiology, Versbacherstr. 7, University of Würzburg, Würzburg, Germany.

*To whom correspondence should be addressed.

It is not clear whether CTLs can be generated in the absence of IL-2. In many model systems, the generation of CTL responses depends on the presence of T_H cells (*13*), which are the major source of IL-2 (*7*), although certain viruses [for example, ectromelia (*14*), LCMV (*15*), and vaccinia virus (*16*)] can induce an efficient CTL response in the absence of T_H cells. In these latter cases, IL-2 from $CD8^+$ T cells rather than T_H cells has been assumed to be involved (*17*).

Vaccinia-specific CTL responses were measured 6 days after intravenous infection with vaccinia virus in three independent experiments. IL-$2^{-/-}$ mice and IL-$2^{+/-}$ littermates or C57BL/6 mice responded equally well (Fig. 1C).

LCMV-specific cytotoxicity was assessed 9 days after infection by primary ^{51}Cr-release assays. The frequency of LCMV-specific CTLs generated by IL-$2^{-/-}$ mice was reduced about threefold as compared with that generated by heterozygous littermates in both independent experiments (Fig. 1, A and B). This decrease in specific CTL frequency in the latter assay was of no biological importance, since the CTL responses in IL-$2^{-/-}$ mice mediated a normal early immunopathological swelling-reaction of the footpads starting 7 to 8 days after local infection with LCMV (Fig. 1, D and E). The early phase of swelling is dependent exclusively on $CD8^+$ T cells (*15*, *18*). The primary CTL response eliminated LCMV below detection levels from spleens and livers by day 10 in both IL-$2^{-/-}$ and control mice (*19*). IL-$2^{-/-}$ mice that had been primed intravenously with LCMV were protected against lethal choriomeningitis