Differential T Cell Costimulatory Requirements in CD28-Deficient Mice

Arda Shahinian,* Klaus Pfeffer,* Kelvin P. Lee,*† Thomas M. Kündig, Kenji Kishihara, Andrew Wakeham, Kazuhiro Kawai, Pamela S. Ohashi, Craig B. Thompson, Tak W. Mak‡

T cell receptor stimulation without costimulation is insufficient for the induction of an optimal immune response. It is thought that engagement of the CD28 molecule with its ligand B7 provides an essential costimulatory signal without which full activation of T cells cannot occur. A mouse strain with a defective CD28 gene was established. Development of T and B cells in the CD28-deficient mice appeared normal. However, T lymphocytes derived from $CD28^{-/-}$ mutant mice had impaired responses to lectins. Lectin stimulation did not trigger interleukin-2 (IL-2) production, IL-2 receptor α expression was significantly decreased, and exogenous IL-2 only partially rescued the CD28 defect. Basal immunoglobulin (Ig) concentrations in CD28-deficient mice were about one-fifth of those found in wild-type controls, with low titers of IgG1 and IgG2b but an increase in IgG2a. In addition, activity of T helper cells in $CD28^{-/-}$ mice was reduced and immunoglobulin class switching was diminished after infection with vesicular stomatitis virus. However, cytotoxic T cells could still be induced and the mice showed delayed-type hypersensitivity after infection with lymphocytic choriomeningitis virus. Thus, CD28 is not required for all T cell responses in vivo, suggesting that alternative costimulatory pathways may exist.

The molecular basis of T cell costimulation is not fully understood but may involve ligands and soluble factors provided by antigen-presenting cells (APCs) that interact with specific T cell surface molecules (*1–3*). One major costimulatory pathway is characterized by the activation of the CD28 receptor (*4, 5*). Cross-linking of CD28 synergizes with T cell receptor (TCR) signals during T cell activation and can prevent the induction of T cell unresponsiveness in vitro (*6*). CD28 and the related CTLA-4 molecule (*7*) share a common ligand (B7), which is expressed on APCs (*4, 7–9*). In the mouse, CD28 molecules are constitutively expressed on almost all $CD4^+$ and $CD8^+$ peripheral T cells (*10*), whereas CTLA-4 is found on activated T cells only (*11*). Murine double positive ($CD4^+CD8^+$) thymocytes express large amounts of CD28, whereas immature double negative ($CD4^-CD8^-$) and mature single positive thymocytes have less CD28 (*10*). The B7 molecule, constitutively expressed on dendritic cells (*12*), is also expressed on other APCs and B cells and is up-regulated after activation (*9, 12–14*). Studies in vitro indicate that CD28-B7 engagement in combination with TCR occupancy delivers important signals for induction of proliferation of $CD4^+$ T cells and for T cell–dependent B cell differentiation (*15*).

The signaling pathways for CD28 have not yet been unraveled; however, tyrosine phosphorylation of cellular proteins changes after CD28 cross-linking (*16*). At transcriptional and posttranscriptional levels, CD28 can synergize with TCR signals (*17, 18*). Signals mediated by CD28 stabilize a set of important mRNAs for cytokines and monokines (*17*). Disruption of CD28 and CTLA-4 interactions with B7 by the introduction of a soluble form of CTLA-4 (CTLA4Ig) impedes cell-mediated immune responses in vitro (*19*) and leads to T cell unresponsiveness (*6, 20, 21*). Experiments in vivo suggest that treatment with CTLA4Ig can reduce IgG1 production in a primary response to keyhole limpet hemocyanin or sheep red blood cells (*22*). Similar treatment also results in long-term acceptance of xenogenic pancreatic islet grafts (*23*) and prolongs the survival of cardiac allografts (*24*). Transfection of B7 into tumor cells induces or augments the generation of a cell-mediated immune response to otherwise nonimmunogenic tumor cells (*25, 26*).

Despite such data, it has not been determined whether CD28 is the only costimulatory signal for T cell activation in vivo. The role of CD28 in viral infections and thymocyte differentiation and its interdependence with CTLA-4 have not yet been elucidated. To address these questions, we have established a mutant mouse strain lacking the CD28 costimulatory molecule.

The CD28 gene (*27*) was disrupted by a partial replacement of exon 2 with a neomycin resistance gene cassette (*28*) (Fig. 1A). Homologous recombination events were screened by polymerase chain reaction (PCR) and verified by Southern (DNA) blot analysis (Fig. 1B). Of the 814 colonies we screened that were G418-resistant, 3 contained the desired mutation. We obtained chimeric mice by injecting mutant embryonic stem cells into C57BL/6 blastocysts (*29, 30*). We tested for transmission of

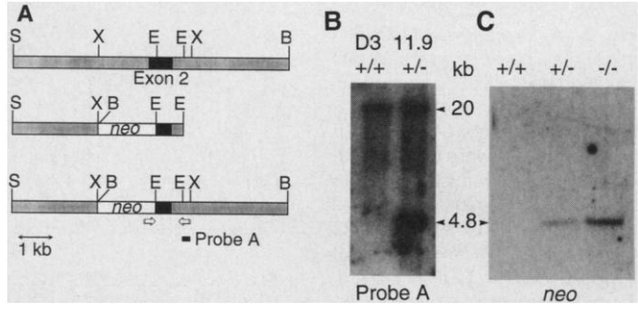

Fig. 1. Gene targeting of the murine CD28 locus. (**A**) A genomic clone of the murine CD28 gene (*41*) was isolated from an EMBL/3 genomic library prepared from BALB/c liver DNA. We used the exon 2 cDNA from murine CD28 as a probe. The 3′ end of intron 1 and the 5′ region of exon 2 were replaced by a pMCIneopA-derived (*28*) neomycin resistance gene (*neo*) cassette in the antisense direction using an Xba I and an Eco RI site. The replacement vector spanned 4.3 kb. Exon 2 is indicated by a black box; B, Bam HI; E, Eco RI; S, Sal I; and X, Xba I. We transfected D3 embryonic stem (ES) cells with the linearized construct as described (*30*). Targeted D3NS ES cell colonies were analyzed for homologous recombination by PCR with primers specific for the thymidine kinase promoter of the *neo* cassette and for an intronic sequence 3′ of the targeting construct (white arrows). Shown are, from top to bottom, partial CD28 gene, targeting vector, and mutant CD28 locus. (**B**) Southern blot analysis of D3 DNA (control) and cell line 11.9 using probe A (0.2 kb Eco RI–Xba I fragment of intron 2) and a *neo* probe to verify homologous recombination events. The ES cell line 11.9 shows the expected sizes of bands (4.8 and 20 kb) after digestion with Bam HI. (**C**) Southern blot analysis of Bam HI–digested genomic tail DNA of wild-type, heterozygous, and homozygous offspring after we mated (C57BL/6 × DBA/2)F_1 mice with chimeric founder mice and bred heterozygous offspring. Additional integration sites of the targeting construct were not detected with the *neo*-cassette probe.

A. Shahinian, K. Pfeffer, K. Kishihara, A. Wakeham, T. W. Mak, Amgen Institute, Department of Medical Biophysics and Immunology, Ontario Cancer Institute, University of Toronto, Toronto M4X 1K9, Canada.
K. P. Lee and C. B. Thompson, Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109.
T. M. Kündig, K. Kawai, P. S. Ohashi, Department of Medical Biophysics and Immunology, Ontario Cancer Institute, Toronto M4X 1K9, Canada.

*These authors have contributed equally to this work.
†Present address: Immune Cell Biology Program, Naval Medical Research Institute, Bethesda, MD 20889.
‡To whom correspondence should be addressed.

the mutated allele by mating the chimeric mice with (C57BL/6 × DBA/2)F_1 mice. Heterozygous offspring were intercrossed to generate mice homozygous for the targeted mutation of the CD28 gene. Homozygous mice were healthy and fertile, and we did not detect gross abnormalities in body weight, organ size, or number of lymphocytes in primary and secondary lymphatic organs. To verify inactivation of the CD28 molecule, we stained the cell surface with phycoerythrin (PE)-conjugated hamster monoclonal antibodies (mAbs) to mouse CD28 (Fig. 2A). Peripheral blood lymphocytes (PBLs) from homozygous targeted mice did not express CD28 molecules, indicating that the CD28 locus was disrupted in $CD28^{-/-}$ mice. The PBLs from heterozygous ($CD28^{+/-}$) mice had decreased expression of cell surface CD28 (Fig. 2A). Thymocyte development in CD28-deficient mice appeared normal, as we judged from the expression of CD4, CD8 (Fig. 2B), CD3, heat-stable antigen, and interleukin-2 receptor α (IL-2Rα) (*31*).

Analysis of the V_β repertoire of CD28-deficient mice showed no defect in the clonal deletion of potentially self-reactive T cells. Mls-1^a–reactive (*32*) $V_\beta6^+$ T cells were efficiently deleted in the peripheral blood of $CD28^{-/-}$ mice (0.2% $V_\beta6^+$ in $CD28^{-/-}$ Mls-1^{a+} versus 7.5% $V_\beta6^+$ in $CD28^{-/-}$ Mls-1^{a-}). Accordingly, $V_\beta11$-bearing T cells (*33*) were also depleted in CD28-deficient mice when I-E and endogenous viral products were present ($CD28^{-/-}$, $I\text{-}E^+$, 0.4% $V_\beta11^+$ cells; $CD28^{-/-}$, $I\text{-}E^-$, 4.7% $V_\beta11^+$ cells). Splenic B cells were observed in normal numbers, and cell surface expression of B7 was not detectable in homozygous $CD28^{-/-}$ animals (Fig. 2B). Staining of splenocytes with mAb against CD3, TCRαβ, TCRγδ, and IL-2 receptor also did not reveal any significant alterations between littermate controls and $CD28^{-/-}$ mice (*31*).

Basal immunoglobulin (Ig) concentrations in $CD28^{-/-}$ mice were only 20% of those of wild-type littermates (Fig. 3). Analysis of Ig isotypes revealed a decrease in IgG1 and IgG2b, whereas IgG2a was increased significantly. Basal IgM and IgG3 titers seemed to be unaffected by the CD28 mutation (Fig. 3).

To evaluate the role of the CD28 molecule on T cell proliferation and cytokine secretion, we stimulated splenocytes from wild-type, heterozygous, and homozygous mice with the T cell lectin concanavalin A (Con A) (Fig. 4A). T cells derived from $CD28^{-/-}$ mice had a significantly reduced proliferative response to Con A. However, $CD28^{-/-}$ cells were not impaired in their growth potential when activated by a combination of phorbol ester [phorbol 12-myristate 13-acetate (PMA)] and calcium ionophore (Fig. 4A). Supernatants of lectin-stimulated cultures contained reduced amounts of T cell growth factors, as we determined by a standard CTLL-2 assay (Fig. 4B). The amount of IL-2Rα expression was much lower on lectin-activated $CD28^{-/-}$ T cells than on T cells derived from wild-type littermate controls (Table 1). Addition of exogenous IL-2 or T cell–conditioned medium to lectin-stimulated cultures only partially restored the proliferative response and IL-2Rα expression of $CD28^{-/-}$ T cells (Fig. 4A and Table 1). These results indicate that lectin-driven T cell responses are critically dependent on a functional costimulatory signal through CD28 and that this signal cannot be replaced completely in vitro by IL-2.

We tested antiviral T and B cell responses by infecting mutant and wild-type mice with lymphocytic choriomeningitis virus (LCMV) or vesicular stomatitis virus (VSV). The $CD28^{-/-}$ mice mounted a normal anti-LCMV cytotoxic T cell (CTL) response in vivo (Fig. 5A). To confirm that the capacity of $CD28^{-/-}$ mice to elicit a LCMV-specific CTL response was unaltered, we injected LCMV into the footpads of mice. The footpads subsequently showed a normal immunopathological swelling reaction (Fig. 5B). The early phase of this swelling reaction is mediated exclusively by $CD8^+$ CTLs (*34*, *35*).

After infecting CD28-deficient mice

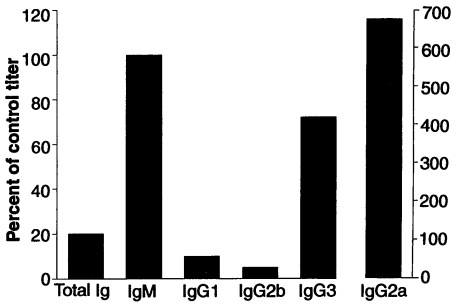

Fig. 3. Decreased basal Ig and altered IgG subclass patterns in $CD28^{-/-}$ mice. Serum from two $CD28^{+/+}$ control mice or $CD28^{-/-}$ mice were pooled and Ig was determined in an enzyme-linked immunosorbent assay with isotype-specific, alkaline-phosphatase–conjugated antibodies (Southern Biotechnology Associates). Titers were determined by fivefold serial dilutions. Wild-type titers were taken as 100%, and $CD28^{-/-}$ titers were calculated accordingly for both charts. Standard deviations were less than 4%. Results shown are representative of three independent experiments.

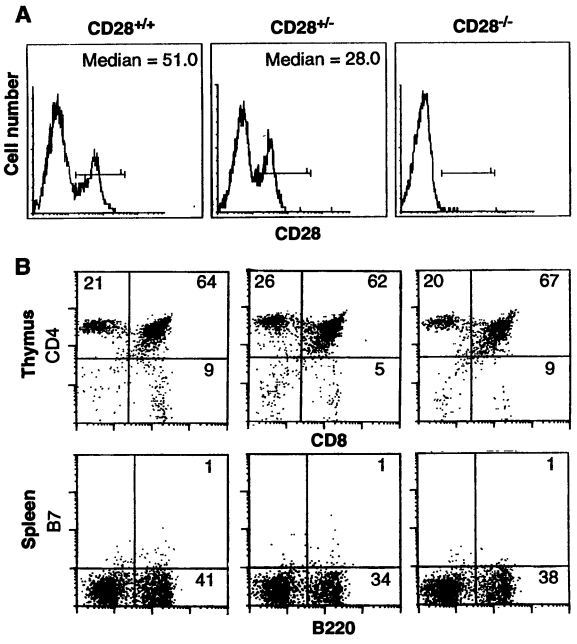

Fig. 2. (**A**) Expression of CD28 in mice that carry a homozygous mutation in the CD28 locus. Flow-cytometric analysis of peripheral blood lymphocytes of CD28 mutant mice. Cells were stained with PE-conjugated hamster mAb to murine CD28 (Pharmingen). We analyzed a total of 5000 cells for each sample. (**B**) Development of T and B cells in $CD28^{-/-}$ mice. Thymocytes were stained with fluorescein isothiocyanate (FITC)–conjugated mAb to CD8 or PE-conjugated mAb to CD4. Splenocytes were stained with FITC-conjugated mAb to B220 and biotin-conjugated mAb to B7 plus streptavidin-PE, as indicated (all mAb from Pharmingen). The numbers represent percentages of cells in each quadrant. We analyzed a total of 10,000 viable cells for each sample.

Table 1. Kinetics of IL-2Rα expression on CD28-deficient splenocytes after mitogen stimulation. Values shown are mean fluorescence intensity values of IL-2Rα expression on Thy1.2-positive cells. Splenocytes of $CD28^{+/+}$ and $CD28^{-/-}$ mice were cultured in the presence of Con A (5 μg/ml) or Con A plus exogenous mouse recombinant IL-2 (5 U/ml) in 24-well plates at 3 × 10^6 cells per well. Expression of IL-2Rα on the cell surface of T lymphocytes was determined at 0, 24, and 48 hours. Cells were harvested and stained consecutively with a biotinylated rat mAb to mouse IL-2Rα (Pharmingen), streptavidin-PE, and Thy1.2-FITC. Cells were analyzed by flow cytometry, and 50,000 viable cells were scored per sample.

Phenotype	Nil	Con A		Con A + rIL-2	
	0	24	48	24	48
$CD28^{+/+}$	<1	6.0	82.0	11.0	102.3
$CD28^{-/-}$	<1	5.1	24.2	10.6	62.0

with VSV, we found neutralizing anti-VSV IgM titers to be normal. However, the class switch to neutralizing antibodies of the IgG class (*34*), which is strictly T helper cell–dependent, was reduced (Fig. 6). Taken together, these data indicate that the generation of $CD8^+$ CTLs in response to LCMV is not dependent on interactions involving CD28, whereas T cell–B cell collaboration appears to be dependent on a functional CD28-B7 costimulation.

We have established a mutant mouse strain deficient for the CD28 gene. Despite expression of CD28 on thymocytes and B7 on APCs in wild-type mice (*9, 10, 13*), development of T lymphocytes in the CD28 mutant mice appears normal, suggesting that this interaction is not essential for thymic maturation of T cells. The ability to delete potentially self-reactive T cell receptors for Mls-1^a and I-E indicates that thymic negative selection of $CD28^{-/-}$ mice is not dependent on CD28-B7 signaling.

Lectins do not activate highly purified T cells in the absence of APCs (*36*), yet the molecular basis of this phenomenon is not completely understood. Our data that the interaction of B7 and CD28 is critical for the mitogenicity of T cell lectins are consistent with preliminary in vitro experiments that showed that CTLA4Ig could block Con A–induced proliferation in murine $CD28^{+/+}$ splenocytes. Lectin-stimulated $CD28^{-/-}$ splenocytes did not secrete T cell growth factors, suggesting that T cell–dependent lectins are operative through CD28 costimulation. This observation is in accordance with previous reports that CD28 costimulation plays a decisive role in the production of IL-2 (*20, 37*). Even in the presence of T cell–conditioned medium (*31*) or exogenous recombinant IL-2, the CD28 defect cannot be overcome. Hence, CD28 signal transduction is an essential prerequisite for lectin-induced T cell activation. In addition, heterozygous $CD28^{+/-}$ splenocytes show a constant reduction in their responses to Con A. The T cells derived from these mice display only about 50% of their CD28 molecules at the cell surface (Fig. 2). This suggests that even the amount of CD28 expressed plays a role in the regulation of T cell immune functions. The defects in mitogen responsiveness of $CD28^{+/-}$ and $CD28^{-/-}$ T cells do not appear to be due to an intrinsic functional abnormality in these T cells, because $CD28^{+/+}$, $CD28^{+/-}$, and $CD28^{-/-}$ T cells responded equivalently to the chemical mitogens PMA and Ca^{2+} ionophore, an activation scheme that bypasses the normal T cell activation pathways that are regulated by cell surface receptors.

In mice, CD28 is expressed on virtually all $CD4^+$ and $CD8^+$ T cells (*10*). It has been reported that the induction of $CD8^+$ CTL responses to tumors depends completely on the interaction of CD28 and B7 (*25*). However, this interaction does not appear to be essential for the induction of an anti-LCMV cytolytic immune response mediated by CD8 T cells. An explanation could be that CD8 T cells can become fully activated by costimulation through alternative pathways, which are induced by a replicating viral pathogen but not by a syngenic tumor cell. Such secondary signals may be provided by a set of cytokines or adhesion molecules (*1, 3, 5, 38*).

Immunoglobulin concentrations are re-

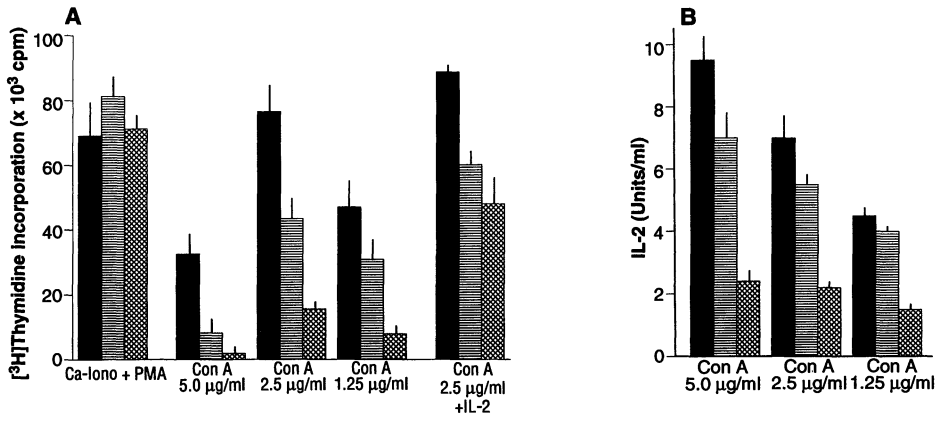

Fig. 4. Response of T cells from CD28-deficient mice to mitogen stimulation in vitro. (**A**) Proliferative response of splenocytes derived from $CD28^{+/+}$ (solid bars), $CD28^{+/-}$ (horizontally hatched bars), and $CD28^{-/-}$ (crosshatched bars) mice. Lymphocytes were plated in 96-well flat bottom plates at 2×10^5 cells per well. Concanavalin A, PMA (15 ng/ml), calcium ionophore A23617 (Ca-Iono, 250 ng/ml), and mouse recombinant IL-2 (50 U/ml) were added as indicated. We determined proliferation by measuring ^{3}H-labeled thymidine uptake after 2 days of culture. Results are compiled from three experiments. (**B**) CTLL-2 assay of supernatants from mitogen-activated splenocytes. Culture supernatants of unstimulated and stimulated splenocytes were harvested after 72 hours of culture and tested on CTLL-2 cells for the presence of T cell growth factors. An aliquot of 50 µl of culture supernatant was removed and serial dilutions were made. We quantified IL-2 content of individual culture supernatants by comparing proliferative responses of CTLL-2 cells with a standard curve that was obtained by addition of defined amounts of mouse recombinant IL-2 to CTLL-2 cells.

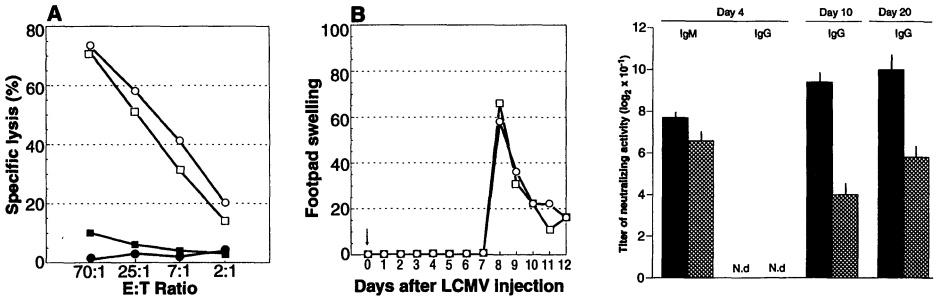

Fig. 5 (left). Response of $CD28^{-/-}$ mice to LCMV infection. (**A**) Mice (H-2^b) were infected intravenously with 200 plaque-forming units (PFU) of LCMV (Armstrong strain), and cytotoxic activities of splenic T cells of wild-type (circles) and CD28-deficient (squares) animals were tested 8 days after infection. Target cells were MC57G cells (H-2^b) either uninfected (closed symbols) or infected (open symbols) with LCMV as described (*40*). The percentage of specific release of ^{51}Cr (specific lysis) was calculated for each effector to target ratio (E:T) [(experimental release − spontaneous release) × 100/(total release − spontaneous release)]. Standard deviation was below 10% for each data point. Data given are compiled from four independent experiments. (**B**) Footpad swelling reaction after local injection of LCMV. Footpads of $CD28^{+/+}$ and $CD28^{-/-}$ mice were injected subcutaneously with 200 PFU of LCMV (Armstrong strain) on day 0. Subsequent swelling was measured with a spring-loaded caliper. Swelling was calculated as percentage of the footpad size before injection. Markers represent the mean swelling of four measurements, standard deviation was less than 20%. Data points for days 0 through 7 are superimposed. **Fig. 6 (right).** Decreased neutralizing anti-VSV response in mice defective for CD28 expression. We infected $CD28^{+/+}$ (solid bars) and $CD28^{-/-}$ (crosshatched bars) mice intravenously with VSV (Indiana strain, 2×10^6 PFU). At the time points indicated, sera from mice were analyzed for neutralizing IgM and IgG antibodies as described previously (*34*). Titers represent twofold dilution steps of sera starting with 1:40. Each bar represents the mean value of a group of five mice, and data are expressed as $\log_2 \times 10^{-1}$ of the neutralizing activity. (N.d., not detectable).

duced in $CD28^{-/-}$ mice. Whereas the amount of IgM is normal, the pattern of IgG subclasses in CD28-deficient mice is altered. The nature of the changes in T cell–dependent IgG subclasses in $CD28^{-/-}$ mice could lie in an altered pattern of cytokines that are involved in Ig isotype regulation (*39*). Disruption of CD28-B7 interaction with soluble CTLA4Ig suppresses T cell–dependent antibody responses to sheep erythrocytes or keyhole limpet hemocyanin (*22*). To investigate whether these results are due to impaired T or B cell function, we infected CD28 mutant mice with VSV. Our data show that B cell responsiveness is unaltered in the absence of CD28, as indicated by the normal titers of neutralizing anti-VSV IgM, which are independent of T cell helper activity (*34*). However, the class switch to IgG, which is dependent on the function of T helper cells (*34*), was significantly reduced, suggesting that T help is diminished in the absence of costimulation by CD28.

In conclusion, our data indicate that CD28 costimulation is differentially required for cell-mediated and humoral immune responses in vivo. The CD28-deficient mice will be a valuable tool to further elucidate the role of costimulatory events by CD28-dependent and -independent mechanisms in the generation of immune responses against pathogens and tumors as well as in the course of autoimmune diseases. The study of these animals could help determine where immunosuppression by disruption of CD28-B7 interaction can be effective as a treatment strategy as well as when T cell activation is dependent on other mechanisms of costimulation.

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Interaction of Activated EGF Receptors with Coated Pit Adaptins

Alexander Sorkin* and Graham Carpenter

The epidermal growth factor (EGF) receptor interacts with plasma membrane–associated adapter proteins during endocytosis through coated pits. Almost 50 percent of the total pool of α-adaptins was coimmunoprecipitated with the EGF receptor when A-431 cells were treated with EGF at 37°C, but not at 4°C. Partial proteolysis of α-adaptin suggested that the amino-terminal domain is the region that associates with the EGF receptor. The extent of receptor-adaptin association was increased in cells depleted of potassium to block endocytosis. These data suggest that receptor-adaptin association occurs in intact cells before coated pits are fully assembled.

A large variety of extracellular molecules (for example, nutrient carriers, growth factors, and peptide hormones) and viruses interact with the surface of mammalian cells and rapidly enter the cell through specialized coated pit regions of the plasma membrane (*1*, *2*). Clathrin-coated pits and vesicles have been implicated in the selective recruitment of membrane proteins in receptor-mediated endocytosis (*1*, *2*). However, the mechanism of this dynamic process, which must involve interactions between the cytoplasmic tails of receptors and coated pit proteins, is poorly understood, particularly for ligand-induced endocytosis. For example, binding of EGF to its receptor on the cell surface accelerates receptor endocytosis and results in a rapid loss of receptors (*3*–*6*). This ligand-induced downregulation is a general phenomenon for all receptor tyrosine kinases (*3*–*7*) and an important part of the signal transduction events initiated by the growth factors (*8*). Growth factor binding allows receptors, which are diffusely distributed at the cell surface, to cluster in clathrin-coated pits and, thereafter, to be rapidly internalized (*5*, *9*).

The main components of plasma membrane coated pits are AP-2, assembly or adapter proteins, and clathrin (*1*, *2*, *10*).

A. Sorkin, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232–0146.

G. Carpenter, Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232–0146.

*To whom correspondence should be addressed.