Limited Role of CD28-mediated Signals in T Helper Subset Differentiation

By Daniel R. Brown,* Jonathan M. Green,* Naomi H. Moskowitz,* Mandel Davis,* Craig B. Thompson,** and Steven L. Reiner**

From the *Department of Medicine, *Committee on Immunology, *Howard Hughes Medical Institute, and **Department of Molecular Genetics and Cell Biology, Gwen Knapp Center for Lupus and Immunology Research, University of Chicago, Chicago, Illinois 60637

Summary

The role of CD28-mediated signals in T helper cell maturation is not fully understood. We tested the requirement for costimulation through CD28 in several systems of CD4+ T cell differentiation. In vivo priming of mice with genetic disruption of CD28 (CD28−/−) yielded normal levels of antigen-specific interferon γ production but markedly diminished levels of interleukin 4 (IL-4) after in vitro restimulation. In response to the pathogenic microbe, Leishmania major, C57BL6 CD28−/− mice were fully capable of controlling infection and exhibited a normal T helper 1 response. BALB/c CD28−/− mice unexpectedly exhibited normal susceptibility to L. major. BALB/c CD28−/− mice developed high levels of IL-4 mRNA and protein induction in the draining lymph nodes. In addition, susceptibility of BALB/c CD28−/− mice was reversed by neutralization of IL-4 in vivo. We also activated transgenic CD28-bearing T cells from the BALB and C57BL background in vitro in the presence of CTLA4Ig. BALB cells had greater IL-4-producing capacity than C57BL cells in the absence of costimulation. Diverse factors including costimulatory signals, genetic polymorphism, and the nature of the immunogen all influence T helper phenotype commitment, but these results provide evidence that CD28 is not an absolute requirement for generating either Th1 or Th2 responses.

Under most circumstances, successful T cell activation requires a signal delivered through the T cell antigen receptor and a second signal referred to as costimulation (1). T cells activated in vitro in the presence of CD28-B7 blockade display characteristics of anergy (2), and blockade in vivo has attenuated many T cell-mediated immune responses, including graft rejection (3, 4) and autoimmunity (5–7). Analysis of CD28-deficient mice revealed relatively normal T cell development, preserved cytolytic T cell responses, but impaired B cell help (8). The role of costimulation in the generation and maintenance of antigen-specific CD4+ T helper (Th) subsets remains controversial. Data supports models in which both Th1 and Th2 responses require CD28-mediated signals (2, 9–11), but some evidence suggests that only certain Th subsets are dependent on costimulation (12–20). Other findings suggest discrete stages of maturation selectively require CD28-mediated costimulation (12), and some current models propose that specific B7 ligands mediate biased Th maturation (5, 6, 21, 22).

In murine infection with Leishmania major, pathogen-specific CD4+ Th effectors mediate the polarized potential outcomes of infection (23). Th1 responses that arise in C57BL/6 mice result in self-limited healing disease through successful macrophage activation by T cell–derived IFN-γ. Th2 responses that arise in mice from the BALB background mediate inexorable susceptibility characterized by uncontrolled parasite dissemination. The unique localization of the parasite to MHC class II-rich intracellular compartments leads to a strong bias in CD4+, class II–restricted immunization, such that polarized anti-leishmanial Th1 and Th2 phenotypes proceed normally, even in the genetic absence of MHC class I–restricted responses (24, 24a). Interventions that reduce the efficiency of CD4+ T cell responses can result in increased susceptibility to infection. Transient suppression of T cell immunity, however, can also yield a default from a Th2 to Th1 response with consequent healing in the BALB/c strain (23).

To address the requirements of CD28-mediated costimulation in generating Th1 and Th2 subset development in vivo, we infected CD28-deficient mice from both C57BL/6 and BALB/c backgrounds with L. major. We also tested antigen-specific cytokine responses to a nonpathogenic antigen, keyhole limpet hemocyanin (KLH). IL-4 and, to a lesser extent, IFN-γ production are augmented by CD28-
mediated signals. Despite reduced levels of Th cytokine production in vitro, genetically driven polarization of Th responses and stereotyped disease phenotypes were observed in *Leishmania*-infected, CD28-deficient mice. Transgenic T cells from the BALB and C57BL backgrounds had reduced IL-4 levels when primed in vitro in the presence of CTLA4Ig. Addition of rIL-4 during priming restored IL-4 levels from BALB cells, but not C57BL T cells. Together, these results provide evidence that CD28 is not absolutely required for generating a Th2 response in vivo. Genetic background, the replicating nature of the microbe, and/or the strength of other second signal activities provided in vivo may overcome the need for a component of activation formerly deemed indispensable for Th cell function.

**Materials and Methods**

**CD28-deficient Mice.** CD28−/− mice were generated as described (8). The animals used in these experiments were from the fifth back-cross to C57BL/6 and fourth or fifth back-cross to BALB/c. Heterozygote and wild-type littermates were used as controls for BALB/c experiments. Commercial wild-type mice (Jackson Laboratories, Bar Harbor, ME) were used as controls for C57BL/6 experiments. Mice were housed in a specific pathogen-free environment. Genotypes were determined by flow cytometry using stains for commercially conjugated fluorescent mAbs against CD28 and CD4 (Caltag Laboratories, South San Francisco, CA) and confirmation by PCR using two oligonucleotide pairs specifically spanning the genomic disruption of CD28 and the neomycin resistance marker. All work was performed in accordance with University of Chicago guidelines for animal use and care.

**KLH Immunization.** BALB/c CD28−/− and C57BL/6 CD28−/− mice plus normal littermate controls were immunized in the hind footpad and base of the tail with 100 µg KLH (Calbiochem-Novabiochem Corp., San Diego, CA) emulsified in complete Freund’s adjuvant (GIBCO BRL, Gaithersburg, MD). 8 days after immunization, draining lymph nodes were harvested and single cell suspensions were prepared in complete DME media (supplemented with 10% FCS, 2-MM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-ME, 10 mM Hepes, and 100 µM nonessential amino acids). For the restimulation assay, 5 × 10⁵ cells were plated in each well of a flat-bottom 96-well microtiter plate (Costar Corp., Cambridge, MA) with either media or KLH at 100 µg/ml. Supernatants for these and all other experiments were harvested at 48 h and analyzed for IL-4 and IFN-γ by ELISA using commercial mAb pairs (PharMingen, San Diego, CA) according to the manufacturer’s instructions.

**Infection with *L. major*.** Infections were performed by injection of 5 × 10³ metacyclic promastigotes (WHOM/IR/173) into each hind footpad. Lesion size was measured weekly with a metric caliper. Select animals were treated with 5 mg mAb 11B11 (rat IgG1 antimurine IL-4) intraperitoneally on day 0. Parasite cultures were quantitated by homogenization of tissue in fixed volumes of parasite media. Aliquots were diluted serially across 96-well plates and scored at 1 and 2 wk for parasite growth. *Leishmania*-infected mice were killed at 6–8 wk after infection and analyzed as individual animals.

Single cell suspensions of popliteal lymph node cells were examined for antigen-specific cytokine production by restimulation in vitro and also for direct ex vivo IL-4 production by ELISPOT. For the restimulation assays, 0.5–1 × 10⁵ lymph node cells per well were cultured in a 96-well round bottom microtiter plate in complete media with and without soluble *Leishmania* antigen (100 µg/ml). MHC class II was blocked using mAb M5/114 (rat IgG2b anti-A-h and -E), while mAb 10-2-16 served as an isotype control (mouse IgG2b anti-A). Supernatants from the restimulation cultures were removed at 48 h and analyzed for IL-4 and IFN-γ production by ELISA.

ELISPOTs were performed as previously described (25) using commercial mAb pairs (PharMingen). Briefly, plates were coated with purified antibody, 10⁵ lymph node cells were plated in two-fold serial dilutions and incubated at 37°C for 4 h. IL-4 spots were detected by sequential addition of biotinylated secondary antibody, streptavidin alkaline phosphatase (Jackson Immunoresearch Laboratories Inc., West Grove, PA), followed by development with 1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M 2-aminoo2-methyl-1-propanol (Sigma Chemical Co., St. Louis, MO) containing 0.6% agarose.

Lymph node cells taken directly from the animal were also placed immediately in RNAzol (Biotecx Laboratories, Houston, TX) and total RNA was extracted according to the manufacturer’s instructions. RNA was reverse transcribed into cDNA. Semiquantitative PCR was performed as described (26). Briefly, cDNA was amplified in the presence of a polynucleotide construct that contains addition mutations of the authentic cDNA. Resolved on agarose gels, the larger molecular weight bands provide an internal standard for the relative amounts of the lower molecular weight experimental cDNAs. Concentrations of cDNAs are adjusted using the housekeeping gene HPRT before assay of lymphokine gene transcription. Results were verified by repetition of both individual samples and whole experiments.

**In vitro Priming of Transgenic T Cells.** Transgenic DO11.10 mice recognizing a chicken ovalbumin-derived (OVA) peptide in the context of I-A^d^ were generously provided by Dr. Dennis Loh (27). Animals used in these experiments were from the fourth back-cross to B10.D2 or the eighth back-cross to BALB/c. Transgenic mice were identified by flow cytometry using stains for commercially conjugated fluorescent mAbs against V88 and CD4 (Caltag). Primary cultures were performed in 24-well plates using 5 × 10⁵ splenocytes from transgenic mice stimulated with 100 µg/ml of a tryptic digest of OVA protein (Sigma). Human CTLA4Ig (20 µg/ml; a generous gift from Gary Gray, Repligen Corp., Boston, MA) was used to block B7-mediated costimulation. After 6 d, cells were washed extensively and counted. Secondary stimulations were repeated in 96-well round-bottom plates in triplicate, using 2 × 10⁵ T cells, 1.2 × 10⁶ irradiated wild-type splenocytes (2,500 rads) as APCs, and 100 µg/ml peptide. No cytokine or CTLA4Ig additions were used in secondary cultures. Supernatants were harvested at 48 h and assayed by ELISA.

**Results**

CD28-deficient Mice Produce Less Antigen-specific IL-4 after In Vivo Immunization with KLH. To determine how signals delivered specifically through CD28 affect Th cell maturation we examined the ability of CD28−/− mice to generate IFN-γ and IL-4-producing cells after immunization. CD28-deficient mice bred onto the BALB/c or...
CD28-deficient C57BL/6 Mice Control Infection with L. major with Normal Th1-Type Responses. To address the requirement for CD28-mediated signals in microbial immunity, we challenged CD28-deficient mice with L. major. CD28+/+ and CD28−/− mice from the resistant C57BL/6 background and susceptible BALB/c mice were infected with L. major promastigotes in the hind footpad and monitored for their response to infection. C57BL/6 CD28−/− mice and C57BL/6 wild-type mice both controlled infection as assessed by footpad lesion size (Fig. 2A). Quantitative parasite cultures revealed equivalently low burdens for all C57BL/6 background animals that were approximately five orders of magnitude less in the feet and three orders of magnitude less in the spleens than susceptible BALB/c mice. CD28-deficient mice also displayed normal delayed-type hypersensitivity responses to Leishmania antigens in comparison to wild-type C57BL/6 mice (data not shown).

Analysis of Th cytokine production in infected animals was performed by restimulating single-cell suspensions of popliteal lymph node cells with soluble Leishmania antigens in vitro. Compared to wild-type C57BL/6 mice, CD28−/− mice had normal levels of antigen-specific IFN-γ production and comparably low levels of antigen-specific IL-4 production (Fig. 2B). In addition, cytokine production could be blocked with anti-class II mAbs in culture (Fig. 2B), suggesting that conventional class II–restricted Th cells are the source of these cytokines. Analysis of cytokine transcripts taken directly ex vivo from lymph nodes of CD28−/− mice also revealed fully mature Th1 responses compared to control-resistant mice (Fig. 2C). Furthermore, C57BL/6 CD28−/− mice healed in an IFN-γ–dependent fashion, as demonstrated by the ability of anti-IFN-γ mAb treatment to abrogate resistance in both the CD28-deficient and wild-type controls (data not shown). We conclude that CD28-mediated costimulation is not required for efficient Th1 responses in vivo against this pathogen.

C57BL/6 background and CD28-positive controls were immunized with KLH. In both genetic backgrounds, the absence of CD28 reproducibly resulted in a 10- to 20-fold decrease in IL-4 production upon antigen restimulation of draining lymph node cells compared to their CD28-positive controls (Fig. 1). In contrast, the levels of IFN-γ were similar in both wild-type and CD28-deficient mice of both strains. These results suggested that Th2 responses were more severely impaired than Th1 responses. Of particular note, there was profound difference in IL-4 production between BALB/c and C57BL/6 backgrounds, with at least 10-fold greater IL-4 levels in BALB/c animals when matched for CD28 expression. The genetic disparity led to levels of IL-4 production that were comparable between BALB/c CD28−/− and wild-type C57BL/6 mice.

Figure 2. C57BL/6 CD28−/− mice control infection with L. major. (A) Disease phenotype in response to infection of BALB/c, C57BL/6 CD28+/+, and C57BL/6 CD28−/− mice is depicted as the mean lesion size for each group with the standard deviations as y-axis error bars. (B) In vitro cytokine production from CD28-deficient mice. Draining lymph node cells were cultured with media alone (open bar), antigen plus an isotype control mAb (black bar, Ag + ctrl Ab), or antigen plus anti-class II blocking mAb (gray bar, Ag + α C II). Supernatants were assayed for IL-4 and IFN-γ production at 48 h by ELISA. Bars depict the mean of triplicate wells with the standard deviation expressed as y-axis error bars. Results are representative of five experiments using a total of 11 C57BL/6 CD28−/− mice. (C) Semi quantitative competitive PCR of draining lymph node cells from representative C57BL/6 CD28−/− (B6), susceptible BALB/c (B/c), and two C57BL/6 CD28−/− mice. Amplification was performed as described in Materials and Methods. Within each reaction, the upper band represents amplification of competitor DNA, while the lower band represents amplification of authentic cDNA. Differences in the level of mRNA between experimental groups is standardized by the relative intensity compared to the internal standard. Input cDNA was equalized by amplification of HPRT. Results are representative of three separate experiments.

Figure 1. Impaired Th2 response after in vivo priming of CD28-deficient mice. C57BL/6 and BALB/c wild-type (+/+) and CD28-deficient (−/−) mice were immunized with KLH, as described in Materials and Methods. Draining lymph node cells were restimulated with KLH in vitro, and supernatants were assayed after 48 h for IL-4 and IFN-γ by ELISA. Bars depict the mean values of duplicate wells with the standard deviation expressed as y-axis error bars. Cytokine levels in wells without antigen were below 10 U/ml. Results are representative of four separate experiments.
Figure 3. BALB/c CD28−/− mice are susceptible to L. major. (A) Disease phenotype in response to infection of C57BL/6, BALB/c CD28+/+, and BALB/c CD28−/− mice is depicted as the mean lesion size for each group with standard deviations as y-axis error bars. (B) In vitro restimulation of cytokine production from BALB/c CD28-deficient (−/−) and CD28-positive (+/+ and +/+−) mice. Draining popliteal lymph node cells were cultured with antigen and supernatants assayed for IL-4 (black bar) and IFN-γ (gray bar) production at 48 h by ELISA. Bars depict the mean of triplicate wells from individual animals with standard deviation expressed as y-axis error bars. Results are representative of five experiments using 24 CD28−/− mice. For some experiments, resistant C57BL background controls were MHC H-2d−congenic B10.D2 mice (sometimes designated only as B10). (C) Ex vivo analysis of cytokine expression using semi-quantitative competitive PCR. cDNA from draining popliteal lymph nodes was amplified as in Fig. 2 C. Each lane represents cDNA from individual animals. Results are representative of three separate experiments. (D) Ex vivo and in vitro comparison of IL-4 production. ELISPOT assays were performed on cells immediately upon isolation or after 2 d of restimulation with antigen in vitro. The number of IL-4-producing cells/well is the fraction of IL-4-producing cells multiplied by the number of viable cells. The bars represent averages of two B10.D2 (B10), two BALB/c CD28+/+, or five BALB/c CD28−/− mice with the standard deviations depicted as y-axis error bars. (E) Susceptibility of BALB/c CD28−/− mice is IL-4 dependent. CD28+/+ (CD28+) and CD28−/− (CD28−) mice were infected with L. major. Designated groups of animals were treated with 5 mg anti-IL-4 on day 0. Disease progression is depicted as mean lesion size for each group with the standard deviations as y-axis error bars. Results are representative of two experiments in which a total of six out of six BALB/c CD28−/− mice were treated and cured with anti-IL-4.
parably to treated wild-type BALB/c mice (Fig. 3 E) and to genetically resistant control animals (data not shown). We conclude that CD28-deficient animals can, under some circumstances, mount fully normal Th2 responses in vivo.

**Th2 Responses can be Induced in BALB/c-transgenic T Cells in the Absence of Costimulation.** Because of the unexpected finding of intact Th2 responses in *Leishmania*-infected BALB/c CD28−/− mice, we examined alternative signals that promote Th2 differentiation. Using naive transgenic T cells, we assessed the effect of genetic background and cytokine environment on Th2 development when T cell activation was initiated without costimulation. D011.10 TCR-transgenic mice were bred onto either the H-2d congenic BALB/c or B10.D2 (C57BL) backgrounds. In these assays, T cells undergo primary activation in the presence of experimental variables such as rIL-4 (to induce Th2 maturation) or CTLA4Ig (to block costimulation). To assess the impact of the initial experimental variables on early T cell activation, cells are restimulated without cytokine or CTLA4Ig addition. For cells from the C57BL background, Th2 maturation was limited by the addition of CTLA4Ig, consistent with published findings (10). In contrast, BALB T cells underwent normal Th2 maturation in the absence of costimulation (Fig. 4). This phenotypic difference between strains parallels and may be under the same genetic control as their tendency toward T helper polarization with no cytokine or CTLA4Ig addition (Fig. 4 and reference 28).

**Discussion**

Since the initial description of reciprocally dominant cell-mediated or humoral immune responses to repeated injections of flagellin (29), it has become increasingly clear that Th responses move toward polarized maturational states. Many factors, including the type of APC, form of antigen, route of immunization, genetic background (28, 30), and, perhaps most notably, the type of cytokines present during activation, can influence biased differentia-

![Figure 4](image-url)

**Figure 4.** Genetic background can alter requirements for costimulation in Th2 development. TCR-transgenic splenocytes from B10.D2 (C57BL) or BALB/c (BALB) mice were stimulated in vitro with OVA peptide with or without (-) the addition of CTLA4Ig. Designated wells received rIL-4 (10,000 U/ml) to induce Th2 maturation. After 5 d, viable cells were washed and restimulated with fresh irradiated splenocytes and peptide. Supernatants were assayed for IL-4 after 48 h by ELISA. Bars depict the mean of triplicate wells with the standard deviation expressed as y-axis error bars.
published results), Th1 responses proceeded normally. This was not unique to the C57BL/6 genetic background, since BALB/c mice infected with *L. major* and given neutralizing anti-IL-4 antibodies also demonstrated normal healing responses. The generation of Th2 responses in CD28-deficient mice is perplexing: the finding that early CTLA4Ig administration can prevent Th2 development in response to *L. major* (13), together with the findings that CD28-/Δ mice have reduced basal levels of IgG1, increased IgG2a and inefficient B cell help (8) might have led to a prediction that costimulation is required for maturation of Th2 responses. In the present study, CD28-deficient mice had decreased IL-4 production in restimulation assays after antigen priming or *L. major* infection. BALB/c CD28-/Δ mice, however, generated sufficient IL-4 to mediate susceptibility as demonstrated by the readily-induced resistance of anti-IL-4-treated mice. Direct measurements of IL-4 gene transcription and protein-producing cells from BALB/c CD28-/Δ mice actually indicate that their Th2 responses against *L. major* are fully intact in vivo. The apparent deficiency of IL-4 from in vitro assays may be caused by physical disruption of the cognate architecture required to compensate CD28-mediated signals in vivo, the inability to deliver important soluble factors in the appropriate paracrine fashion, or the inability of CD28-deficient cells to survive (37) or proliferate normally despite normal cytokine production. These findings raise the possibility that many of the biologic properties observed after in vitro manipulation of costimulation may have limited predictive value for immune responses in vivo.

Our data illustrates that genetic factors can influence the need for costimulation. Although there was a general trend toward preserved Th1 and impaired Th2 development in CD28-/Δ mice, variation in genetic background may contribute to phenotype commitment as much as costimulation. In the presence of CTLA4Ig, C57BL transgenic T cells could not be induced to make IL-4 even with the addition of rIL-4 in the primary cultures, consistent with reports using other TCR-transgenic mice on the C57BL (B10.A) background (10). In contrast, BALB/c T cells incubated with rIL-4 during primary activation were able to generate IL-4-producing effectors despite the presence of CTLA4Ig. There was also a background contribution to IL-4 production in response to KLH immunization. BALB/c mice produced 10-fold higher IL-4 than C57BL/6 mice, consistent with reports of others (38). The BALB/c genetic predisposition resulted in comparable levels of IL-4 between BALB/c CD28-/Δ and C57BL/6 CD28+/Δ mice. It is unlikely, however, that genetic polymorphism is the sole reason for preserved Th2 responses to *L. major* challenge, since anti-IFN-γ treatment of C57BL/6 CD28-/Δ mice also resulted in increased IL-4 mRNA (Brown, D., unpublished results). In addition, C57BL/6 CD28-/Δ mice are able to mount normal Th2 responses after challenge with an intestinal helminth (Gause, W., personal communication).

BALB/c CD28-/Δ mice were able to generate a functional Th2 response to *L. major* despite decreased IL-4 production after KLH immunization. The reduced restimulation of IL-4 seen after KLH immunization may not represent generation of KLH-reactive Th2 cells in vivo, since a marked discrepancy was seen between the in vitro restimulation assays and the ex vivo analyses of BALB/c CD28-/Δ mice infected with *L. major*. Assuming that the impaired Th2 response of CD28-/Δ mice was indeed representative of the generation of KLH-reactive IL-4-producing cells in vivo, other differences between KLH immunization and *Leishmania* infection could account for the discrepancy. Features of the microbial immunogen may obviate the need for costimulation. The replicating nature of the organism may increase the effective dose of antigen that could compensate the absence of costimulation. Studies in CTLA4Ig transgenic mice show that repeated immunization with KLH overcame a deficiency in IL-4 production seen after a single immunization (39). In addition, unique second signals induced by the microbe may be important in influencing the strength and type of immune response generated. An early burst in IL-4 and IL-2 transcription is induced by *L. major* shortly after infection (40), and these responses are entirely preserved in CD28-deficient mice (Brown, D., unpublished results).

An intact Th2 response in BALB/c CD28-/Δ mice infected with *L. major* infection was surprising given that a single dose of CTLA4Ig can evoke a curative Th1-type response in infected wild-type BALB/c mice (13). One potential explanation for this discrepancy is that developmental compensatory mechanisms have endowed CD28-/Δ cells with the capacity to be activated in a manner not possible in CD28-bearing cells. Compensation could manifest as overproduction of soluble factor(s) or overexpression of surface molecule(s). The current in vitro finding that rIL-4 could correct impaired Th2 differentiation caused by CTLA4Ig might predict that a compensatory increase in autocrine IL-4 production in vivo could overcome the genetic absence of costimulation. Even without invoking compensatory abnormalities, there are significant differences between the infection of BALB/c CD28-/Δ mice compared to the treatment of infected BALB/c mice with CTLA4Ig. The transient nature of blockade using CTLA4Ig may allow selective outgrowth of Th1 cells similar to other curative interventions in BALB/c mice that are based on transient reduction in CD4+ T cell responses (25). Sustained absence of costimulation in CD28-/Δ mice could favor genetically or microbiobially driven outgrowth of Th2 responses if maintenance of Th2 responses is less CD28-dependent than maintenance of Th1 responses (2, 12, 20). This model would be consistent with the finding that either sustained or delayed blockade with CTLA4Ig was far less efficacious than early treatment in *L. major*-infected mice (13). CD28-deficient mice also differ from CTLA4Ig-treated wild-type mice in the extent of blockade. The additional loss of CTLA4-mediated signals in treated mice may have distinct effects on Th development compared to the absence of CD28 alone. Treatment of CD28-/Δ mice with CTLA4Ig, however, failed to abrogate Th2 responses (Brown, D., unpublished results), consistent with data that...
T cell activation in CD28-deficient mice is not B7-dependent (41). The maturation of Th precursors into biased Th1 or Th2 subsets is affected by numerous factors. The present results demonstrate that CD28 is not an absolute requirement for generating either of the polarized subsets. BALB/c-transgenic T cells activated in the presence of rIL-4 can be primed to become Th2 cells in the absence of costimulation through CD28. Conversely, studies with human T cells have demonstrated an ability to generate Th2 cells despite neutralization of IL-4 if CD28-mediated costimulation was provided (17, 18). Together, these findings support a complex model of Th maturation in which there are distinct sufficient signals for Th2 development rendering no signal an absolute necessity depending on the presence of the other determinants. Th1 clones can also be cooperatively costimulated by B7 and rIL-12 (42). This finding, together with recent descriptions of novel Th1 costimulatory molecules (43) and Th1-inducing cytokines (44), suggests similar redundancy in the Th1 pathways. Models of Th cell differentiation will need to account for the complexity of factors that influence polarized Th responses.

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Address correspondence to Craig B. Thompson or Steven L. Reiner, Gwen Knapp Center, University of Chicago, 924 E. 57th St., Chicago, IL 60637-5420. The current address for Jonathan M. Green is Department of Medicine, Washington University School of Medicine, 660 S. Euclid Ave., Box 8052, St. Louis, MO 63110.

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