Brief Definitive Report

CD28-independent Induction of T Helper Cells and Immunoglobulin Class Switches Requires Costimulation by the Heat-stable Antigen

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Summary

It is well established that B7-CD28/CTLA4 interactions play an important role in the induction of T helper cells for T-dependent antibody responses. However, targeted mutation of CD28 does not significantly affect production of IgG and activation of CD4 T helper cells in response to infections by some viruses and nematode parasites. To test whether the CD28-independent induction of Ig class switches requires costimulation by the heat-stable antigen (HSA), we compared T helper cell induction and antibody response in mice deficient for either HSA, CD28, or both genes. We found that after immunization with KLH-DNP, mice deficient for both CD28 and HSA lack DNP-specific IgA and all subtypes of IgG. This deficiency corresponds to a reduced number of effector helper T cells that rapidly produce IL-2, IL-4, and IFN-γ after in vitro stimulation with carrier antigen KLH. In contrast, priming of T helper cells and Ig class switch are normal in mice deficient with either HSA or CD28 alone. IgM responses are not affected by any of these targeted mutations. These results demonstrate that CD28-independent induction of T helper cells and Ig class-switches requires costimulation by the HSA.

Immunological help from T cells to B cells is essential for the induction of antibody class switching and B cell memory. Since induction of T cell help requires costimulation by the antigen-presenting cells (1), lack of proper costimulation leads to defective T cell–dependent antibody response (2, 3). For some antigens (2, 4), substantial defects in Ig class switches and memory of T cell–dependent antibody responses have been reported in mice with a targeted mutation of CD28 gene that encodes for a major receptor for B7 family of costimulatory molecules (5). Interestingly, the extent of defects varies depending on the types of antigens used. For instance, IgG responses to NIP (hydroxy-iodo-nitrophenyl-acetyl)–coupled chicken gamma globulin and goat anti–mouse IgD are severely reduced in CD28-deficient mice (2, 4), whereas blockade of B7-CD28/CTLA4 interaction only marginally affects IgG responses in mice infected with viruses such as lymphocytic choriomeningitis virus (LCMV) (6, 7) and vesicular stomatitis (VSV) (7), or a nematode parasite, *Heligmosomoides polygyrus* (4).

Two hypotheses can be invoked to explain the CD28-independent IgG responses. First, T cells specific for the antigens can be activated by TCR ligand in the absence of costimulation. Second, activation of T cells specific for these antigens requires costimulation provided by other costimulatory molecules. Recent studies from several laboratories including ours have demonstrated that multiple costimulatory molecules, such as the heat-stable antigen (HSA; references 8–13), CD48 (14, 15), CD44H (16), intercellular adhesion molecule 1 (ICAM-1; references 17, 18), and 4-1BB ligand (19, 20) can promote T cell activation in a number of experimental models. It is therefore plausible that these costimulators may compensate for the CD28-deficiency. We are especially interested in the role of HSA in CD28-deficient mice, as we and others have established that this molecule plays a critical role in the induction of T cell clonal expansion (8–10), CTL maturation (10–12), and induction of CD8 T cell memory (12, 13). To test whether CD28-independent induction of immunological help for CD4 T cells requires costimulation by HSA, we compared activation of T helper cells as well as T-dependent antibody responses in wild-type mice to those in mice that are deficient of CD28 alone, HSA alone, and both HSA and CD28. Our results demonstrated that after immunization with DNP-coupled KLH, mice deficient for both HSA and CD28 failed to produce DNP-specific IgG1, IgG2a, IgG2b, IgG3, and IgA. This deficiency correlates with a defective induction of antigen-specific cytokine-producing cells. In contrast, substantial IgG1, IgG2a, and IgG2b responses and cytokine-producing cells are present in mice that are deficient for either CD28 or HSA.

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Thus, CD28-independent induction of T helper function and Ig class switches require costimulation by the HSA.

Materials and Methods

Experimental Animals. Mice deficient for CD28 gene (6) were provided by Dr. Tak Mak (University of Toronto, Toronto, Ontario, Canada); those deficient for HSA (21) were a gift from Dr. Peter Nielsen (Max Planck Institut fur Immunologie, Freiburg, Germany), and those deficient for both HSA and CD28 were produced as previously described (12). CD28-deficient mice have been backcrossed to C57BL6/j for six generations, while the HSA-deficient mice were produced using ES cells from C57BL6/j mice, as described. C57BL6/j mice purchased from the National Cancer Institute (Rockville, MD) were used as controls. Mice between 6 and 16 wk of age were used for the study.

Immunization and Measurement of DNP-specific Antibody Responses. Age- and sex-matched mice were immunized intraperitoneally with 50 μg/mouse of DNP-KLH (Sigma Chemical Co., St. Louis, MO) in CFA. Sera were collected at day 7 after the primary immunization. The immunized mice were boosted with 50 μg of KLH-DNP in IFA at 2 wk after the primary immunization, and the sera were collected at 2 wk after the second immunization, as indicated in Fig. 1. 1 mo after the second immunization, mice were killed and the spleen cells were harvested for ELISA spot assay (Fig. 1).

DNP-specific antibodies were detected by an indirect ELISA. In brief, MicroTest III™ Flexible Assay Plates (Falcon®, Oxnard, CA) were coated with 1 μg/ml of either BSA or BSA-DNP (50 μl/well) at 4°C overnight, after blocking with 10% FCS in PBS, the plates were washed five times with PBS containing 0.1% Tween 20. Varying dilutions of mouse sera were added and incubated for 2 h. The classes and subclasses of the DNP-specific antibodies, namely, IgM, IgA (Sigma Chemical Co.), IgG1, IgG2a (PharMingen, San Diego, CA), IgG2b, and IgG3 (Southern Biotechnology Associates, Birmingham, AL) were determined using horseradish peroxidase (HRP)–conjugated, subtype-specific antibodies according the manufacturer's instructions.

ELISA Spot Assay to Detect Cytokine-producing Cells. We used antibody pairs from PharMingen to detect cytokine-producing cells using Multiscreen™ 96-well filtration plates (MAHA S45 10; Millipore, France). In brief, the filtration plates were coated with purified mAbs specific for either IL-2 (JES6-1A12), IL-4 (BVD4-1D11), or IFN-γ (R46A2) (2 μg/ml, 100 μl/well) at 4°C overnight. After blocking with 10% FCS-PBS and washing with PBS, varying numbers of spleen cells from either naive mice or mice that had been immunized twice with KLH-DNP were added to the wells. For APCs we used T cell–depleted, mitomycin C–treated spleen cells from C57BL6/j mice (2×10^5/well). KLH or medium control was added before overnight incubation. The cells were washed away using PBS-0.1% Tween 20. Biotinylated mAbs specific for either IL-2 (JES6-5H4), IL-4 (BVD6.24G2), or IFN-γ (XMG1.2) were added. HRP-labeled avidin (PharMingen) and its substrate 3-amino-9-ethylcarbazole were used to visualize the cytokine-producing spots.

Induction of CD40L on CD4 T Cells from Different Strains of Mice. We used a previously published protocol (22) to detect maximal level of induced CD40L on CD4 T cells. In brief, spleen cells (3×10^6/ml) from primed mice were stimulated with anti-CD3 mAb for 4 h in the presence of PE-labeled anti-CD40L mAb MR-1 (1μg/ml, PharMingen). The unbound mAb was washed away, while the CD4 T cells were marked using FITC-labeled anti-CD4 mAb (PharMingen). To verify the specificity of the PE-MR1 staining, we used 100-fold excess of unlabeled MR-1 or control HB224 mAb to block the staining.

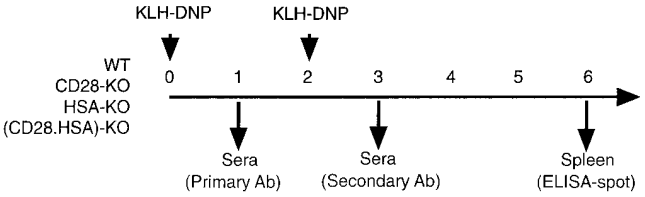

Figure 1. Experimental scheme. Age- and sex-matched mice were immunized intraperitoneally with 50 μg/mouse of DNP-KLH in CFA. Sera were collected at 1 wk after the primary immunization. The immunized mice were boosted with 50 μg of KLH-DNP in IFA at 2 wk after the primary immunization, and the sera were collected 2 wk after the second immunization. At 1 mo after the second immunization, mice were killed and the spleen cells were harvested for ELISA spot assay.

Results and Discussion

Targeted Mutation of HSA Ablates CD28-independent Antibody Class Switching. Previous studies from several laboratories have established that although targeted mutation of CD28 ablates germinal center formation and Ig class switching to some antigens (2, 4), IgG responses to infectious antigens tested, such as LCMV, VSV, and nematode parasites, were largely unaffected (4, 6, 7). Moreover, priming of CD4 T cells by KLH is only marginally affected (2). To determine whether CD28-independent Ig class switch depends on the HSA, we immunized congenic mice with KLH-DNP and measured the quantity and isotypes of DNP-specific antibodies, as depicted in Fig. 1. As shown in Fig. 2 *A*, 1 wk after primary immunization, strong anti-DNP IgM responses were detected in all four strains of mice. Thus, mutations of HSA and/or CD28 do not significantly affect the T cell–independent antibody responses. Moreover, since the DNP-specific B cells are present in all four strains of mice, neither HSA nor CD28 is required for the development of B cells specific for DNP. In wild-type mice and those with a targeted mutation of either HSA or CD28, IgG2b and IgG3 responses were quite substantial after one immunization. However, IgG2b and IgG3 were not detectable in mice deficient for both CD28 and HSA (Fig. 2, *B* and *C*). DNP-specific IgG1, IgG2a, and IgA were barely detectable in all four strains of mice tested after the primary immunization (data not shown).

After a boost, IgM, IgA, and all subclasses of IgG were present at high levels in wild-type mice and in mice with a targeted mutation of either HSA or CD28. However, no anti-DNP IgG and IgA antibodies were detected in mice deficient for both HSA and CD28 (Fig. 3). Thus, CD28-independent Ig class switch requires costimulation by the HSA.

HSA Is Necessary for CD28-independent Induction of Carrier Specific, Cytokine-producing Helper T Cells. Since Ig class switch requires T cell help, we investigated the defects in T

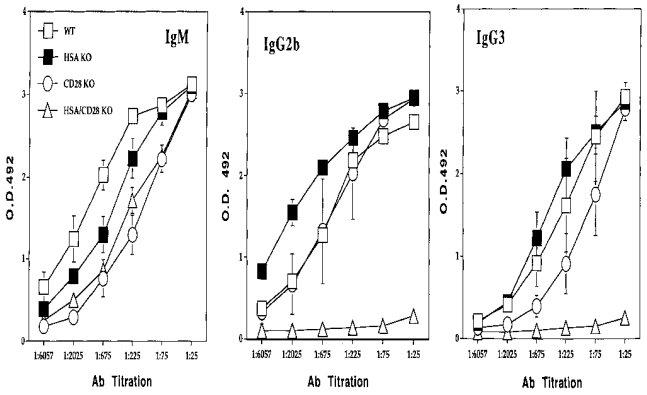

Figure 2. Quantity and isotypes of DNP-specific antibodies 1 wk after primary immunization, as determined by ELISA. Data presented are means and SD of OD 492 as a relative measure for the amount of antibodies that binds to DNP-BSA–coated plates. Antibody bindings to BSA-coated plates were insignificant and were not presented. Five age- and sex-matched mice were used in each group. Data are representative of five independent experiments with two to five mice per group.

helper cell induction in mice with targeted mutation of both HSA and CD28. Previous studies have established that T cells help antibody class switch by at least two mechanisms. First, antigen-specific T cells express CD40L upon engagement of antigen, and the CD40L then interact with CD40 on B cells to deliver immunological help (23). Second, activated T cells produce a number of cytokines to induce antibody class switch in B cells. Deficiency in CD40 or CD40L leads to a severely reduced antibody class-switch (for review see reference 24), whereas lack of response to given cytokines affects selective isotypes of antibodies produced (for review see references 24–26). To determine whether the CD40L induction on CD4 T cells is affected by deficiency in HSA and CD28, we stimulated T cells by anti-CD3 in the presence of PE-labeled anti-CD40L mAb. We have previously demonstrated that this method allows rapid detection of high levels of CD40L after engagement of TCR–CD3 complex (22). As shown in Fig. 4, *a-d*, anti-CD3 induced a comparable amount of CD40L on CD4 T cells from wild-type and HSA-deficient mice. Although

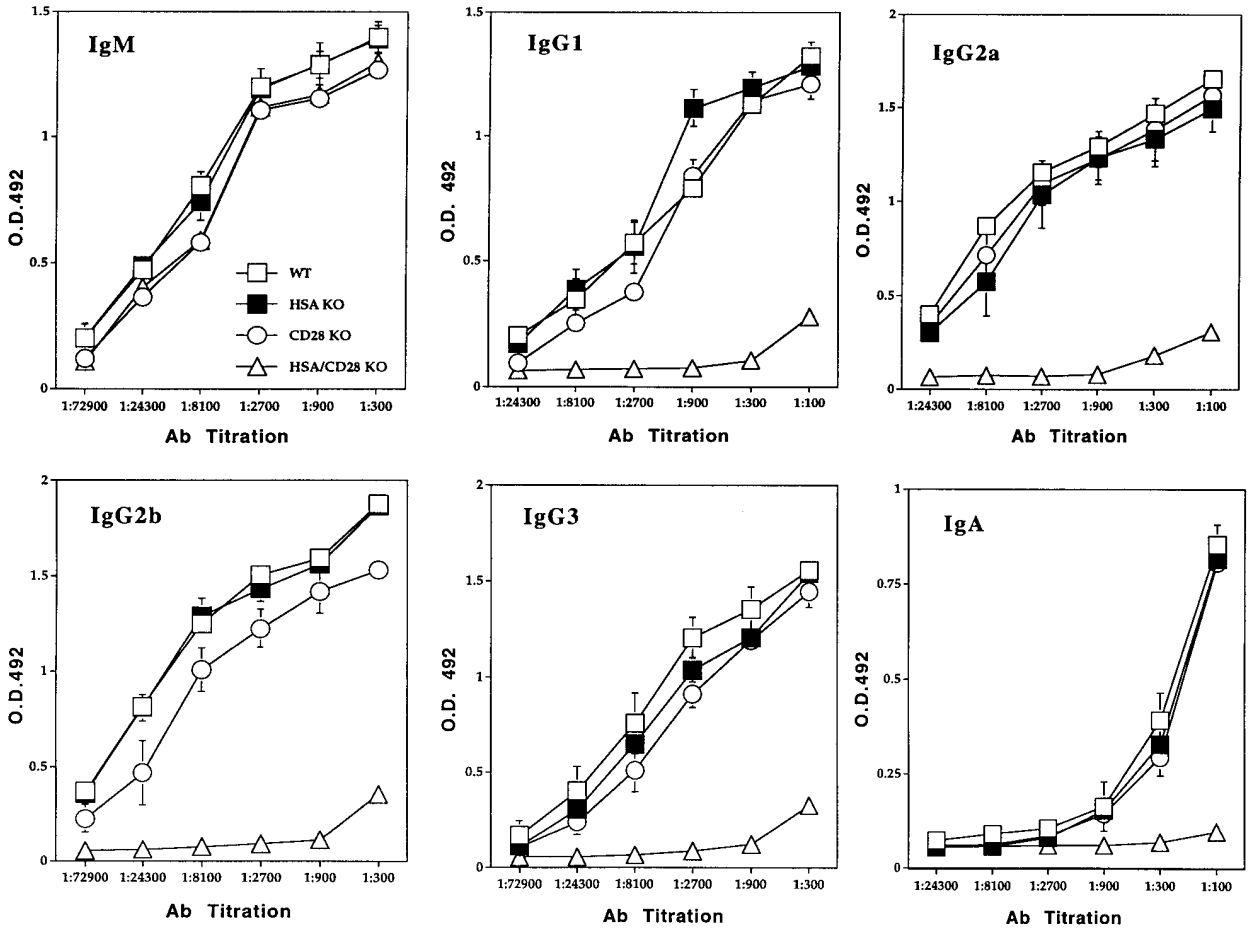

Figure 3. Quantity and isotypes of DNP-specific antibodies 1 wk after second immunization, as determined by ELISA. Mice used in experiments described in Fig. 2 were boosted, and their sera Ig determined. See Fig. 2 legend for details. Data are representative of five independent experiments with two to five mice per group.

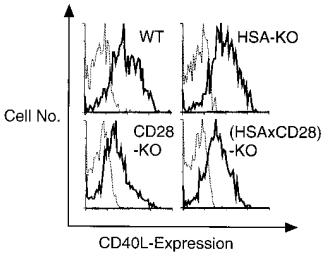

Figure 4. Induction of CD40L on CD4 T cells from wild-type (*WT*), CD28-KO, HSA-KO, and CD28/HSA double KO. Spleen cells from each strain of mice were incubated with 0.1 μg/ml of anti-CD3 mAb at 37°C for 2 h in the presence of 1 μg/ml of PE-labeled anti-CD40L (MR-1) in the presence of 100-fold–excess of a control mAb HB224. The spleen cells were then stained with FITC-conjugated anti-CD4 mAb and analyzed by flow cytometry. Data presented were histograms depicting CD40L expression of gated CD4 T cells (*solid lines*); the autofluorescences of gated CD4 T cells in the absence of anti-CD40L mAb were depicted in dotted lines.

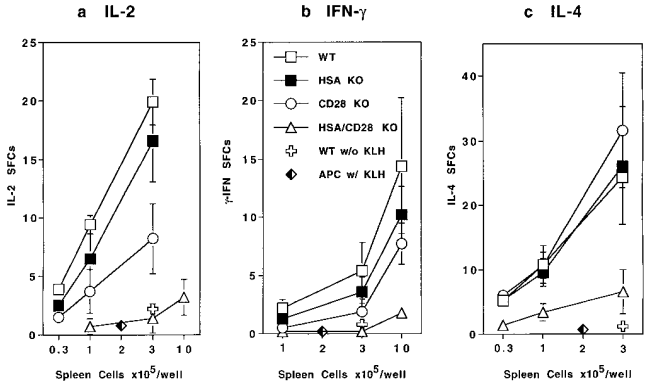

Figure 5. Quantitation of the number of (*a*) IL-2–, (*b*) IFN-γ– (WT), and (*c*) IL-4–producing cells by ELISA spot. Mice were killed at one month after the second immunization. Their spleen cells were cultured in antibody-coated plates in the presence of 1 μg/ml of KLH for 16 h; after lysis and washing away the cell debris, cytokine-producing spots were detected by biotinylated mAbs and HRP-conjugated avidin. T-depleted naive spleen cells from wild-type mice (2×10^5/well; *WT*) were treated with mitomycin C and added to all wells as feeders. These feeder cells (*half-filled diamonds*) do not contain cytokine producers as expected. In the absence of antigen (*unfilled crosses*), no cytokine producing spot can be detected in primed wild-type mouse spleen. Data presented are summary of five independent experiments with one to two spleens per group.

the expression of CD40L is somewhat lower in CD28-deficient mice, mutations of both HSA and CD28 did not lead to further reduction in CD40L expression. The specificity of the CD40L staining on CD4 T cells is verified, as unlabeled anti-CD40L mAb significantly blocked the binding of PE-labeled anti-CD40L (data not shown). Thus, costimulation by HSA is neither necessary nor sufficient for CD40L induction on CD4 T cells. The partial reduction of CD40L in CD28-knockout (KO) mice is consistent with recent observations by others (26). Since no difference is observed between CD28-deficient mice and those deficient for both HSA and CD28, it is unlikely that the suboptimal induction of CD40L can be responsible for the severe defects in DNP-specific Ig class switches in the HSA-CD28 double-deficient mice.

We determined the number of cytokine producing cells in the four congenic strains of mice, and the results from five independent experiments are summarized in Fig. 5. Targeted-mutations of both HSA and CD28 eliminated induction of KLH-specific IL-2 producers, IL-4 producers, and IFN-γ producers. Although CD28-deficiency caused a threefold reduction in the number of IL-2–producing cells, it did not significantly affect the number of IL-4 and IFN-γ producers. Targeted mutation of HSA had no significant effect on the number of cytokine-producing cells. Given the critical function of cytokine-producing cells in Ig class switches, the reduction of the cytokine producers is likely to be responsible for the lack of Ig class switches in mice deficient for both HSA and CD28.

Taken together, we have demonstrated that for KLH-DNP, an antigen that can induce Ig class switches by a CD28-independent mechanism, costimulation by either HSA or CD28 is required for the induction of T cell help. Although the CD28-independent IgG responses were not observed in experiments using chicken gamma globulin as carrier (2), they are substantial in CD28-deficient mice when infected by viruses such as LCMV and VSV, and by nematodes. Thus, two classes of antigens may exist: one requires CD28 to activate T helper cells, whereas the other does not. The molecular basis for such distinction remains to be determined. Given the recent findings by Kündig and colleagues that CD28-mediated costimulation can be bypassed by prolonged exposure to antigen (28), one parameter that may distinguish two classes of antigens is their persistence in vivo.

How does repeated antigen stimulation bypass the requirement for costimulation by CD28? We have demonstrated that in the absence of CD28–B7 interaction, induction of memory CTL and elicitation of effector from memory cells are not significantly affected, whereas induction of effector from naive T cells is abrogated (12). If the conclusion is also applicable to CD4 T cell activation, this suggests that the CD28-independent helper effectors are not directly induced from naive T cells; rather, they are elicited from memory cells induced by a HSA-dependent mechanism. This requirement for restimulation would explain the need for persistent antigenic stimulation.

HSA- and CD28-mediated costimulatory pathways appear redundant in the sense that, for some antigens, each can induce Ig class switches in the absence of the other. However, the immunological mechanism for helper induction by the two pathways can be distinct. An important difference lies in their ability to induce germinal center formation: although costimulation by CD28 is necessary for induction of germinal center (2), that by HSA is neither necessary nor sufficient (data not shown). Given the general requirement of germinal center for Ig hypermutation and affinity maturation (29), it is unlikely that T cells costimulated by HSA can promote Ig hypermutation. Although this may potentially limit the biological function of HSA-induced T helper cells, it should be noted that a recent study by Kalinke et al. has demonstrated that high-affinity neutralizing IgG antibodies against VSV can be produced without hypermutations (30).

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