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Role of CD28 in Polyclonal and Specific T and B Cell Responses Required for Protection against Blood Stage Malaria

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The role of B7/CD28 costimulatory pathway in the polyclonal and specific lymphocyte activation induced by blood stages of Plasmodium chabaudi AS was investigated in CD28 gene knockout (CD28−/−) and C57BL/6 (CD28+/+) mice. Analysis of the spleen during the acute infection revealed a similar increase in T and B cell populations in both groups of mice. Moreover, CD28−/− mice were able to develop a polyclonal IgM response to P. chabaudi. On the contrary, the polyclonal IgG2a response was markedly reduced in the absence of CD28. Production of IFN-γ; up-regulation of CD69, CD40L, CD95 (Fas), and CD95L (Fas ligand); and induction of apoptosis were also affected by the lack of CD28. Interestingly, the ability to control the first parasitemia peak was compromised in acutely infected CD28−/− mice, but CD28−/− mice failed to eradicate the parasites that persisted in the blood for >3 mo after infection. In addition, drug-cured CD28−/− mice were unable to generate memory T cells, develop an anamnestic IgG response, or eliminate the parasites from a secondary challenge. The incapacity of CD28−/− mice to acquire a full protective immunity to P. chabaudi correlated with an impaired production of specific IgG2a. Moreover, reinfected CD28−/− mice were protected by the adoptive transfer of serum from reinfected CD28+/+ mice containing specific IgG2a. Our results demonstrate that the polyclonal lymphocyte response is only partially affected by the absence of CD28, but this coreceptor is essential to generate specific T and B cell responses required for complete protection against P. chabaudi malaria. The Journal of Immunology, 2005, 174: 790–799.

Malaria is one of the most prevalent parasitic diseases in tropical countries, with 500 million clinical cases reported yearly, resulting in 1 million deaths. The pathology of malaria is caused by the asexual blood stages of the parasite, which have been the focus of many studies concerning protective immunity. Because of the difficulty of analyzing the human disease, murine models of malaria have been adopted to evaluate the immune responses against Plasmodium. Among them, infection with Plasmodium chabaudi chabaudi has been extensively analyzed, evidencing the complexity of the immune response against these parasites.

In the early phase of blood stage P. chabaudi malaria, a polyclonal activation of splenic T and B cells is observed, with huge production of type 1 effector molecules, such as IFN-γ and IgG2a (1–4). CD4+ αβ T cells (5) as well as γδ T cells (6) and NK cells (7) have been implicated in mouse resistance during this phase through an effector mechanism mediated by IFN-γ (8, 9). Acute phase Abs may also contribute to limit the disease (10) despite the fact that most of them have parasite-unrelated specificities (2). As the disease progresses, the majority of polyclonally activated lymphocytes are eliminated by apoptosis (11, 12), and a specific T and B cell response is generated, guaranteeing complete parasite clearance and protection against subsequent infection. The control of P. chabaudi malaria is mediated by both CD4+ and CD8+ T cells (5). Concomitant with parasite elimination, a switch from type 1 to type 2 response is observed, with dominant production of IL-4 and IgG1 (1, 13), a pattern also characteristic of the anamnestic response to a secondary infection (1, 3). To acquire resistance to P. chabaudi malaria, the humoral immunity is indispensable, and protection can be conferred by the adoptive transfer of specific Abs (14–16) or IgG1-inducing Th2 clones (17).

The magnitude of immune response to P. chabaudi malaria makes this model suitable to evaluate the role of the B7/CD28 costimulatory pathway during polyclonal lymphocyte activation (PLA)³ and specific T and B cell responses. Two different signals contribute to T cell activation (18–20). The first signal is provided by TCR interacting with peptide-bound MHC complexes. The balance of costimulatory and inhibitory molecules expressed on APCs provides the second signal, through interaction with their coreceptors on T cells. The B7/CD28 interaction is the best-characterized costimulatory pathway (21), with particular relevance for activation of naive T cells (22). Even if a strong TCR signal may activate T cells in the absence of CD28, signaling through this coreceptor reduces the number of TCRs that need to be engaged, lowering the lymphocyte activation threshold and allowing the response to low affinity peptides (23).

Recent studies have shown that P. chabaudi-infected erythrocytes can directly activate dendritic cells and up-regulate the expression of several molecules, including MHC class II and B7.2 (CD86) (24). Therefore, it is attractive to hypothesize that in

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³ Abbreviations used in this paper: PLA, polyclonal lymphocyte activation; 7-AAD, 7-aminoactinomycin D; LCMV, lymphocytic choriomeningitis virus; NRBC, normal RBC; PRBC, parasitized RBC.
acutely infected mice, the affinity of TCR/MHC peptide interaction required for T cell activation may be reduced as a consequence of the high expression of costimulatory molecules on APCs. In other words, the polyclonal T cell activation observed during acute malaria could result from the abundance of costimulatory signals. Moreover, as ligation of B7 with CD28 results in CD40L expression by CD4+ T (25), this costimulatory pathway could also be involved in the polyclonal B cell activation induced by P. chabaudi, a process that comprises both T cell-dependent and -independent components (1).

The generation of specific responses and memory could also rely on the B7/CD28 pathway. Signaling though CD28 might not be an essential requirement for activation of memory T cells, particularly those of the Th2 subtype that respond to signals provided by ICOS/ICOS ligand interaction (26), but it could be important to rescue T cells from activation-induced cell death, allowing their long-term survival (27). Moreover, CD28-mediated signal is important to avoid T cell anergy, defined as the failure to proliferate and produce IL-2 (28–30), two elements involved in memory T cell generation. Finally, the B7/CD28 interaction could also be required during P. chabaudi infection for switching the pattern of response from type 1 to type 2, an idea supported by the fact that blockade of this costimulatory pathway expands the population of IFN-γ-producing cells (31).

The involvement of the B7/CD28 pathway in the response to infectious diseases was demonstrated in mice infected with Schistosoma mansoni, the CD28-mediated signal necessary for the production of type 2 cytokines and Abs (32). In Toxoplasma gondii infection, however, CD28 is not necessary to resolve the primary infection, even though it has been implicated in the generation of memory responses essential for the control of secondary infections (33). In infections by many other pathogens, including lymphocytic choriomeningitis virus (LCMV) (34), vaccinia virus (30), Heligmosomoides polygyrus (35), and Leishmania major (36), efficient type 1 and type 2 responses are triggered in CD28-deficient mice. In addition, studies with influenza virus and H. polygyrus have led to the idea that the induction of T cell memory is not dependent on CD28 (35, 37). These findings open the possibility that in many pathogenic infections, but not all of them, other costimulatory signals, such as those provided by ICOS/ICOS ligand interaction, could bypass the CD28 requirement.

Hence, analyzing the development of the immune response to P. chabaudi malaria in CD28 gene knockout (CD28−/−) mice may add useful information about the molecular mechanisms underlying PLA and its regulation by apoptosis. In addition, determining how the specific immune response develops in the absence of CD28 may contribute to understanding the role of B7/CD28 costimulation in the Th1/Th2 imbalance and in the generation of memory T and B cells required for protection against this parasite.

**Materials and Methods**

**Mice, parasites, infection, and chloroquine treatment**

Six- to 8-wk-old CD28−/− (CD28 knockout) (30) and CD28+/+ (C57BL/6) female mice were bred under specific pathogen-free conditions at the Isogenic Mice Facility, Instituto de Ciências Biomédicas/University of Sao Paulo (Sao Paulo, Brazil). P. chabaudi AS was maintained as previously described (5). In the primary infection, mice were inoculated i.p. with 108 parasitized RBC (PRBC). To ensure a complete parasite elimination, mice were treated i.p. with chloroquine (Sigma-Aldrich; 10 mg/kg body weight dissolved in 200 μL of PBS daily) from days 30 to 40 postinfection. In the secondary infection, 109 PRBC were given i.p. 3 mo after the primary infection. Parasitemias were determined by microscopic examination of Giemsa-stained blood smears.

**Adaptive serum transfer**

To test whether CD28−/− mice could be protected by the serum of CD28+/+ mice, on day 110 postinfection (day 20 after the secondary challenge with 109 PRBC), three reinfected CD28−/− mice were killed, and 0.6 ml of its serum was injected i.p. into reinfected CD28−/− mice. As controls, reinfected CD28−/− mice were treated with serum obtained from noninfected CD28+/+ mice or from reinfected CD28−/− mice.

**Spleen cell suspensions**

Spleen cells were cultured in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 2-ME (50 μM), L-glutamine (2 mM), sodium pyruvate (1 mM), and 3% heat-inactivated FCS. All supplements were purchased from Invitrogen Life Technologies. The number of cells in the spleen was determined in a Newbauer chamber.

**Phenotypic analysis of spleen cells**

Spleen cells (106) were stained with FITC- , PE-, or CyChrome-labeled mAbs to CD4 (H129.19), CD8 (53–67), CD45R (B220), RA3-6B2, CD69 (HL.2F3), CD95L (Fasl., MFL3), CD95 (Fas, Jo2), CD154 (CD40L, MR1), Ly6G (1A8), CD11b (M1/70), CD11c (HL3), TCRγδ (GL3), NK1.1 (PK39E), and CD138 (Syndecan-1, 281–2) from BD Pharmingen and F4/80 was obtained from Serotec. Cells were analyzed by flow cytometry using a FACS Calibur with CellQuest software (BD Biosciences). The numbers of large cells in each population were determined according to forward light scatter, using gates defined in histograms of nonactivated splenocytes.

**IFN-γ and Ig intracellular detection**

For intracellular IFN-γ, spleen cells (106) were cultured with GolgiStop overnight at 37°C in a 5% CO2 atmosphere according to the manufacturer’s instructions. After washing, cells were surface-stained with FITC- or CyChrome-labeled mAb to CD4 and CD8. Cells were then fixed with Cytofix/Cytoperm buffer and incubated with PE-labeled mAb to IFN-γ (XM-12, 1:2 diluted) in Perm/Wash buffer. All reagents were purchased from BD Pharmingen. For intracellular Ig, spleen cells (106) were surface-stained with PE-labeled mAb to CD138 (Syndecan-1) and CyChrome-labeled mAb to B220, fixed with Cytofix/Cytoperm buffer, and incubated with goat anti-mouse IgG or IgG2a biotinylated Abs (Southern Biotechnology Associates), followed by an FITC-avidin conjugate (BD Pharmingen) diluted in Perm/Wash buffer. In control samples, cells were surface-stained with biotinylated anti-Ig Abs, and conjugate was diluted in PBS. The analysis was performed by flow cytometry using a FACS Calibur.

**7-Aminoactinomycin D (7-AAD) and annexin V labeling for apoptosis**

For analysis of total dead cells, spleen cells (106) were stained with 7-AAD (BD Pharmingen). For quantification of apoptotic T and B cells, annexin V labeling was used as previously described (38). In brief, spleen cells (106) were incubated in labeling buffer (10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, and 5 mM CaCl2) with FITC-conjugated annexin V (BD Pharmingen) and CyChrome- or PE-labeled mAb to CD4, CD8, or CD45R. The samples were immediately analyzed on a FACS Calibur. Propidium iodide (Sigma-Aldrich) staining was used to exclude necrotic cells.

**ELISPOT assay for Ig-secreting cells**

The ELISPOT assay has been described in detail previously (2). In brief, 96-well, flat-bottom microtest plates (Costar) coated overnight (4°C) with goat anti-mouse total Ig (10 μg/ml) were saturated with 1% gelatin (Merck) in PBS for 120 min. Titrated numbers of spleen cells (105 to 5 × 105 cells/well) were added in RPMI 1640 medium with 1% FCS and cultured for 6 h at 37°C in a 5% CO2 atmosphere. The spots were developed by adding goat anti-mouse IgM, IgG1, or IgG2a biotinylated Abs, followed by a phosphatase alkaline-avidin conjugate (all Abs and conjugates were purchased from Southern Biotechnology Associates). 5-Bromo-chloro-3-indolyl phosphate (Sigma-Aldrich) diluted in AMP (Merck) was used as substrate. From the titration plots (numbers of cells plated vs spots) and the total number of splenocytes, the number of total Ig-secreting cells per spleen was calculated.

**ELISA test for parasite-specific Abs**

Anti-P. chabaudi Abs were quantified by ELISA as previously described (16). In brief, 96-well, flat-bottom microtest plates (Costar) were coated overnight (4°C) with an intraerythrocytic P. chabaudi extract (8 μg/ml). Plates were saturated with 1% BSA for 1 h. After washing, 50 μl of mouse
serum samples (diluted from 1/50 to 1/3200) were added and left for 1 h at room temperature. A positive serum (hyperimmune serum) diluted in the same manner was included in all plates. Assays were developed by adding goat anti-mouse IgM, IgG peroxidase-conjugated Abs (Southern Biotechnology Associates), and IgG2a and IgG1 biotinylated Abs (BD Pharmingen), followed by a peroxidase-avidin conjugated (Invitrogen Life Technologies) and O-phenylenediamine (Sigma-Aldrich). The enzymatic reaction was developed for 10 min and blocked with citric acid (0.2 N, 50 μl/well). A Dynatech reader using a 450-nm wavelength filter quantified the absorbance values.

CSFE proliferation assay for the analysis of memory CD4+ T cells

Spleen cells were stained with the vital dye CSFE (Molecular Probes) as previously described (39). Briefly, 4 × 10⁶ cells/ml resuspended in PBS with 0.1% BSA were incubated with CSFE at a final concentration of 5 μM for 20 min at 37°C. Cells were washed twice in cold PBS and resuspended at 10⁶ cells/ml complete RPMI 1640. Cells (10⁶) were then cultured in 96-well plates (Costar) with PRBC (10⁶), 5 μg/ml plate-bound anti-CD3 mAb (clone 145-2C11; BD Pharmingen), or medium for 96 h at 37°C in a 5% CO₂ atmosphere. Human rIL-2 (Proleukin; Chiron) was used at a final concentration of 0.5 U/ml. After incubation, cells were stained with PE-labeled mAb to CD4 and analyzed by flow cytometry using a FACSCalibur.

Statistical analysis

Statistical analysis was generally performed with ANOVA and Tukey’s multiple comparison test. Parasite-specific Ab curves of different groups were compared with the nonparametric Mann-Whitney U test. GraphPad PRISM 3 software was used in both tests. Differences between two groups were considered significant at p < 0.05 (5%).

Results

Development of splenomegaly during P. chabaudi malaria in the absence of CD28

To evaluate the role of CD28 in P. chabaudi-induced splenomegaly, CD28−/− and CD28+/+ mice were infected i.p. with 10⁶ PRBC, and 7 and 11 days later, spleen weight and cellularity were determined. The results showed a notable increase in these parameters in both groups of infected mice, even though the changes were less pronounced in CD28−/− mice (Fig. 1A). Moreover, the analysis of T and B cells during P. chabaudi infection suggests a minor role for CD28 in the expansion of spleen lymphocyte populations. In infected CD28−/− and CD28+/+ mice, CD4+ and CD8+ T and B cell populations were similarly increased on day 7 of infection, decreasing to values close to those in controls 4 days later, when the majority of nucleated cells had a CD4+CD8−B220− phenotype (Fig. 1B). In fact, CD4+CD8−B220− cells constituted the only population whose expansion was statistically different between infected CD28−/− and CD28+/+ mice. The comparative analysis of Ly6G, CD11b, CD11c, F4/80, TCRγδ, NK1.1, and CD138 expression in spleen cells of both groups of infected mice revealed that the difference between the groups resides in the number of CD138+ (Syndecan-1) cells (Fig. 1C). Within this population, probably plasmocytes that express low levels of B220, the percentage of cells stained for intracellular IgG2a was significantly reduced in infected CD28−/− mice compared with infected CD28+/+ mice.

Early T and B cell responses to P. chabaudi in the absence of CD28

CD69 is one of the earliest cell surface Ags expressed by lymphocytes after activation. Once expressed, CD69 acts as a costimulatory molecule for cell activation and proliferation (40). Therefore, to further characterize the involvement of CD28 in the PLA induced by P. chabaudi, the surface expression of CD69 was determined in spleen lymphocytes of CD28−/− and CD28+/+ mice on day 7 of infection with 10⁶ PRBC. As shown in Fig. 2A, a substantial CD69 up-regulation was observed in CD4+ and CD8+ T and B cells of infected CD28+/+ mice, but a very limited response
FIGURE 2. Role of CD28 in the PLA induced by P. chabaudi. A, Histograms of the expression of CD69 in CD4+, CD8+, and B220 (CD45R)+ spleen cells of CD28−/− and CD28+/+ mice on day 7 of infection with 10⁶ PRBC (●). Noninfected mice were used as controls (▲). The numbers in the histograms represent the mean ± SD (n = 4–5) percentage of positive cells in each population. B, Percentages of large cells in CD4+, CD8+, and B220+ spleen cells of CD28−/− and CD28+/+ mice on day 7 of infection with 10⁶ PRBC. Noninfected CD28−/− and CD28+/+ mice were used as controls. Data represent the mean ± SD (n = 3–4). C, Percentages of IFN-γ+ cells in CD4+ and CD8+ spleen cell populations of CD28−/− and CD28+/+ mice on day 7 of infection with 10⁶ PRBC. Noninfected mice were used as controls. Each point represents a single mouse (n = 2–3). D, Numbers of IgM-, IgG1-, and IgG2a-producing cells in the spleens of infected CD28−/− and CD28+/+ mice on day 7 of infection with 10⁶ PRBC. Noninfected mice were used as controls. Data represent the mean ± SD (n = 3–4). *p < 0.05, comparing values from CD28−/− and CD28+/+ mice. The data shown in this figure are representative of two or three separate experiments.

To determine whether CD28 was providing the costimulatory signal required for polyclonal lymphocyte proliferation, the percentages of large cells were determined in T and B cell populations on day 7 of infection with 10⁶ PRBC. As shown in Fig. 2B, marked increases in large cells bearing a CD4+, CD8+ and B220+ phenotype were detected in infected CD28+/+ mice. In contrast, with the exception of CD8+ cells, the increase in large cell frequencies was much less intense in infected CD28−/− mice. On days 9 and 11 of infection, the percentages of both CD69+ and large cells were still significantly higher in CD28+/+ mice compared with CD28−/− mice (data not shown), ruling out the possibility that the proliferative response to acute infection was delayed in mice lacking CD28. These results are consistent with the idea that CD28-mediated signals are involved in the polyclonal lymphocyte proliferation induced by P. chabaudi infection.

To determine whether the type 1 response generated during acute P. chabaudi malaria depends on CD28, intracellular IFN-γ was quantified in spleen T cells of CD28−/− and CD28+/+ mice 1 wk after infection with 10⁶ PRBC. In infected CD28+/+ mice, the percentages of CD4+ and CD8+ IFN-γ+ cells showed 10- and 5-fold increases compared with noninfected controls, respectively, with the majority of positive cells exhibiting a CD4+ phenotype (Fig. 2C). In contrast, in infected CD28−/− mice, only a limited increase in the percentages of IFN-γ+ cells was observed. A similar result was obtained on day 4 of infection, when 2 and 6% of CD4+ cells were positive for IFN-γ in CD28−/− and CD28+/+ mice, respectively (data not shown). Moreover, the frequency of CD4+ and CD8+ IFN-γ+ cells progressively decreased on days 9 and 11 of infection, with CD28+/+ mice still presenting significantly higher numbers of IFN-γ+ cells compared with CD28−/− mice (data not shown). Stimulation with plate-bound anti-CD3 mAb also revealed a different production of IFN-γ by T cells obtained from both groups of infected mice (data not shown). Confirming the major role of CD28 in the early IFN-γ response to P. chabaudi, we found that IFN-γ levels in 24- and 48-h supernatants of Con A-stimulated spleen cells of acutely infected CD28−/− mice were similar to those in noninfected mice, a result clearly different from that obtained with cells of acutely infected CD28+/+ mice that produced high amounts of this cytokine (data not shown).

To evaluate whether the polyclonal Ab response to acute P. chabaudi infection is influenced by CD28-mediated signals, the spleen cells secreting IgM and IgG2a were quantified in CD28−/− and CD28+/+ mice on day 7 of infection with 10⁶ PRBC, which represents the peak of the response for both groups of mice (data not show). IgG1 was also analyzed because production of this Ig isotype characterizes a shift toward a type 2 response. A similar, although statistically different, increase in the number of IgM-secreting cells per spleen was observed in both groups of infected mice, although for IgG2a-secreting cells, a poor increase occurred in the absence of CD28 (Fig. 2D). When compared, the numbers of IgM- and IgG2a-secreting cells in infected CD28−/− mice represented 80 and 25% of those detected in infected CD28+/+ mice, respectively. In addition, the number of IgG1-secreting cells was also lower in infected CD28−/− mice, indicating that the absence of CD28 did not result in a bias to Th2-induced IgG subclasses. Because the Ig isotype switch depends on T-B cell cooperation through a CD40/CD40L interaction, CD40L expression was also evaluated in spleen T cells 1 wk after infection. A higher increase in the percentage of CD40L+ cells was observed in CD28+/+ mice (from 8.7 ± 1.0 to 19.9 ± 2.8 in CD4+ cells and from 11.0 ± 2.8 to 33.2 ± 4.8 in CD8+ cells) compared with CD28−/− mice (from 5.3 ± 0.3 to 13.3 ± 2.1 in CD4+ cells and from 5.5 ± 0.9 to 11.1 ± 8.8 in CD8+ cells). For both CD4+ and CD8+ populations, data from infected CD28−/− and CD28+/+ mice were statistically different compared with each other and with those from noninfected controls (n = 3; p < 0.05). Taken together, the results for the early immune response to P. chabaudi show that although polyclonal IgM production is only marginally affected by the absence of CD28, other activation and differentiation parameters, such as up-regulation of CD69 in T and B cells, up-regulation of CD40L in T cells, and secretion of polyclonal IgG2a and IFN-γ, are notably diminished in mice lacking this coreceptor.
Induction of T and B cell apoptosis during acute P. chabaudi malaria in the absence of CD28

To analyze the role of CD28 in the apoptosis of T and B cells during acute malaria, spleen cells from CD28−/− and CD28+/+ mice on day 7 of infection with 10⁶ PRBC were stained with 7-AAD or annexin V. 7-AAD labels the nuclei of dead cells, and annexin V binds to phosphatidylserine externalized at the initial process of apoptosis. Because the CD95/CD95L (Fas/FasL) interaction is involved in caspase activation and cell death (41), we also quantified the expression of these molecules on T and B cells of infected CD28−/− and CD28+/+ mice. Conferring previous reports (11, 12), the analysis of 7-AAD+ cells in the spleens of CD28+/+ mice revealed a consistent increase in the percentages of dead cells after P. chabaudi infection (Fig. 3A). These results, however, sharply contrasted with the limited increase in the number of 7-AAD+ cells observed in infected CD28−/− mice. Staining of cells with annexin V extended these findings by showing that apoptosis of CD4+ and CD8+ T and B cells was affected by the absence of CD28 (Fig. 3B). When CD95L (FasL) expression was analyzed in CD4+ and CD8+ T and B cells, up-regulation of this molecule was observed in all lymphocyte subsets of infected CD28+/+ mice, whereas in lymphocytes of infected CD28−/− mice it remained the same as in noninfected controls (Fig. 3C). Analysis of the percentages of CD95+ (Fas+) T and B cells from both groups of infected mice revealed an expression pattern similar to that observed for CD95L: that is, a higher increase after infection of CD28+/+ mice (from 5.9 ± 2.2 to 34.6 ± 3.1 in CD4+ cells, 3.8 ± 1.9 to 26.7 ± 1.7 in CD8+ cells, and 14.8 ± 2.8 to 34.9 ± 3.5 in B220+ cells) compared with CD28−/− mice (from 4.0 ± 0.7 to 14.9 ± 4.2 in CD4+ cells, 2.4 ± 0.1 to 16.1 ± 3.8 in CD8+ cells, and 9.6 ± 0.8 to 17.7 ± 3.2 in B220+ cells). For CD4+, CD8+, and B220+ populations, data from infected CD28−/− and CD28+/+ mice were statistically different compared with each other and with noninfected controls (n = 3; p < 0.05). Differences between the groups did not result from a delay in the spleen cell response, because on days 9 and 11 of infection the changes in these apoptosis-associated parameters were still less evident in CD28−/− mice compared with CD28+/+ mice (data not shown). Based on these results, we concluded that the induction of apoptosis in splenic T and B cells during acute P. chabaudi malaria is influenced by the absence of CD28-mediated signals.

Requirement of CD28 for parasite control

To determine to what extent the inadequate immune response to P. chabaudi observed in the absence of CD28 affects the ability to control infection, we compared the parasitemia curves in CD28−/− and CD28+/+ mice injected with 10⁶ PRBC. Interestingly, mice from both groups were able to control the first parasitemia peak (Fig. 4). Nevertheless, although CD28+/+ mice cleared blood parasites within 20 days, CD28−/− mice developed a recrudescence parasitemia, with peaks attaining 10–15% of erythrocyte levels, which persisted at least for 3 mo after infection when animals were killed. Taken together, these results indicate that CD28 is not necessary to control the acute infection, but this coreceptor is essential for acquisition of full-protective immunity to P. chabaudi malaria.

Role of CD28 for the generation of parasite-specific Ab responses

To investigate the role of CD28 in specific Ab responses to P. chabaudi, we analyzed the serum of CD28−/− and CD28+/+ mice obtained 3 mo after the primary infection. First, we confirmed that all CD28−/− and CD28+/+ mice were positive for IgH allotype b (IgHb) and negative for IgH allotype a (IgHa). As shown in Fig. 5, specific IgM was detected in infected CD28−/− mice, but the levels were lower than in infected CD28+/+ mice. It is remarkable, however, that mice lacking CD28 failed to produce specific IgG and showed serum OD values similar to those in noninfected controls. When the IgG subclasses were evaluated, we observed that infected CD28−/− and CD28+/+ mice sharply differed in their
ability to produced specific IgG2a, whereas serum levels of specific IgG1 were negligible in both groups of infected mice (data not shown). These results indicate that a specific IgM response to \textit{P. chabaudi} malaria can be generated in the absence of CD28, but the production of IgG2a is totally dependent of this coreceptor.

**Role of CD28 for the acquisition of full protective immunity to \textit{P. chabaudi} malaria**

To confirm the major role of CD28 in the acquisition of full protective immunity against \textit{P. chabaudi}, 1 mo after infection with \(10^6\) PRBC, CD28\(^{-/-}\) and CD28\(^{+/+}\) mice were treated with chloroquine for 10 consecutive days to eliminate the parasites, and 50 days later, the animals were challenged with \(10^6\) PRBC. Although reinfeected CD28\(^{+/+}\) mice were able to clear the parasitemia in a few days, reinfeected CD28\(^{-/-}\) mice remained positive for \(>30\) days after the second challenge (Fig. 6). To test whether CD28\(^{-/-}\) mice can be protected by Abs from CD28\(^{+/+}\) mice, on day 110 postinfection (day 20 after the secondary challenge with \(10^6\) PRBC), three reinfeected CD28\(^{+/+}\) mice were killed, and 0.6 ml of serum was injected i.p. in reinfeected CD28\(^{-/-}\) mice. As shown in Fig. 6B, this procedure was able to clear the parasitemia in reinfeected CD28\(^{-/-}\) mice for the next 5–8 days after serum transfer. When reinfeected CD28\(^{-/-}\) mice were treated with serum obtained from either noninfected CD28\(^{+/+}\) mice (Fig. 6B) or reinfeected CD28\(^{-/-}\) mice (data not shown), the parasitemias remained positive. These data corroborate the idea that CD28 is required for the acquisition of full protective immunity to \textit{P. chabaudi} malaria. In addition, the experiments with serum transfer suggest that the necessity of CD28 for parasite eradication may reside in the central role of this coreceptor for the generation of specific IgG Abs.

**Role of CD28 for the generation of secondary Ab responses to \textit{P. chabaudi}**

The next experiments were designed to verify whether CD28 is required for secondary Ab responses to \textit{P. chabaudi}. Three months after infection, drug-cured CD28\(^{-/-}\) and CD28\(^{+/+}\) mice were challenged with \(10^6\) PRBC, and specific Ab serum levels were quantified 7 and 24 days later. As shown in Fig. 7, reinfection of CD28\(^{+/+}\) mice led to an increase in both IgM and IgG, with maximum levels on day 7 (day 97), whereas the secondary Ab response in mice lacking CD28 was restricted to IgM, with maximum levels on day 24 (day 114). Analysis of IgG subclasses in reinfeected CD28\(^{+/+}\) mice revealed that IgG2a, not IgG1 (data not shown), was enhanced. We concluded that CD28 is necessary for a secondary IgG response to \textit{P. chabaudi}, whereas an anamnestic IgM response occurs in the absence of this coreceptor.

**Role of CD28 in generation of memory T cells in \textit{P. chabaudi} malaria**

To determine whether CD28 is required for the generation of memory CD4\(^+\) T cells in \textit{P. chabaudi} malaria, we analyzed the in vitro proliferative response of splenocytes obtained from CD28\(^{-/-}\) and CD28\(^{+/+}\) mice 3 mo after a drug-cured infection. In these assays CSFE-labeled cells were incubated with normal RBC (NRBC), PRBC, or plate-bound anti-CD3 mAb. After \(96\) h of PRBC stimulation, a huge fraction of CD4\(^+\) cells of drug-cured CD28\(^{+/+}\) mice was proliferating (Fig. 8). In contrast, only 1.7% of PRBC-stimulated CD4\(^+\) cells of drug-cured CD28\(^{-/-}\) mice proliferated, a value similar to that of lymphocytes cultured with NRBC. The unresponsiveness of CD4\(^+\) cells from drug-cured CD28\(^{-/-}\) mice was not due to a general deficiency in their capacity to proliferate, because in both groups of mice an intense response to plate-bound anti-CD3 mAb was observed. Moreover, the proliferative response in drug-cured CD28\(^{-/-}\) mice was partially restored by adding rIL-2 to PRBC-stimulated cultures, indicating that specific CD4\(^+\) cells could be generated in the absence of CD28, but the cells remained unresponsive or anergic. When rIL-2 was added to PRBC-stimulated cultures of drug-cured CD28\(^{+/+}\) mice, a reduction in the proliferative response was observed (data not shown). Moreover, in cultures of noninfected CD28\(^{+/+}\) or CD28\(^{-/-}\) mice, the percentage of dividing cells was negligible.
FIGURE 6. Role of CD28 in the control of a secondary P. chabaudi infection. Parasitemia was determined in CD28−/− and CD28+/+ mice during the primary and secondary infections. A, To eliminate the parasites from a primary infection with 10⁶ PRBC, all mice were treated i.p. with chloroquine once a day from days 30 to 40 postinfection. Three months after the primary infection, three of six drug-cured mice in each group were reinfected with 10⁹ PRBC (□). Nonchallenged, drug-cured mice were used as controls (○; n = 3). B, On day 110 postinfection (day 20 after the secondary challenge with 10⁹ PRBC), three reinfected CD28+/+ mice were killed, and 0.6 ml of serum was injected i.p. in reinfected CD28−/− mice. As a control, reinfected CD28−/− mice were treated with serum from noninfected CD28+/+ mice. Each curve corresponds to a single mouse. Data are representative of two separate experiments.

even in the presence of rIL-2 (data not shown). In conclusion, these findings suggest that CD28 is required to generate competent memory CD4+ T cells that can be recalled by a second contact with the parasite.

Discussion

The immune response to P. chabaudi involves a complex network of molecular and cellular interactions that ensures not only a rapid and somehow imprecise identification of the parasite at the early phase of infection, but also a transition to a detailed recognition of its molecular constitution as the disease progresses. This shift in parasite perception goes together with the selection of a restricted cohort of effector mechanisms, which improve parasite destruction and contribute to maintain host integrity. With this perspective in mind, in this study we analyzed the role of CD28 in the early and late immune responses to P. chabaudi, focusing on PLA and the generation of specific responses.

Analysis of spleen at the early phase of P. chabaudi malaria revealed a huge increase in the numbers of T and B cells in both CD28−/− and CD28+/+ mice, suggesting that CD28-mediated signaling is not a prerequisite for the expansion of lymphocyte populations during PLA. Nevertheless, this analysis also showed that in the absence of CD28, the proliferative response is less intense, as evidenced by the low numbers of large cells and CD69+ T and B cells in infected CD28−/− mice. The apparent contradiction between these findings can be explained by considering that the increase in spleen lymphocyte populations in response to infection is the final result of T and B cell proliferation and elimination of activated cells by apoptosis. In other words, because lymphocytes submitted to increasing signal strength not only proliferate and acquire effector functions, but also die by activation-induced cell death (42), a similar expansion of spleen cell populations could occur as a consequence of either a moderate or an intense proliferative response. Supporting this idea, T and B cell proliferation in acutely infected CD28−/− and CD28+/+ mice directly correlates with the level of apoptosis. Our study also suggests that the mechanisms responsible for the elimination of polyclonally activated lymphocytes are not impaired in mice lacking CD28, because the expansion of spleen lymphocytes is rapidly controlled in both groups of infected mice. It is worth mentioning, however, that the striking proapoptotic effect of CD28 observed in this study obfuscates any antiapoptotic effect this molecule might have on specific B and T cell clones.

Concerning the production of effector molecules, the absence of CD28 affected the early T and B cell responses to acute malaria with a certain hierarchy. A week after infection, the number of IgM-secreting cells in the spleen of CD28−/− mice was 80% that in CD28+/+ mice, whereas for IFN-γ+ T cells and IgG2a-secreting cells, this percentage dropped to 50 and 25%, respectively. The major role of CD28 in the generation of IgG2a-secreting cells was also evident in the analysis of CD138+ (Syndecan-1) cells in the spleens of acutely infected mice. These results indicate that the polyclonal IgM response to acute P. chabaudi malaria is mostly independent of CD28, but the production of type 1 effector molecules is largely influenced by this coreceptor. These findings add useful information to the previous study reported by Taylor-Robinson and Smith (31) showing an increase in IFN-γ production in P. chabaudi-infected mice treated with anti-CD80/86 Abs. Thus, the fact that the blockage of B7 interactions, which also impairs the negative signals provided by CTLA-4, results in an increase in IFN-γ production, whereas the lack of CD28 that facilitates the binding of B7 to CTLA-4 results in a decrease in IFN-γ production, raises the possibility that CTLA-4 participates in the control of IFN-γ response to acute P. chabaudi infection.

Our observation that CD28 is required for the early IgG2a response to P. chabaudi malaria extends a previous report in athymic BALB/c mice, showing that the polyclonal IgM response to infection is relatively independent of T cell help, whereas the polyclonal
IgG2a response is mostly T cell dependent (1). A T cell-dependent mechanism for induction of polyclonal B cell activation has been recently described in LCMV infection, in which polyclonal IgG production arises when specific Th cells recognize B cells that have processed viral Ags regardless of their BCR specificity (43). If a similar mechanism operates during acute malaria, the major role of CD28 in P. chabaudi-induced polyclonal IgG2a production could be associated with the fact that T cell signaling by this co-receptor up-regulates CD40L, an essential molecule for Ig isotype switching (25). This concept is supported by the present data, which show a lower increase in CD40L in CD4⁺ T cells of acutely infected CD28⁻/⁻ mice compared with CD28⁺/⁺ mice.

The ability of CD28⁻/⁻ mice to resolve the acute phase of infection with kinetics similar to those of CD28⁺/⁺ mice was unexpected. Because IFN-γ has a major role in the control of acute P. chabaudi malaria (8, 9), and spleen cells from infected CD28⁻/⁻ mice produced lower amounts of this cytokine, it was predictable that CD28 would be important for the initial control of the parasite. However, it is worth nothing that even if parasitemias have been shown to be more intense in IFN-γ-deficient mice (9), ~60% of the females succeed to resolve the acute infection, suggesting the existence of protective effector mechanisms other than those mediated by IFN-γ. Therefore, it is possible that the low levels of IFN-γ secreted by CD4⁺ and CD8⁺ cells in infected CD28⁻/⁻ mice acting together with polyclonal IgM or other effector molecules of the innate immune system could have been sufficient to allow control of the first wave of parasites.

The most remarkable observation of the present study was that parasites from primary and secondary challenges could not be eliminated in mice lacking CD28, suggesting that signaling through this co-receptor is an absolute requirement for the acquisition of full protective immunity to P. chabaudi malaria. The inability to resolve the chronic P. chabaudi infection was also observed after treatment with anti-CD80/86 Abs (31). These findings are of particular interest because CD28⁻/⁻ mice have been shown to efficiently heal diseases caused by LCMV (34), vaccinia virus (30), H. polygyrus (35), L. major (36), and T. gondii (33), which have led to the conclusion that in many infections CD28-mediated signals can be substituted by other costimulatory signals. The idea that ICOS and CD28 are parallel costimulatory pathways, either of which is sufficient to promote resistance to pathogens, has emerged from a recent study showing that ICOS has a critical role in the induction of protective type 1 response required for resistance to T. gondii (44). Hence, the present study contributes to current knowledge by showing that CD28 cannot be substituted by other costimulatory co-receptors in resistance to P. chabaudi malaria. Similar to the present findings, the involvement of CD28 in defense against pathogens is also suggested by the observations that CD28⁻/⁻ mice fail to generate memory responses to T. gondii (33) and have an impaired type 2 response to S. mansoni (32).
Finally, our results strongly indicate that the deficiency of CD28−/− mice in eliminating the primary and secondary P. chabaudi infections is mostly due to their incapacity to produce specific IgG, notably that of the IgG2a subclass. This conclusion is based on the following observations. First, 3 mo after infection, the serum of CD28−/− mice was negative for specific IgG, and these mice failed to eradicate the parasites. Second, reinfection of CD28−/− mice did not result in a secondary IgG response, and the parasites were not eliminated. Third, serum of reinfected CD28+/+ mice containing specific IgG2a was able to transfer protection to reinfected CD28−/− mice. Thus, concerning the production of specific IgG required for protection against P. chabaudi malaria, our data suggest that the absence of CD28-mediated signals cannot be replaced by other costimulatory signals. Despite this, mice lacking CD28 are able to develop primary and secondary specific IgM responses that, although not sufficient to eliminate the parasite, might have contributed to maintain the parasitemia below 20% and ensure mouse survival. It is worth mentioning that in these experiments we did not observe the switch from a type 1 to a type 2 response described by several authors, including ourselves (1, 3). This difference could be a matter of time, because in this study the late response was only evaluated by the analysis of serum IgG2a, and these Abs are known to persist for a long period.

Our results also showed that the incapacity of CD28−/− mice to resolve the secondary infection correlates with their failure to generate memory CD4+ T cells. In fact, the observation that the proliferative response of drug-cured CD28−/− mice was partially re-stored with rIL-2 indicates that specific lymphocytes were generated and maintained in the absence of CD28, but these cells probably went through a tolerance/anergy program (45). A priori, defects in T cell function can affect both cell-mediated and humoral immunities. However, the direct contribution of T cells to the acquired immunity against P. chabaudi infection is minimized by data showing that mice deficient in IFN-γ receptor can develop full protective responses upon reinfection (46). Moreover, in our experiments the numbers of IFN-γ-producing spleen cells were not increased in the first days after reinfection (data not shown), when CD28+/+ mice eliminated the parasites, suggesting that T cells are not directly involved in clearance of the secondary challenge. Nevertheless, we believe that the impaired T cell response of drug-cured CD28−/− mice may be directly related to the incapacity of these mice to produce specific IgG required for the acquisition of full protective immunity.

The role of CD28 for in vivo Ab production has been previously approached in two different models of type 2 immune responses (35). Although IgG1 and IgE responses induced by treating mice with anti-IgD mAb were impaired in the absence of CD28, H. polygyrus infection resulted in Ab production in both CD28−/− and CD28+/+ mice. Because B cells are the primary APCs in the anti-IgD response and dendritic cells are the most likely APCs in the anti-nematode response, Gause et al. (35) proposed that the capacity to bypass the CD28 requirement resides in the APC’s ability to provide additional costimulatory signals. Based on this interpretation, it is tempting to speculate that P. chabaudi might not signalize APCs to up-regulate the expression of costimulatory molecules that substitute for the B7/CD28 pathway in the generation of acquired immunity. If this is the case, manipulations aiming to amplify the range of costimulatory signals provided by APCs may improve the immune response to P. chabaudi malaria.

The data presented in this study provide evidence that CD28 is absolutely required for the acquisition of full protective immunity to P. chabaudi malaria. We believe that this analysis will help in understanding why the protective immunity to human malaria is so difficult to achieve and so rapidly lost, knowledge that will certainly improve our strategies for vaccine development.

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References


