Role of B7 Costimulatory Molecules in Immune Responses and T-Helper Cell Differentiation in Response to Recombinant HagB from *Porphyromonas gingivalis*

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In addition to antigen-specific signals mediated through the T-cell receptor, T cells also require antigen nonspecific costimulation for activation. The B7 family of molecules on antigen-presenting cells, which include B7-1 (CD80) and B7-2 (CD86), play important roles in providing costimulatory signals required for development of antigen-specific immune responses. Hemagglutinin B (HagB) is a nonfimbrial adhesin of the periodontopathic microorganism *Porphyromonas gingivalis* and is thought to be involved in the attachment of the bacterium to host tissues. However, the immune mechanisms involved in responses to HagB and their roles in pathogenesis have yet to be elucidated. Therefore, the purpose of this study was to determine the role of B7 costimulatory molecules on T-helper-cell differentiation for the induction of immune responses to HagB. Mice deficient in either or both of the costimulatory molecules B7-1 and B7-2 were used to explore their role in immune responses to HagB after subcutaneous immunization. B7-1^{-/-} mice had levels of immunoglobulin G (IgG) anti-HagB antibody activity in serum similar to those of wild-type mice, whereas lower serum IgG anti-HagB antibody responses were seen in B7-2^{-/-} mice. Moreover, significantly lower numbers of IgG antibody-secreting cells and lower levels of CD4⁺-T-cell proliferation were observed in B7-2^{-/-} mice compared to wild-type mice. No serum IgG response to HagB was detected in B7-1/B7-2^{-/-} mice. Analysis of the subclass of the serum IgG responses and the cytokines induced in response to HagB revealed that B7-2^{-/-} mice had significantly lower IgG1 and higher IgG2a anti-HagB antibody responses compared to wild-type mice. The B7-2^{-/-} mice also had significantly reduced levels of interleukin-4 (IL-4) and IL-5 and enhanced level of gamma interferon. Furthermore, assessment of B7-1 and B7-2 expression on B cells and macrophages derived from wild-type BALB/c mice after *in vitro* stimulation with HagB revealed a predominant upregulation in the expression of the B7-2 costimulatory molecule on B cells and macrophages. Essentially no change was seen in the expression of B7-1. Taken together, these results suggest a critical role for B7, especially B7-2, for the preferential induction of a Th2-like response to HagB.

Porphyromonas gingivalis, a gram-negative, black-pigmented anaerobic bacterium, has been implicated as a major etiological agent of human adult periodontitis, a chronic inflammatory disease characterized by the destruction of the supportive tissues surrounding teeth (38). A variety of putative virulence factors of *P. gingivalis* thought to be important in the disease process have been identified (18). Nonfimbrial adhesins such as the hemagglutinin B (HagB) are thought to be potential virulence factors involved in mediating the attachment of the bacterium to host cells (11, 17, 21–23, 30). In terms of its potential as a protective vaccine antigen against periodontal disease, we have previously shown that systemic immunization with recombinant HagB (rHagB) results in protection from *P. gingivalis* infection in an experimental rat model (20). However, there is a lack of information on the immune mechanism(s) involved in host responses to HagB.

Activation and expansion of T cells is the central event in the development of specific immune responses against protein antigens. Recent studies by Yang et al. (42) showed that immunization with HagB induced an immunoglobulin G1 (IgG1)

and IgG2a immune response, reflecting the involvement of Th1 and Th2 cells. Other studies have reported that the lipopolysaccharide (LPS) from *P. gingivalis* when given with OVA induced clonal expansion of OVA-specific CD4⁺ T cells with a cytokine profile reflective of a Th2-like response (31). This was in contrast to that seen with the LPS from *Escherichia coli*, where a Th1-like cytokine profile was observed. In general, naive CD4⁺ T cells require two distinct stimuli to promote T-cell clonal expansion, cytokine secretion, and effector function. The first is an antigen-specific signal provided by interactions between the T-cell receptor and a processed antigenic peptide presented by a syngeneic major histocompatibility molecule. A second T-cell-receptor-independent signal is mediated by the engagement of T-cell surface molecules with costimulatory molecules expressed on antigen-presenting cells (APC) (28, 39, 41). The best-defined costimulatory molecules are B7-1 (CD80) and B7-2 (CD86). B7-1 and B7-2 are ligands for the T-cell membrane proteins CD28 and CTLA-4 and have been implicated as important determinants on professional APC that play a major role in murine and human CD4⁺-T-cell activation (9, 15, 34, 37).

In vitro and *in vivo* model systems have demonstrated the importance of B7 costimulation in the generation of antigen-specific immune responses (39). Some studies (6, 25, 26, 39, 40)

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have shown that both ligands have a critical role in the activation of CD4⁺ T cells in vivo and in vitro, whereas other studies (1, 13, 14, 33) have provided evidence for a preferential role for B7-1 or B7-2 in T-cell activation. Moreover, it has been suggested that the relative expression of B7-1 and B7-2 on APC may have different functional consequences, such as driving T-cell differentiation into either the Th1 or the Th2 pathway, respectively (10, 24, 27, 28). Other studies, however, have shown that B7-1 and B7-2 have distinct effects on the generation of T-cell responses to different antigens and can compensate for each other in T-cell activation and proliferation (40).

The purpose of the present study was to investigate the role of B7-1 and B7-2 in the induction of humoral immune responses to rHagB and to determine the role of the B7 costimulatory molecules in T-helper cell differentiation in response to HagB. Finally, in vitro expression of B7-1 and B7-2 on B cells and macrophages after rHagB stimulation was characterized. Evidence is provided that B7-2 is the major costimulatory molecule for the preferential induction of a Th2-like response to HagB.

MATERIALS AND METHODS

Antigen. rHagB was purified as previously described (42). Briefly, the *hagB* gene was cloned from *P. gingivalis* 381 into a pET vector with a *lac* promoter and histidine tag and expressed in *Escherichia coli* JM109 (kindly provided by Ann Progulski-Fox and Thomas Brown, University of Florida, Gainesville). A culture of *E. coli* was grown overnight at 35°C in Luria-Bertani broth containing carbenicillin and kanamycin. The overnight culture was transferred to Luria-Bertani broth with antibiotics and incubated at 35°C with vigorous shaking. When the concentration of bacteria reached an optical density at 600 nm of 0.6, 0.36 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added, and the culture was incubated for an additional 3 h. The culture was centrifuged, and the pellet was suspended in 5 mM imidazole–0.5 M NaCl–20 mM Tris-HCl (pH 7.9) and then stored at –70°C. The next day, the cells were thawed and sonicated, and the supernatant was passed through a 0.45-μm-pore-size low-protein-binding filter. rHagB was purified from the supernatant by using a His-Bind resin column according to the manufacturer's instructions (Novagen, Madison, Wis.). The fraction containing rHagB was dialyzed against phosphate-buffered saline (PBS). The purity of rHagB was confirmed by silver stain and Western blot analysis with a rat anti-rHagB antibody. The concentration of rHagB was estimated by bicinchoninic acid protein determination assay (Pierce, Rockford, Ill.) using bovine serum albumin (BSA) as the standard.

Mice and immunization. BALB/c wild-type (wt), B7-1^{–/–}, B7-2^{–/–}, and B7-1/B7-2^{–/–} mice used in the present study were bred and maintained in a pathogen-free colony within the animal facility at the University of Alabama at Birmingham. Groups of mice were immunized by the subcutaneous (s.c.) route with 20 μg of rHagB on days 0 and 14, a regimen that induces maximum responses (44). In one series of experiments, blood samples were collected prior to and at 2, 4, and 6 weeks after the first immunization and used to quantitate levels of antibody production. In a second series of experiments, mice were sacrificed 7 to 10 days after the second immunization, and spleen cells were assessed for the number of antibody-secreting cells (ASC), proliferative responses, and cytokine production. All studies were performed according to National Institutes of Health guidelines, and protocols were approved by the UAB Institutional Animal Care and Use Committee.

Quantification of antibody responses. Serum was obtained after centrifugation of blood samples collected with a heparinized capillary pipette from the retroorbital plexus. All samples were stored at –20°C until analyzed for antibody activity.

The level of specific antibodies against rHagB in serum was quantitated by an enzyme-linked immunosorbent assay as previously described (19, 43). Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with rHagB (1 μg/ml) at 4°C overnight. Plates were blocked for 2 h at room temperature with 1% BSA in borate-buffer saline (100 mM NaCl, 50 mM boric acid, 1.2 mM Na₂B₄O₇, 0.02% azide [pH 8.2]). Serial twofold dilutions of serum were added in duplicate to wells, and the plates were incubated overnight at 4°C. Peroxidase-conjugated goat anti-mouse IgG or IgG subclass-specific antibodies (Southern Biotechnology Associates, Inc., Birmingham, Ala.) were used for detection, followed by the addition of *o*-phenylenediamine substrate with H₂O₂. The concentrations of antibodies in test serum samples were calculated by interpolation on standard

curves generated by using a mouse immunoglobulin reference serum (ICN Biomedicals, Aurora, Ohio) and constructed by a computer program based on four-parameter logistic algorithms (Softmax/Molecular Devices Corp., Menlo, Calif.).

Splenic lymphoid cells preparation. Spleens were aseptically removed from euthanized mice and single-cell suspensions were prepared by mechanically dispersing the tissues through 40-μm cell strainers (Falcon BD Labware, Franklin Lakes, N.J.) into Hanks balanced salt solution supplemented with 10% fetal calf serum. Erythrocytes were removed from the single cell suspensions by using M-lyse buffer (R&D Systems, Inc., Minneapolis, Minn.). Cells were then washed, suspended in RPMI 1640 complete medium (10% fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 1.5 mg of sodium bicarbonate/ml, 50 μg of gentamicin sulfate/ml, 25 mM HEPES, 50 U of penicillin/ml, 50 μg of streptomycin/ml), and counted in a hemacytometer with trypan blue to estimate viability.

ELISPOT assay. Cells secreting IgM, IgG, or IgA antibodies to HagB were enumerated in spleen cell suspensions by enzyme-linked immunosorbent (ELISPOT) assay. Multiscreen HA 96-well sterile plates (MAHAS4510; Millipore, Bedford, Mass.) were coated with rHagB (5 μg/ml at 100 μl/well) in PBS at 4°C overnight. Plates were blocked with 1% BSA in PBS for 1 h at 37°C. The spleen cell suspensions in complete RPMI medium were plated at 1 × 10⁶, 2 × 10⁵, and 4 × 10⁴ cells/well in duplicate and incubated for 4 h at 37°C in a humidified CO₂ incubator. Biotin-conjugated goat anti-mouse IgM, IgG, and IgA immunoglobulin isotype reagents (Southern Biotech) and streptavidin-alkaline phosphatase conjugate (Southern Biotech), followed by BCIP/NBT Plus substrate (Moss, Inc., Pasadena, Mass.) were used for detection. ASC were counted by using a stereo dissecting microscope. The results are expressed as the number of ASC per 10⁶ cells.

Proliferation assay. Proliferative responses to HagB of purified CD4⁺ T cells from each group of mice were assessed 7 to 10 days after the second immunization. CD4⁺ T cells were purified from spleen cell suspensions by negative selection by using mouse T-cell CD4 subset column kit, according to the manufacturer's protocol (R&D Systems). The purity of the recovered CD4⁺ T cells was >85%, as determined by flow cytometry. The purified CD4⁺ T cells (2 × 10⁶ cells/ml) were cocultured with irradiated (2,200 rads) splenic feeder cells (5 × 10⁶ cells/ml) in complete medium in 96-well flat-bottom plates. Medium alone, concanavalin A (2.5 μg/ml), or rHagB (20 μg/ml) was added to appropriate culture wells in triplicate. The cultures were incubated for 48 h (concanavalin A) or 96 h (rHagB) in a humidified CO₂ incubator at 37°C. CD4⁺ T-cell proliferation was measured by adding 0.5 μCi of [³H]thymidine for the last 18 to 20 h of culture. The amount of [³H]thymidine incorporation was measured in a liquid scintillation counter. The proliferative responses are expressed as a stimulation index calculated as the ratio of the mean counts per minute (cpm) in HagB-stimulated cultures to the mean cpm in unstimulated control cultures.

Cytokine analysis. Supernatants from cultures of purified splenic CD4⁺ T cells (prepared as described above) were harvested after 5 days of incubation with (20 μg/ml) or without rHagB. The levels of interleukin-4 (IL-4), IL-5, and IFN-γ in the culture supernatants were determined by enzyme-linked immunosorbent assay according to the manufacturers' instructions (IL-4 and IFN-γ; eBioscience, San Diego, Calif.; IL-5, BD Biosciences, San Diego, Calif.).

Flow cytometry analysis. The expression of B7-1 and B7-2 on splenic B cells (B220⁺) and macrophages (CD11b⁺) from naive BALB/c wt mice was analyzed after in vitro stimulation with rHagB. Briefly, single-spleen-cell suspensions, prepared as described above, were cultured in duplicate either with 1, 5, 10, or 20 μg of rHagB/ml or without rHagB for 24 or 48 h. Cholera toxin (CT; 1 μg/ml) was used as a positive control. Cells were then suspended in fluorescence-activated cell sorting (FACS) buffer and stained with biotin-conjugated anti-B220 or anti-CD11b monoclonal antibody (MAb), together with phycoerythrin-labeled B7-1 and fluorescein isothiocyanate-labeled B7-2 MAbs (eBioscience) for 30 min on ice. The cells were washed three times and then stained with streptavidin-PerCP (BD Biosciences) for 30 min on ice. Cells were then washed twice and suspended in 500 μl of FACS buffer and immediately analyzed by using a FACSCalibur (BD Biosciences).

Statistical analysis. Statistical significance between groups was evaluated by analysis of variance and the Tukey multiple-comparison test by using the InStat program (Graphpad Software, San Diego, Calif.). Differences between groups were considered significant at the level of *P* < 0.05.

RESULTS

Antibody responses. To determine the role of B7-1 and B7-2 for antibody responses to HagB, groups of wt, B7-1^{–/–}, B7-

FIG. 1. Serum IgG anti-HagB responses in wt, B7-1^{-/-}, B7-2^{-/-}, and B7-1/B7-2^{-/-} mice immunized by the s.c. route with 20 µg of rHagB/ml on days 0 and 14. The results are expressed as the arithmetic mean $\pm$ the standard error of the mean (SEM; $n = 6$). Values were significantly different from those of wt mice at P values of <0.05 (*) and <0.01 (**).

2^{-/-}, and B7-1/B7-2^{-/-} mice were immunized with rHagB by the s.c. route, and serum samples were collected and assessed for anti-HagB antibody activity. B7-1^{-/-} mice had levels of serum IgG anti-HagB antibody similar to those of wt mice, whereas a lower serum IgG anti-HagB response was seen in B7-2^{-/-} mice after the first immunization (Fig. 1). A 51% decrease in the specific antibody level was observed at week 4 in B7-2^{-/-} mice compared to wt mice. No serum IgG anti-HagB antibody activity was observed in B7-1/B7-2^{-/-} mice. No serum IgG anti-HagB antibody response was observed in pre-

immune samples (day 0) from the four experimental groups or from samples from nonimmunized control mice (data not shown). These results suggest that the B7 costimulatory molecules play a critical role in the HagB-specific IgG responses; the IgG anti-HagB antibody activity in wt mice was mainly attributed to B7-2.

To better understand the nature of the antibody response to HagB and to gain insight into the Th subsets involved in the anti-HagB response, serum samples were further assessed for the IgG subclass distribution. Comparable levels of serum IgG1 anti-HagB antibody activity were seen in B7-1^{-/-} mice and wt mice (Fig. 2A). In contrast, B7-2^{-/-} mice exhibited a 75% lower specific serum IgG1 response ($P < 0.05$) at week 4, thus suggesting a profound defect in Th2 responses. Lower responses were also seen in B7-2^{-/-} mice at weeks 2 (50%) and 6 (63%). These results suggest that B7-2 is required for an IgG1 response to HagB. Interestingly, a different pattern was seen in terms of the IgG2a anti-HagB response (Fig. 2B). B7-1^{-/-} mice had higher levels of serum IgG2a anti-HagB antibody responses than wt mice after the first immunization; however, the difference was not significant. The specific IgG2a responses in B7-2^{-/-} mice were significantly higher ($P < 0.05$) than that seen in wt mice at weeks 4 (340% increase) and 6 (275% increase). Taken together, these results suggest a critical role for B7 costimulatory molecules in IgG responses to HagB after s.c. immunization. B7-2 appears to play a predominant role in the IgG1 response toward HagB, which is reflective of a type 2 response. Furthermore, the results suggest that B7-2 may also play a role in the downregulation of the IgG2a response, which is reflective of a type 1 response to HagB.

ASC. To further identify the requirement for B7-1 and B7-2 for the development of antibody responses to HagB, spleens from the four experimental groups were collected and analyzed for ASC by an ELISPOT assay. High numbers of IgM-

FIG. 2. Serum IgG1 (A) and IgG2a (B) anti-HagB responses in wt, B7-1^{-/-}, B7-2^{-/-}, and B7-1/B7-2^{-/-} mice immunized by the s.c. route with 20 µg of rHagB/ml on days 0 and 14. The results are expressed as the arithmetic mean $\pm$ the SEM ($n = 6$). Values were significantly different from those of wt mice at $P < 0.05$ (*) and $P < 0.001$ (***)

FIG. 3. IgM (A) and IgG (B) ASC specific for HagB in spleens from wt, B7-1^{-/-}, B7-2^{-/-}, and B7-1/B7-2^{-/-} mice 7 to 10 days after the second immunization. The results are expressed as the number of ASC per 10⁶ cells (arithmetic mean $\pm$ the SEM; $n = 6$). Values were significantly different from those of wt mice at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

specific ASC were detected in the spleens from all groups 7 days after the second immunization (Fig. 3A). However, a significantly higher number of IgM ASC ($P < 0.001$) was seen in B7-1/B7-2^{-/-} mice compared to wt mice and B7-1^{-/-} mice. Moreover, significantly higher numbers of HagB-specific IgM ASC ($P < 0.01$) were also found in B7-2^{-/-} mice compared to wt mice. In contrast, the highest number of HagB-specific IgG ASC was detected in wt mice, a finding that was significantly different ($P < 0.05$) from that seen in B7-2^{-/-} mice and B7-1/B7-2^{-/-} mice but not in B7-1^{-/-} mice (Fig. 3B). Few specific IgA ASC were detected in spleens from all of the four groups of mice (data not shown). These findings support the serum IgG antibody results, which suggested a primary involvement of B7-2 for humoral responses to rHagB.

CD4⁺ T-cell proliferative responses. To assess the effect of the B7 costimulatory molecules on responses of CD4⁺ T cells to HagB, purified splenic CD4⁺ T cells from immunized mice were incubated with rHagB in vitro, and the proliferative responses were assessed. Significantly higher proliferative responses were observed with CD4⁺ T cells from wt mice ($P < 0.001$) and B7-1^{-/-} mice ($P < 0.01$) compared to B7-1/B7-2^{-/-} mice (Fig. 4). No significant difference in CD4⁺ T-cell proliferative responses was observed between wt and B7-1^{-/-} mice. However, the HagB-specific proliferative activity of CD4⁺ T cells from B7-2^{-/-} mice was >58% decreased ($P < 0.05$) compared to the responses of CD4⁺ T cells from wt mice. Similar results were also seen with T cells from B7-1/B7-2^{-/-} mice. These results suggest that the responses of CD4⁺ T cells seen in wt mice after HagB stimulation were mainly due to the expression of the B7-2 costimulatory molecule.

Cytokine production. HagB-specific CD4⁺ T cells from wt, B7-1^{-/-}, B7-2^{-/-}, and B7-1/B7-2^{-/-} mice also revealed distinct differences in Th1- and Th2-derived cytokine production. CD4⁺ T cells from wt mice produced the highest amounts of IL-4 and IL-5 upon restimulation with HagB (Fig. 5A and B). A 45% decrease in IL-4 production and a 53% decrease in IL-5 production ($P < 0.01$) were seen with CD4⁺ T cells from

B7-1^{-/-} mice compared to T cells from wt mice. Moreover, CD4⁺ T cells from B7-2^{-/-} mice had significantly lower levels of IL-4 (82% decrease) and IL-5 (57% decrease) compared to T cells from wt mice ($P < 0.01$). In marked contrast, the production of gamma interferon (IFN- γ) was significantly enhanced by CD4⁺ T cells from B7-1^{-/-} mice ($P < 0.05$) and B7-2^{-/-} mice ($P < 0.01$) than by CD4⁺ T cells from wt mice (Fig. 5C). Low levels of IL-4, IL-5, and IFN- γ cytokine pro-

FIG. 4. Proliferative responses to HagB of splenic CD4⁺ T cells from wt, B7-1^{-/-}, B7-2^{-/-}, and B7-1/B7-2^{-/-} mice 7 to 10 days after the second immunization. Purified splenic CD4⁺ T cells (2×10^6 cells/ml) were incubated with irradiated splenic feeder cells (5×10^6 cells/ml) and rHagB (20 μ g/ml). The results are expressed as the stimulation index, which is calculated as the ratio of mean cpm counts in HagB-stimulated cultures to mean cpm counts in nonstimulated control cultures (arithmetic mean $\pm$ the SEM; $n = 6$). The mean counts for control cultures were as follows: wt, 244 cpm; B7-1^{-/-}, 184 cpm; B7-2^{-/-}, 410 cpm; and B7-1/B7-2^{-/-}, 134 cpm. Values were significantly different from those of wt mice at $P < 0.01$ (**) and $P < 0.001$ (***).

FIG. 5. Th1- and Th2-type cytokine production by splenic CD4⁺ T cells from wt, B7-1^{-/-}, B7-2^{-/-}, and B7-1/B7-2^{-/-} mice 7 to 10 days after the second immunization. Cultures were incubated with (20 μ g/ml) or without rHagB for 5 days, and then the supernatants were assessed for IL-4 (A), IL-5 (B), and IFN- γ (C) production. The results are expressed as the arithmetic mean $\pm$ the SEM ($n = 6$). Values were significantly different from those of wt mice at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

duction were detected in B7-1/B7-2^{-/-} mice. These results provide additional evidence that the absence of B7-2 results in the downregulation of type 2 responses and in the upregulation of type 1 responses to rHagB. Furthermore, the results suggest an involvement of B7-1 in IL-4 and, especially, IL-5 and IFN- γ production.

Effect of HagB on the expression of costimulatory molecules.

To assess the effect of HagB on B7-1 and B7-2 expression by B cells (B220⁺) and macrophages (CD11b⁺), splenic cells derived from naive wt mice were analyzed by FACS after HagB stimulation. A range of rHagB concentrations (1, 5, 10, and 20 μ g/ml) were used to exclude the possibility that differential sensitivity of B7-1 and B7-2 expression to rHagB may affect the effect of rHagB on B7-1 and B7-2 expression by B cells or macrophages. Expression of both B7-1 and B7-2 on B cells and macrophages without rHagB stimulation was low (data not shown). When the spleen cells were cultured in the presence of 1 μ g of rHagB/ml for 24 h, the number of B7-2-expressing B cells and macrophages increased threefold and onefold, respectively (Fig. 6). The percentage of B7-2-positive B cells and

macrophages increased progressively with increasing concentrations of rHagB in the culture. After incubation with 20 μ g of rHagB/ml for 24 h, an $\sim$ 8.5-fold increase in B7-2-expressing B cells and a 2-fold increase of B7-2-expressing macrophages were observed. In contrast, we only observed a <1 -fold increase in the number of B7-1-expressing B cells after 24 h of stimulation with 1 μ g of rHagB/ml, and an $\sim$ 1-fold increase was observed after stimulation with 20 μ g of rHagB/ml. The percentage of macrophages expressing B7-1 was not affected by incubation with any concentration of rHagB for 24 h. CT (1 μ g/ml), which has been shown to potentiate the expression of B7-2, but not of B7-1, on mouse bone marrow macrophages in vitro (8, 9), was used as a positive control in the present study and exhibited a selective enhancement of B7-2 expression on B cells and macrophages. An $\sim$ 7-fold increase and an $\sim$ 4-fold increase in the level of B7-2-expressing B cells and macrophages were induced after 24 h of incubation, respectively (data not shown). No increase in the percentage of B cells and macrophages expressing B7-1 by CT was found. To determine whether B7-1 expression was induced by rHagB at a later time

FIG. 6. Expression of B7-1 and B7-2 on B220⁺ (A) and CD11b⁺ (B) cells from spleens of naive BALB/c mice after in vitro incubation with rHagB for 24 h. The results are expressed as the percentage increase in the arithmetic mean of positive cells expressing B7-1 and B7-2 after rHagB stimulation over the mean of unstimulated cells. The data were obtained from two separate experiments.

point, we also analyzed B7-1 and B7-2 expression after 48 h of stimulation. We found that the optimal time for inducing the expression of B7 molecules by rHagB or CT was in 24 h. However, we still observed a greater increase in the level of B7-2- than B7-1-expressing B cells and macrophages 2 days after stimulation (data not shown). To verify that the observed responses to HagB were not due to LPS contamination, an additional set of cell cultures were incubated with 20 μ g of boiled HagB (100°C for 5 min)/ml for 24 and 48 h. No upregulation of B7-1 or B7-2 expression on B cells or on macrophages was observed (data not shown). The distinct levels of B7-1 and B7-2 expression on the surface of B cells and macrophages upon HagB stimulation *in vitro* further support our *in vivo* observations that B7-2 is the major costimulatory molecule involved in immune responses to HagB.

DISCUSSION

The importance of B7 molecules on APC in providing costimulatory function for T-cell activation and differentiation has been demonstrated by several studies. However, whether B7-1 and B7-2 have identical, overlapping, or distinct functions is under active investigation. Putative differences in the functions of B7-1 and B7-2 have been suggested to be potentially due to differences in the patterns of expression, as well as differences in the binding kinetics of the costimulatory molecules to CD28 and CTLA-4 (7, 28, 40). Furthermore, marked differences in the individual roles of B7-1 or B7-2 *in vitro* and *in vivo* have been observed, indicating the importance of examining the costimulatory molecule requirement in the context of the specific immunogens (40, 41). In the present study, we have examined the role of B7 costimulatory molecules in immune responses to *P. gingivalis* HagB after s.c. immunization. Our study provides new insights into the function of these molecules on regulation of the immune response to a virulence factor of a periodontal pathogen.

Mice deficient in both B7-1 and B7-2 costimulatory molecules failed to generate HagB-specific serum IgG, IgG1, and IgG2a responses. We also found essentially no specific IgG ASC in rHagB-immunized B7-1/B7-2^{-/-} mice. A previous study by Borriello et al. (2) showed that mice deficient in both B7-1 and B7-2 failed to generate antigen-specific IgG1 and IgG2a responses and lacked germinal centers when immunized by a number of routes even in the presence of complete Freund adjuvant. These authors found that B7-mediated signaling plays a critical role in germinal center formation and immunoglobulin class switching *in vivo*. Our results demonstrate the dependence of antigen-specific IgG responses to rHagB on B7-mediated signaling. Moreover, a striking elevated level of HagB-specific IgM ASC was observed in spleens from B7-1/B7-2^{-/-} mice. A similar observation was made by Borriello et al. (2), who showed a threefold increase in the basal IgM level in unimmunized B7-1/B7-2^{-/-} mice compared to wt mice. These authors demonstrated that the lack of B7-1 and B7-2 did not markedly affect antigen-specific IgM production, indicating that this B-cell response was not dependent on B7 expression. However, class switching to IgG, which is dependent upon the function to T helper cells through T-B cell interactions, was markedly affected, suggesting that T-cell help was critically diminished by the absence of B7-1 and B7-2.

In terms of the individual function of B7-1 or B7-2 on the specific antibody responses to rHagB, we found that the HagB-specific antibody responses were comparable in B7-1^{-/-} and wt mice, whereas mice deficient in B7-2 had a low level of HagB-specific IgG antibody activity. We also found that the level of specific IgG ASC in mice deficient in B7-2 was significantly lower than in wt mice. Moreover, mice lacking B7-2 costimulatory molecules had dramatically decreased levels of CD4⁺-T-cell proliferative responses to rHagB compared to wt mice. These results suggest that B7-2 is more important in the induction of specific IgG responses to HagB than B7-1. Several studies (2, 5) have indicated a dominant role for B7-2 in mediating T-cell-dependent responses. This may be due to both the constitutive expression of B7-2 and its more rapid kinetics of induction relative to B7-1 (41).

With regard to the subclass of the serum IgG antibody responses, high IgG1 and low IgG2a responses against rHagB were observed in wt mice, suggesting that rHagB predominantly induces a type 2 response after s.c. immunization. This view is consistent with results from our previous studies that showed a preferential IgG1 or type 2 response after s.c. immunization with HagB (44). The costimulatory signal provided to T cells through interactions with CD28/CTLA-4 plays an important role in the *in vivo* Th cell effector function associated with cytokine production. However, it is uncertain whether B7-1 and B7-2 differentially influence the development of a type 1 or type 2 responses, since controversial results have been obtained concerning the individual contributions of B7-1 versus B7-2 signals in different experimental models both *in vivo* and *in vitro* (16, 34–36). To address this question in the present model, we examined the effect of B7 costimulatory molecules in modulating the nature of the immune response to rHagB. A significantly lower level of serum IgG1 antibody activity was seen in B7-2^{-/-} mice than in wt mice. This finding indicates that B7-2 mainly contributed to the IgG1 response to rHagB, reflective of a type 2 response. Our data are consistent with studies with *Leishmania major*-infected B7-2^{-/-} BALB/c mice that also showed a profound defect in their ability to produce high titers of parasite-specific IgG1 antibodies (4), suggesting that B7-2 provides the dominant costimulatory signal necessary to drive Th2 development *in vivo*. Our finding that the B7-2^{-/-} mice also had significantly higher serum IgG2a anti-HagB antibody responses than wt mice suggests that the IgG2a antibody response was suppressed by the presence of B7-2 molecule.

The observed cytokine profiles induced by rHagB in CD4⁺-T-cell cultures helped to understand the effect of B7 costimulatory molecules on T-cell differentiation. A significantly decreased level of Th2-associated cytokines (IL-4 and IL-5) was found in B7-2^{-/-} mice. This effect is consistent with previous studies in which it was shown that IL-4 production is preferentially induced by B7-2 (14, 32). In addition, we found that B7-1 contributed less to the production of IL-4; however, it contributed significantly to IL-5 production. This finding was in contrast to previous studies (24), suggesting opposing functions for B7-1 and B7-2 for the induction of cytokines. However, our results are consistent with studies demonstrating that the production of Th-2 like cytokines is dependent on both B7-1 and B7-2 (3, 16, 27). Furthermore, increased levels of the Th1-associated cytokine IFN- γ in B7-1^{-/-} and B7-2^{-/-} mice

compared to wt mice were also observed. These data support our IgG subclass response results, which showed an increase in the specific IgG2a responses in B7-1^{-/-} and B7-2^{-/-} mice. However, other studies observed a reduced production of IFN- γ in the absence of B7-2 and, to a lesser extent, in the absence of B7-1 (28, 35). One possible explanation for the difference in the results observed is that an initial B7-1/B7-2 deficiency may lead to a relative increase in Th1 responses by preventing Th2 development (35). Furthermore, a study by Schweitzer et al. (35) did not support a simple Th1/Th2-determining role for either B7-1 and B7-2. Their data showed that B7-1/B7-2-mediated costimulation significantly contributes to both Th1 and Th2 cytokine production, especially when T cells are primed under suboptimal conditions, with a greater influence of B7-2 than of B7-1. However, neither B7-1 nor B7-2 played an obligatory role in priming for production of a particular cytokine when T cells were primed under optimal or supraoptimal conditions. Other studies have also reported no specific associations between the B7-1/B7-2 and Th1/Th2 phenotypes (1, 12, 26, 29), suggesting a redundant or compensatory role for B7 molecules in T-helper subset differentiation. This may be accounted for by differences in the efficiency of T-cell priming as a result of the distinct timing and the level of expression of B7-1 and B7-2. In our study, the greater contribution of B7-2, compared to B7-1, in cytokine production by CD4⁺ T cells after stimulation with rHagB further reflects the predominant role of B7-2 in T-cell differentiation. Different experimental conditions need to be studied to further understand the association between B7-1/B7-2 and T-helper cell differentiation.

The constitutive or early expression of B7-2 has led to the hypothesis that B7-2 may be more important in initiating an immune response, whereas B7-1, being expressed later, may serve to amplify or regulate the immune response (15). In the present study, rHagB predominantly upregulated B7-2 expression on the B cells and macrophages in a dose-dependent manner, whereas little effect was observed on B7-1 expression. This observation explains the *in vivo* immunogenic properties of HagB, showing that the specific IgG responses were affected only in B7-2-deficient mice and not in B7-1-deficient mice. Our results indicate that the more potent effect of B7-2 than of B7-1 on the T-cell activation and differentiation reflects the differential patterns of B7-1 and B7-2 expression upon rHagB stimulation.

In summary, our results suggest a critical role for B7, especially B7-2, for the preferential induction of a Th2-like response to rHagB. Further studies in our laboratory investigating the signaling pathways and other regulatory molecules that may play a role in host responses to HagB are under way.

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