7 and 8). On the contrary, such inefficient selector, I-A^q molecule was apparently inhibitory to the generation of mature $CD8^+$ Id^{high+} cells, as shown in Figs. 7 and 8.

These asymmetric properties of class I and class II MHC in thymic positive selection have become evident for the first time in the system of dual class-restricted TCR-Tg mouse, albeit the precise mechanisms have remained unknown. However, these mechanisms, in addition to other mechanisms (25, 39), could underlie the higher proportion of $CD4^+$ T cells than $CD8^+$ T cells in normal animals of normal conditions. It is also possible that these mechanisms may contribute to explain the influence of class II MHC on the differentiation of some pathogenic $CD8^+$ T cells (42, 43), as recently suggested by Logunova et al. (44).

Although cross-reactivities of other Tg TCRs upon thymic selection have not been investigated so intensively, TCRs responsive to both classes of MHC might exist also in normal animal more frequently than generally appreciated. Some T cell clones were in fact demonstrated to show specificity to both classes of MHC molecules (41–47). When positively selected, those cells with TCRs of dual class specificity should tend to differentiate into $CD4^+$ T cells rather than $CD8^+$ T cells, as observed in the present study. It is expected, however, that some of those cells should emerge in $CD8^+$ lineage more efficiently when the recognition of class II MHC is absent, or is hindered by the absence of CD4 molecules. In fact, Shimizu and Takeda (48) reported that $CD8^+$ T cells from MHC class II-deficient mouse frequently respond to syngeneic as well as allogeneic class II MHC. Interestingly, the authors also reported that they could not detect reciprocal reactivity, i.e., the reactivity of $CD4^+$ T cells from $\beta_2m^{-/-}$ mouse to class I MHC. These findings argue that DP cells indeed include a significant portion of cells bearing TCR responsive to both classes of MHC, and that those cells could be much more apparent in class II $MHC^{-/-}$ than in $\beta_2m^{-/-}$ mice.

With regard to the CD4-deficient mice, Tyznik et al. (49) and Pearce et al. (50) demonstrated that class II-restricted $CD8^+$ T cells were readily detectable after primary bacterial or viral infections in $CD4^{-/-}$ mice. In these studies, the generation of class II-restricted $CD8^+$ T cells was interpreted as a result of misdirection of class II MHC-restricted thymocytes (into $CD8^+$ lineage) due to decreased affinity of TCR/MHC (+ peptide) interaction by the absence of CD4. This was based on the previous interesting report by Matechak et al. (11) showing that in some Tg mice bearing class II-restricted TCR, T cells differentiate into $CD8^+$ T cells in CD4-deficient background. From these observations, the authors have proposed that the lineage fate would be determined by the strength of the TCR signal. Our present study has raised another possibility that those Tg TCR could possibly be restricted with both class I and class II MHCs. In fact, it was shown that, in class II MHC-restricted AND TCR-Tg mouse, which generates a large number of $CD8^+$ T cells in the absence of CD4, a small population of $CD8^+$ T cells existed even in the presence of CD4 and in the absence of endogenous TCR α-chain (11). This situation is reminiscent of H-2^q TCR^{QM11}-Tg $RAG2^{-/-}$ mouse in which both $CD4^+$ and $CD8^+$ subsets could mature into periphery with the predominant maturation of $CD4^+$ subset, and the removal of the selecting class II ligand results in optimal CD8 differentiation.

Implications for $CD4^+$ vs $CD8^+$ lineage commitment

Although the exact frequency of dual class-restricted T cells awaits further investigations, the TCR-Tg system, in which DP thymocytes expressing the Tg TCR can recognize both classes of MHC, provided us with an opportunity to evaluate several models of the mechanism of $CD4^+$/$CD8^+$ lineage choice. Importantly, this could be done by simple genetic analyses of Tg mice without further artificial techniques.

At present, our results appear most consistent with the notion that $CD4^+$ vs $CD8^+$ lineage choice was determined by the duration of TCR signals into DP thymocytes in such a way as proposed in the kinetic signaling model (4, 24, 25), and supported by numbers of studies (12, 15, 18, 28, 51, 52). Brugnera et al. (24) showed that TCR signaling in DP thymocytes responding to intrathymic MHC ligands decreases the CD8 gene expression before the lineage commitment, regardless of the ligand MHC classes. When DP thymocytes react with both classes of intrathymic MHC molecules, they would once lose their CD8 expression. In many of them, their differentiation into $CD8^+$ lineage would be inhibited by the persistent engagement of class II MHC by TCR and CD4 on them. This may be the reason that selecting class II MHC was inhibitory to class I-dependent $CD8^+$ differentiation of dual class-restricted T cells. Regarding the ability of selecting class I ligand to increase the efficiency of $CD4^+$ differentiation, it could be because class I ligand-induced signal helped to augment the number of thymocytes undergoing positive selection (that are subject to the lineage determination), thereby increasing the number of both lineages of mature T cells. This hypothesis was supported by the observation that the proportion of $CD69^+$ cells among DP thymocytes was larger in H-2^{bq4xq} mice as compared in H-2^{bxq} mice when examined on RAG2-deficient background (our unpublished data).

One concern would be that the kinetic signaling model, in theory, does not allow the differentiation of DP thymocytes expressing the dual class-restricted TCRs into $CD8^+$ lineage cells, which were constantly observed in periphery of H-2^q TCR^{QM11}-Tg mice as a significant population. We are currently thinking two possibilities that are not mutually exclusive. First, it is possible that not all DP cells may be able to be selected to mature even when they express TCRs that could potentiate the positive selection, because the positive selection may be somewhat an inefficient process mediated by low avidity interactions between TCRs and MHC ligands (53). It could thus be possible that even in the presence of both classes of selecting MHCs, TCRs (and CD4) on some DP cells might not be engaged by class II ligands. Some of such DP cells would be induced to apoptosis before maturation, but some of them could be rescued by the class I MHC-mediated positive selection to differentiate into $CD8^+$ lineage. The second possibility is that among thymic stroma cells that are capable of mediating positive selection, there might be cells with very low or no expression of class II MHC molecules, or cells with low/no expression of CD83, which was shown to be critical in CD4 (but not CD8) lineage differentiation (54). Those cells, if they exist, would function as specific CD8-selector cells, although the presence of those cells may not be apparent in a normal condition. Thus, it is possible that the DP thymocytes expressing dual class-restricted TCR may differentiate into $CD8^+$ lineage when they encounter such stroma cells for thymic positive selection.

Further studies are required to investigate these possibilities, and some of them are currently being addressed.

Acknowledgments

We cordially thank Drs. T. Saito (RIKEN Research Center for Allergy and Immunology) and M. Takahashi and Y. Takagaki (Kitasato University) for the encouraging support. We also thank Dr. M. Shimamura for providing $RAG\text{-}2^{-/-}$ and $\beta_2m^{-/-}$ mice.

Disclosures

The authors have no financial conflict of interest.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 37, Issue of September 10, pp. 38701–38709, 2004
Printed in U.S.A.

Impaired IgG Production in Mice Deficient for Heat Shock Transcription Factor 1*

Received for publication, May 28, 2004
Published, JBC Papers in Press, June 29, 2004, DOI 10.1074/jbc.M405986200

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Heat shock factor 1 (HSF1) is a major transactivator of heat shock proteins in response to heat shock, and it is also involved in oogenesis, spermatogenesis, and placental development. However, we do not know the molecular mechanisms controlling developmental processes. In this study, we found that HSF1-null mice exhibited a significant decrease in the T cell-dependent B cell response. When mice were immunized intraperitoneally with sheep red blood cells, the sheep red blood cell-specific IgG production, especially IgG2a production, in HSF1-null mice was about 50% lower than that in wild-type mice at 6 days after the immunization, whereas IgM production was normal. The number of bromodeoxyuridine-incorporated spleen cells in immunized HSF1-null mice was one-third that in immunized wild-type mice, indicating reduced proliferation of the spleen cells. We analyzed levels of cytokines and chemokines in spleen cells and in peritoneal macrophages stimulated with lipopolysaccharide and interferon-γ and found that expression levels of interleukin-6 and CCL5 were significantly lower in HSF1-null cells than those in wild-type cells. Furthermore, we demonstrated that the *IL-6* gene is a direct target gene of HSF1. These results revealed a novel molecular link between HSF1 and a gene related to immune response and inflammation.

Members of the heat shock transcription factor (HSF)[1] family bind to heat shock element (HSE), which is composed of at least three inverted repeats of a consensus sequence nGAAn (1). Among three HSFs (HSF1, HSF2, and HSF4) in mammals, HSF1 plays a crucial role in inducing heat shock proteins (Hsps), which is required for acquisition of thermotolerance (2, 3). In addition, HSF1 is known to be involved in normal development. In *Drosophila*, a single HSF is necessary for oogenesis and early larval development (4). Mouse HSF1 is required for oogenesis, placental development, and normal growth (5, 6). Furthermore, HSF1 may regulate genes involved in spermatogenesis because spermatogenesis is completely blocked in mice deficient for both HSF1 and HSF2 (7–9). Moreover, HSF1 is involved in eliminating injured male germ cells when these cells are exposed to heat stress (10, 11). However, molecular mechanisms controlling these developmental processes are unclear as yet.

Here we performed microarray analysis using mouse embryo fibroblasts (MEFs) (12) to discover genes regulated by HSF1. We found that constitutive expression of many genes related to the immune response and inflammation was lower in HSF1-null MEFs than in wild-type cells. Furthermore, we found that the serum immunoglobulin level was lower in HSF1-null mice than in wild-type mice. Therefore, we analyzed the T cell-dependent B cell response by analyzing immunoglobin production in response to the immunization with sheep red blood cells (SRBC). It revealed that SRBC-specific IgG production is impaired in HSF1-null mice, which is associated with reduced expression of interleukin-6 (IL-6) and chemokine CCL5. We identified that the *IL-6* gene is a direct target gene of HSF1.

EXPERIMENTAL PROCEDURES

Microarray Analysis—We generated HSF1-null mice by injecting HSF1−/− (C57BL/6 × CBA) F_1 ES cells, TT2 (12) into ICR mouse embryos in the morula stage. Established chimeric male mice (F_0) were bred further with ICR females, and the genotype of the offspring (F_1) was determined by PCR. Homozygous male and female mice were crossed, and the genotypes of a litter of embryos at embryonic day (E) 15.5 were determined. MEFs were prepared by mixing three wild-type or three HSF1-null embryos. To analyze gene expression in wild-type and HSF1-null MEFs, we used an oligonucleotide microarray, GeneChip Murine Genome U74Av2 (Affymetrix, Santa Clara, CA), which contained 12,422 probe sets. Target cRNA preparations from total RNA, hybridization to the microarray, washing and staining with the antibody amplification procedure, and scanning were all carried out according to the manufacturer's instructions. The expression value (average difference) of each gene was calculated and normalized using Affymetrix Microarray Suite software version 4.0, so that the mean of expression values in each experiment was 100 to adjust for minor differences between the experiments. The change values (-fold changes) were calculated by comparison analysis of the software.

Northern Blot Analysis—RNA isolation and Northern blot analysis were performed as described previously (10). cDNA probes for mouse Hsp90α, Hsp90β, Hsp70, and actin were described previously (11). cDNA probes for IL-6, IL-1α, IRF-9, STAT1, and Isg15 were generated by reverse transcription-PCR using total RNA isolated from MEFs. The primers used were as follows: IL-6, 5′-GAC AAA GCC AGA GTC CTT CAG-3′ and 5′-CAA GAA AGG ATC TGG CTA GG-3′; IL-1α, 5′-GTG

* This work was supported by grants-in-aid for scientific research, and on priority area-cell cycle, life of proteins, and cancer, from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and by the Naitoh Foundation and the Mochida Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HSF, heat shock transcription factor; BrdUrd, 5-bromo-2′-deoxyuridine; ChIP, chromatin immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; HSE, heat shock element; Hsp, heat shock protein; IFN, interferon; IL, interleukin; IRF, interferon-regulatory factor; LPS, lipopolysaccharide; Isg, interferon-stimulated gene; MEF, mouse embryo fibroblast; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; RANTES, regulated on activation normal T cell expressed and secreted; SRBC, sheep red blood cells; STAT, signal transducers and activators of transcription.

AGA CCT TCA CTG AAG ATG ACC-3′ and 5′-CAT ACA GAC TGT CAG CAC TTC C; Isg15, 5′-CAA TGG CCT GGG ACC TAA A-3′ and 5′-ATC CCA AAG TCC TCC ATA CCC C. The amplified DNA fragments were inserted into pCR2.1-TOPO vector (Invitrogen), and DNA fragments were isolated after digestion with EcoRI. cDNA probes for STAT1 and IRF-9 were kindly provided by Dr. T. Fujita (Tokyo Metropolitan Institute of Medical Science).

Immunization of Mice—The mice were crossed more than six generations into ICR or C57BL/6 mice. Mice 6–8 weeks old were immunized by intraperitoneal injection of 1×10^8 SRBC (Nippon Bio-Test Laboratory, Tokyo) and then boosted with the same dose at 21 days after the first immunization. Blood samples were collected at 6, 14, and 21 days after the first immunization and at 7 days after the second immunization, and sera were separated. All experimental protocols were reviewed by the Committee for Ethics on Animal Experiments of Yamaguchi University School of Medicine.

Determination of Immunoglobulin Titers—SRBC-specific immunoglobulins in sera were determined by sandwich enzyme-linked immunosorbent assay (ELISA) using plates coated with SRBC (13). SRBC-bound antibodies were detected by alkaline phosphate-conjugated goat antibody specific for mouse IgM, IgG, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Alabama). Serum immunoglobulin levels were measured using a mouse immunoglobulin isotyping ELISA kit (BD Pharmingen) according to the manufacturer's instructions. The relative concentrations of immunoglobulins in individual samples were calculated by comparing the mean optical densities obtained from triplicate wells with a positive control antigen mixture.

Analysis of BrdUrd Incorporation—The spleen was dissected, embedded in Tissue-Tek compound (Sakura, Tokyo), and frozen at −80 °C. Cryosections of 10 μm thick were stained with hematoxylin. To examine DNA replication, incorporation of BrdUrd was examined by immunohistochemical analysis and flow cytometric analysis. At 6 days after the immunization with SRBC, mice were injected intraperitoneally with 50 μg/ml BrdUrd (Sigma) in phosphate-buffered saline (PBS). 12 h after the first injection, mice were injected again. 1 h after the second injection, the spleen was dissected, and cryosections were immunostained as described previously (14).

To quantify levels of BrdUrd-incorporated spleen cells, red blood cell-depleted spleen cells were fixed in 70% ethanol at 4 °C for 30 min and soaked in 2N HCl containing 0.5% Triton X-100 at room temperature for 30 min. After washing with PBS containing 1% bovine serum albumin, the cells were incubated with fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (1:100 dilution, Biomeda Co., CA) in PBS and 1% bovine serum albumin at room temperature for 30 min. After washing, the cells were incubated with 5 μg/ml RNase at 37 °C for 20 min and then were suspended in PBS and 1% bovine serum albumin containing 25 μg/ml propidium iodide and analyzed using an Epics XL flow cytometer (Coulter). BrdUrd-positive cells were counted, and the means ± S.D. of percentages of BrdUrd-positive cells from three experiments were determined.

MTT Assay—Spleen cells were prepared from dissected spleen, and erythrocytes were removed by Ack lysis buffer (Bio Whittaker, Walkersville, MD). T cells and B cells are purified from total splenocytes using AutoMACS (Miltenyi Biotech) with anti-CD4 and anti-CD8α (for T cells) or anti-B220 (for B cells) antibodies and streptavidin-coated beads. Purities of the cells were greater than 85%. Macrophages were collected as adherent peritoneal cells. Purified T or B cells (1×10^5/well) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, nonessential amino acid, and 2-mercaptoethanol for 3 days with various stimuli, and cell proliferation was determined by MTT assay using a CellTiter 96 proliferation assay kit (Promega). T cells were incubated with 2 μg/ml anti-CD3 and 2 μg/ml anti-CD28 antibodies (Pharmingen) or 2.5 μg/ml concanavalin A (Sigma) for 3 days, and B cells were incubated with 10 μg/ml anti-IgM antibody (Jackson ImmunoResearch), 1 μg/ml LPS, or 1 μg/ml anti-CD40 antibody (Pharmingen) for 3 days.

Measurement of Cytokine, Chemokine, and Nitric Oxide Production—To determine IL-6 and IFN-γ expression, spleen cells (2×10^6/ml) were cultured for 48 h in medium containing 1 μg/ml LPS (Sigma) and 100 units/ml IFN-γ (PeproTech Inc., Rocky Hill, NJ), or 2 μg/ml anti-CD3 and 2 μg/ml anti-CD28 antibodies (Pharmingen), respectively. Determinations of IL-6 and IFN-γ levels in culture media were performed in triplicate using IL-6 and IFN-γ ELISA kits (BioSource International, Inc., Camarillo, CA) according to the manufacturer's instructions. To estimate chemokine levels in culture media, spleen cells (2×10^6/ml) were cultured for 24 h in medium containing 1 μg/ml LPS and 100 units/ml IFN-γ. Levels of CCL2 and CCL5 were assayed using mouse cytokine array I (Ray Biotech, Inc., Norcross, GA) according to the manufacturer's instructions. Macrophages were stimulated with 1 μg/ml LPS and 100 units/ml IFN-γ for 48 h and the nitric oxide produced in the culture medium was measured by the Griess method (15). Briefly, the medium was incubated with an equal amount of Griess reagent (1% sulfanilamide in H_3PO_4 and 0.1% *N*-1-naphthylethylenediamine dihydrochloride) for 10 min, and then the absorbance at 550 nm was measured.

Western Blot Analysis—To examine phosphorylation of STAT1 and STAT3, spleen cells were stimulated with 100 units/ml IFN-γ, 10 ng/ml, or 1 μg/ml LPS for 15 min, and whole cell extracts were subjected to Western blot analysis as described previously (16) using anti-STAT1, anti-pSTAT1, anti-STAT3, anti-pSTAT3 (BD Biosciences), or anti-actin antibody.

Reverse Transcription-PCR—Total RNA was isolated from spleen cells after incubation with 1 μg/ml LPS and 100 units/ml IFN-γ for 24 h. cDNAs were synthesized from 1 μg of total RNA using avian myeloblastosis virus reverse transcriptase (Invitrogen) and random hexamer primers as described previously (17). PCR was performed as described using mouse gene-specific primers (18, 19). The amplified DNA fragments were stained with ethidium bromide and photographed using Epi-Light UV FA1100 (Aisin Cosmos R&D Co., Japan). Expression of S16 ribosomal protein was examined as a control (17).

Gel Shift Assay—Spleen cells isolated from mice at 6 days after the immunization with SRBC were frozen at −80 °C until use. Whole cell extracts were prepared in buffer C (16). Aliquots containing 10 μg of proteins were subjected to gel shift assay using an ideal HSE-oligonucleotide or an HSE2-oligonucleotide corresponding to the sequence of mouse *IL-6* gene (−684 to −659) (20). A binding reaction was performed in the presence or absence of antiserum specific for each HSF (α-HSF1γ, α-HSF2δ, and α-HSF4b) (2 μl of 1:10 diluted antiserum with PBS) (21). To determine the specificity of the HSF1 binding activity to HSE2, 10 μg of extract from HeLa cells overexpressing human HSF1 was used. Binding reactions were performed using ^{32}P-labeled HSE2 in the presence or absence of increasing amounts (10- or 100-fold molar excess) of a nonlabeled HSE2, an ideal HSE, or a mutated HSE2-oligonucleotide. The sequences of oligonucleotides are shown in Fig. 5*B*.

Chromatin Immunoprecipitation (ChIP)—Spleen cells (1×10^6 cells) were treated with 10 ml of 1% formaldehyde and RPMI containing 10% fetal calf serum at 37 °C for 10 min. After washing with PBS twice, ChIP was performed using a ChIP assay kit (Upstate Biotechnology) essentially according to the manufacturer's instructions. Primers used to amplify ChIP-enriched DNA are: mIL-6F, 5′-GCA ACT CTC ACA GAG ACT AAA GG-3′; mIL-6R, 5′-GGA CAA CAG ACA GTA ATG TTG C-3′; mHsp70F, 5′-CAA CAG TGT CAC TAG TAG CAC C-3′; mHsp70R, 5′-CTC TGG ATG GAA CCA GAT TTG G-3′.

Statistical Analysis—Unless otherwise indicated, results are expressed as the means ± S.D. of data obtained from triplicate experiments. Significant values were determined by analyzing data with the Mann-Whitney *U* test using StatView version 4.5J for Macintosh (Abacus Concepts, Berkley, CA). Differences at $p < 0.05$ were considered statistically significant.

RESULTS

Constitutive Expression of Many Genes Is Reduced in HSF1-null MEFs—To search for genes regulated by HSF1, we studied the profiles of gene expression in primary cultures of MEFs using a mouse microarray containing 12,422 probe sets. Total RNAs were isolated from control and heat-shocked (42 °C for 1 h) MEFs and from control and heat-shocked HSF1-null MEFs. We performed cluster analysis of genes whose expression changes more than 3-fold compared with those control wild-type or HSF1-null cells (Fig. 1*A*). We divided the genes into four classes. Expression of class a genes (27 genes) decreased after heat shock in both wild-type and HSF1-null MEFs. Expression levels of class b genes (49 genes) were lower in HSF1-null cells than those in wild-type cells but were constant after heat shock. Expression of class c genes (7 genes) increased after heat shock in wild-type cells but did not increase in HSF1-null cells. Expression of class d genes (13 genes) increased after heat shock in both wild-type and HSF1-null cells. Classical heat shock genes such as Hsp70-1 and Hsp70-3 belong to the class c. Class d genes are induced in response to heat shock independently of HSF1. The existence of genes belonging to class d was reported previously (22). It was

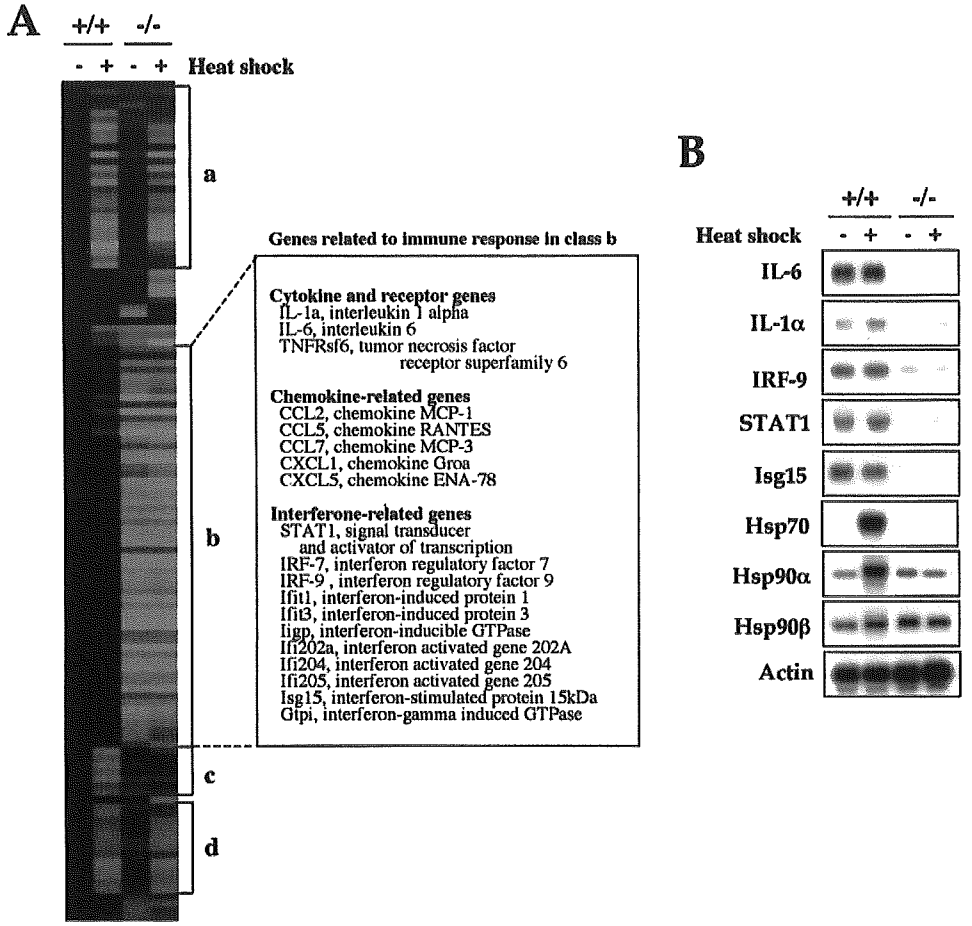

FIG. 1. **Changes in mRNA levels in wild-type and HSF1-null MEF cells.** *A*, total RNAs were isolated from wild-type (+/+) and HSF-null (−/−) MEF cells maintained at 37 °C (−) or heat-shocked at 42 °C for 1 h (+). Genes undergoing more than 3-fold change are cluster analyzed based on microarray analysis. We divided the genes into four classes. Class a contains 27 genes whose expression decreased after heat shock in both wild-type and HSF1-null MEFs. Class b contains 49 genes whose expression was lower in HSF1-null cells than that in wild-type cells. Expression of class b genes is constant after heat shock. Class c contains 7 genes whose expression increased after heat shock in wild-type cells but did not increase in HSF1-null cells. Class d contains 13 genes whose expression increased in both wild-type and HSF1-null cells. The names of 19 genes related to immune response in class b are shown. *B*, mRNA levels of genes related to the immune response and major heat shock genes were examined by Northern blot analysis using each specific probe.

unexpected that constitutive expression of many genes (class b genes) was reduced by lacking HSF1. We found that many genes in this class (at least 19 genes) are related to the immune response and inflammation. The genes include inflammatory cytokine genes (*IL-1α* and *IL-6*), chemokine-related genes (*CCL2*, *CCL5*, *CCL7*, *CXCL1*, and *CXCL5*), and interferon-related genes (*STAT1*, *IRF-7*, *IRF-9*, and interferon-regulated factors) as shown in Fig. 1*A*. We confirmed decreased mRNA levels of IL-6, IL-1α, IRF-9, STAT1, and Isg15 in HSF1-null MEF cells by Northern blot analysis (Fig. 1*B*). These genes were not induced in response to heat shock at all, unlike Hsp70 and Hsp90 genes.

Impaired Serum IgG Induction in HSF1-null Mice—In addition to the reduced expression of cytokine and chemokine genes in HSF1-null MEFs, we found that the serum globulin level was lower in HSF1-null mice (1.43 ± 0.26 g/dl) compared with that in wild-type mice (1.97 ± 0.21 g/dl) without any treatment. Therefore, we expected that the immune response might be abnormal in HSF1-null mice. To examine whether HSF1 play roles in regulating the humoral immune response, we analyzed the T cell-dependent B cell response by immunizing mice intraperitoneally with SRBC. Serum SRBC-specific IgG increased more than 100-fold in wild-type mice, whereas that in HSF1-null mice was less than 50-fold at 6 days after the immunization (Fig. 2*A*), indicating that HSF1-null mice showed a significant decrease in an antigen-specific IgG response. However, the SRBC-specific IgG level in HSF1-null mice reached the same level in wild-type mice at 14 days after the immunization and was similar after the secondary immunization. In contrast to impaired IgG induction in HSF1-null mice, there was no detectable difference in the IgM response. We next compared serum levels of total immunoglobulin isotypes (Fig. 2*B*). Without the immunization, the serum level of total IgG2a was significantly lower in HSF1-null mice than that in wild-type mice, whereas levels of other isotypes were normal. At 6 days after the immunization with SRBC, serum levels of IgG2a and IgG1 were much lower in HSF1-null mice. Levels of light chain κ and λ were the same. Remarkably, the level of SRBC-specific IgG2a in HSF1-null mice was much lower at an early time point after the immunization compared with that in wild-type mice and did not reach normal levels even at 14 days (Fig. 2*C*). Furthermore, SRBC-specific IgG2a did not increase in response to the second immunization. These results indicate that induction of serum IgG2a and IgG1 in response to SRBC is impaired in HSF1-null mice.

Proliferation of Spleen Cells Is Poorly Promoted in HSF1-null Mice in Response to SRBC Immunization—In response to the immunization with SRBC, B cells proliferate in germinal centers in the spleen and produce immunoglobulins (23). Therefore, we next analyzed B cell proliferation in the spleen at 6 days after the immunization. The number and size of the germinal center in the immunized HSF1-null mice were re-

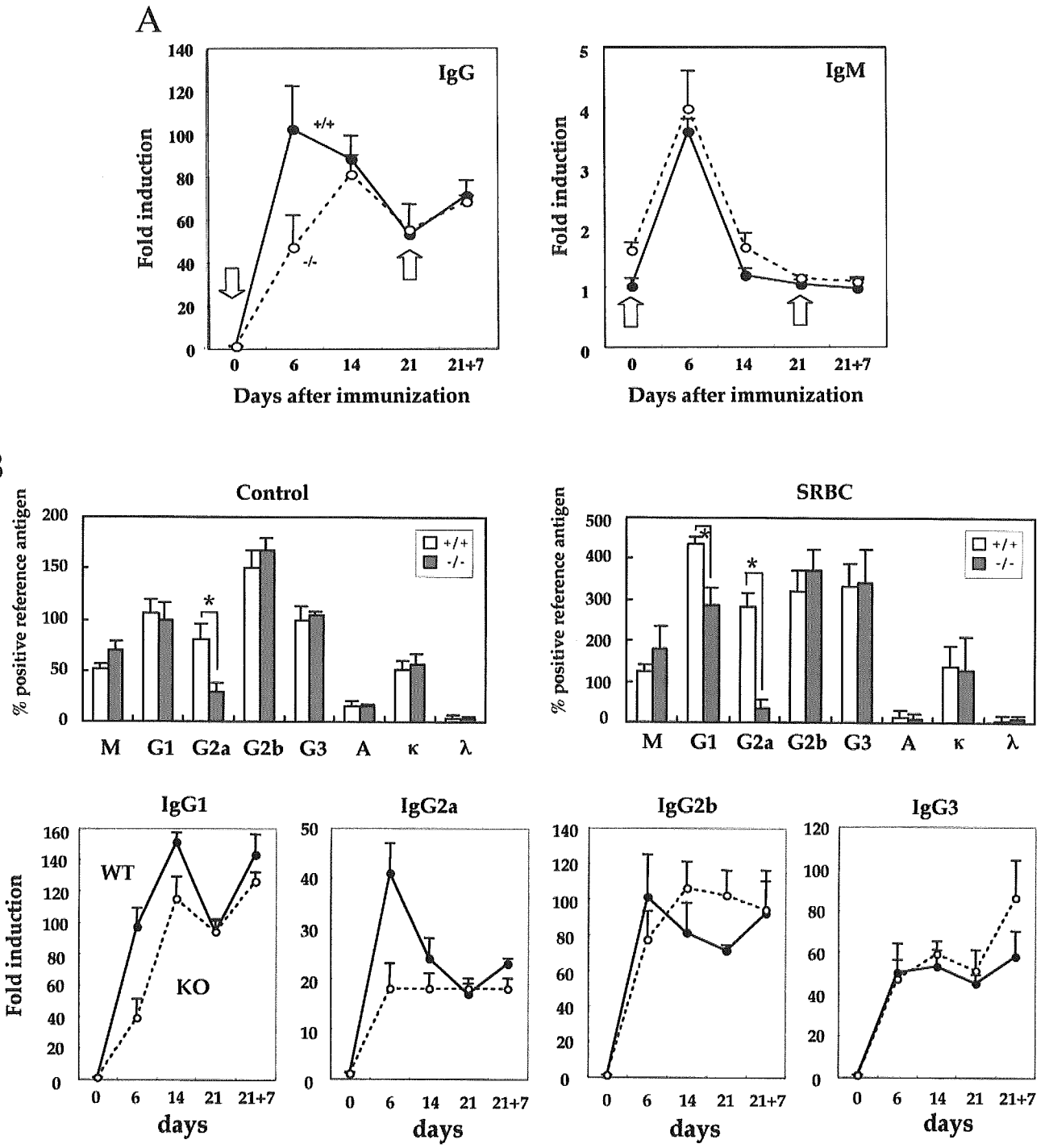

FIG. 2. **Serum immunoglobulin levels in response to SRBC immunization in wild-type and HSF1-null mice.** *A*, 5 wild-type (*closed circles*) and five HSF1-null mice (*open circles*) were immunized at day 0 and boosted at day 21 (*open arrows*) and were bled at the indicated time points. Levels of SRBC-specific IgG and IgM were measured by ELISA, and -fold inductions are shown. *B*, levels of immunoglobulin isotypes before and after SRBC immunization. Wild-type (*open bars*) and HSF1-null (*gray bars*) mice before immunization (*Control*) and at 6 days after immunization with SRBC (*SRBC*) were bled, and levels of immunoglobulin were determined by ELISA using a positive reference antigen as a standard (see "Experimental Procedures"). *C*, changes of levels of SRBC-specific IgG isotypes. All plots are the means ± S.D. from three mice. *Stars* in *B* indicate $p < 0.05$.

duced significantly compared with those in wild-type mice (data not shown). To quantify proliferating cells in the spleen, BrdUrd was injected, and BrdUrd-incorporated cells were examined by immunostaining or flow cytometric analysis. BrdUrd-positive cells were rich in the germinal center of wild-type mice, whereas these cells were poor in HSF1-null mice (Fig. 3*A*, *a–d*). The percentage of BrdUrd-positive cells in HSF1-null spleen (0.9 ± 0.5%) was reduced significantly compared with that in wild-type spleen (2.9 ± 0.8%). These results indicate that proliferation of spleen cells is promoted poorly in HSF1-null mice in response to the immunization with SRBC.

We next examined *in vitro* proliferation of splenic B and T cells after various stimuli. B cells were incubated in the presence of anti-IgM antibody, LPS, or anti-CD40 antibody for 3 days, and MTT assays were carried out. There was no difference in proliferation of B cells isolated from wild-type and HSF1-null mice (Fig. 3*B*). Similarly, analysis of proliferation of T cells in the presence of anti-CD3 and anti-CD28 antibodies or

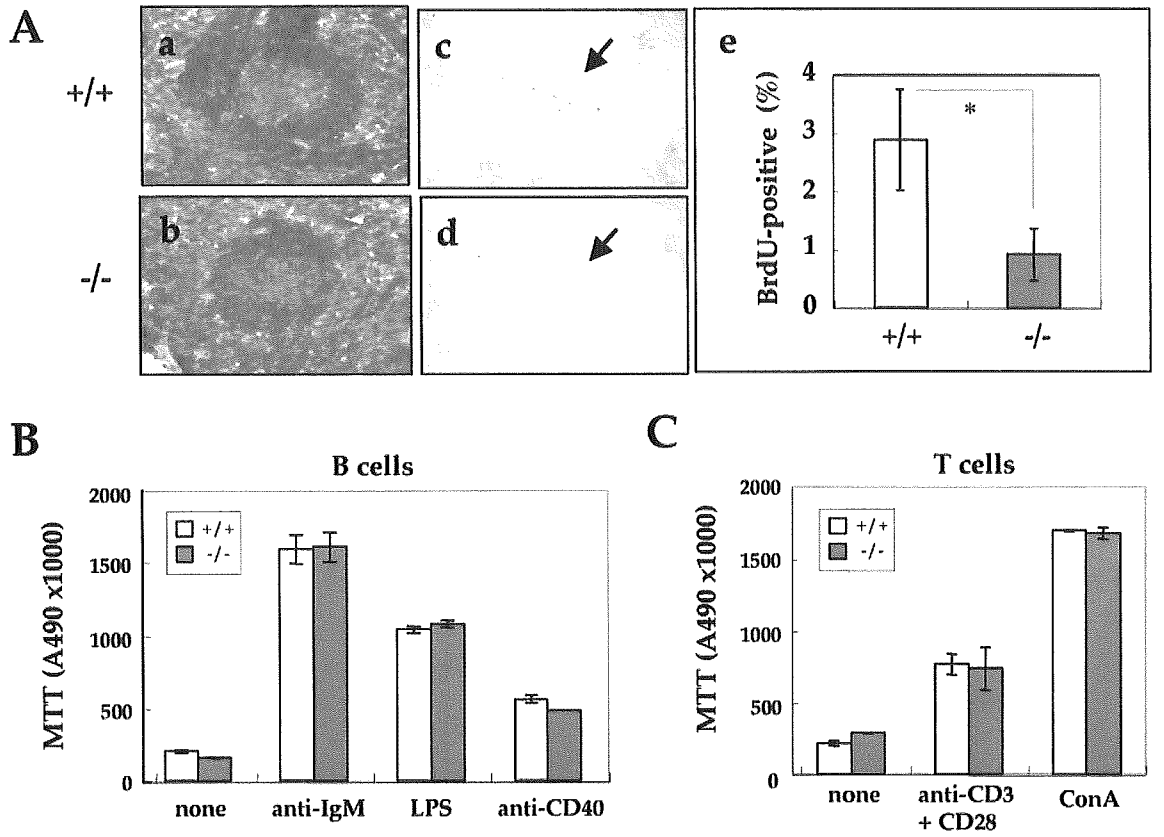

FIG. 3. **Proliferation of spleen cells *in vivo* and *in vitro*.** *A*, after BrdUrd injection, the spleen was dissected, and cryosections were stained with hematoxylin (*a* and *b*). Serial sections were stained with an anti-BrdUrd antibody (*c* and *d*). Clusters of BrdUrd-positive cells are indicated by *arrows*. Percentages of BrdUrd-incorporated cells in total spleen cells were determined by flow cytometry. An *asterisk* indicates $p < 0.05$. Magnification, ×100. *B*, *in vitro* proliferation of splenic B cell isolated from wild-type (*open bars*) and HSF1-null (*closed bars*) mice. B cells were incubated with anti-IgM antibody, LPS, or anti-CD40 antibody for 3 days, and an MTT assay was carried out. *C*, *in vitro* proliferation of splenic T cell isolated from will-type and HSF1-null mice. T cells were incubated with anti-CD3 and anti-CD28 antibodies or concanavalin A for 3 days, and an MTT assay was carried out. The means ± S.D. from three independent experiments are shown.

concanavalin A showed no difference between wild-type and HSF1-null cells (Fig. 3*C*). These results indicate that proliferation of B and T cells isolated from HSF1-null mice is promoted properly in response to extracellular growth signals.

Decreased Expression of IL-6 and CCL5 in Spleen Cells of HSF1-null Mice—Because proliferation and differentiation of B cells are regulated by cytokines produced by spleen cells such as B and T cells and macrophages (23, 24), we examined cytokine gene expression in spleen cells. Spleen cells were stimulated with LPS and IFN-γ for 24 h, and the expression of genes related to immunoglobulin production and class b genes in MEF microarray (Fig. 1*A*) were examined by semiquantitative reverse transcription-PCR (Fig. 4, *A* and *B*, and data not shown). We found that mRNA levels of IL-6 and CCL5/RANTES were significantly lower in HSF1-null spleen cells, whereas the expression of other genes was similar in wild-type and HSF1-null spleen cells. We examined further the levels of cytokines in culture medium. IL-6 accumulation increased by the treatment of LPS and IFN-γ, but the level of IL-6 was 40% lower in HSF1-null spleen cells compared with wild-type cells (Fig. 4*C*). The level of CCL5 in HSF1-null mice was also 45% lower than that in wild-type mice, whereas the levels of CCL2 were similar (Fig. 4*D*). Other cytokines including IL-2, IL-3, IL-4, IL-5, IL-9, IL-13, IL-17, monocyte chemotactic protein-5, and tumor necrosis factor-α were not detected (data not shown). IFN-γ, which is important for B cell maturation (25, 26), was induced in spleen cells stimulated by anti-CD3 and anti-CD28 antibodies, and the induced levels were same in both wild-type and HSF1-null spleen cells (Fig. 4*E*). These results indicate that expression of IL-6 and CCL5 genes was specifically repressed in HSF1-null spleen cells after stimulation.

IL-6 and IFN-γ activate STAT1 and STAT3 (27), and STAT1 expression was reduced in HSF1-null MEF cells (Fig. 1*A*). Therefore, we examined whether the Janus kinase-STAT signaling pathway is intact in HSF1-null spleen cells. It was revealed that expression levels of STAT1 and STAT3 and levels of phosphorylated forms were similar in wild-type and HSF1-null spleen cells in response to IFN-γ, IL-6, or LPS (Fig. 4*F*). These results exclude a possibility that impaired expression of IgG in HSF1-null cells may be caused by the lack of STAT1 and STAT3 phosphorylation.

SRBC Immunization Activates HSF1, Which Binds Directly to the IL-6 Gene—We next examined activation of HSF1 in response to immunization with SRBC. Whole cell extracts were prepared from the spleen cells before and 6 days after the immunization, and a gel shift assay was performed using an ideal HSE-oligonucleotide as a probe. We found that HSE binding activity was induced in wild-type spleen cells, whereas the activity was not induced in HSF1-null cells (Fig. 5*A*). The mobility of HSE binding activity was retarded in the presence of antiserum against HSF1, indicating that HSF1 is activated in response to the immunization with SRBC.

To determine whether HSF1 binds directly to the *IL-6* gene, we searched HSE consensus sequences on the *IL-6* gene. We detected three HSE consensus sequences (HSE1, HSE2, and HSE3) within −1,000 bp from a transcription start site of the mouse *IL-6* gene (20). Among them, an HSE2 sequence (−684

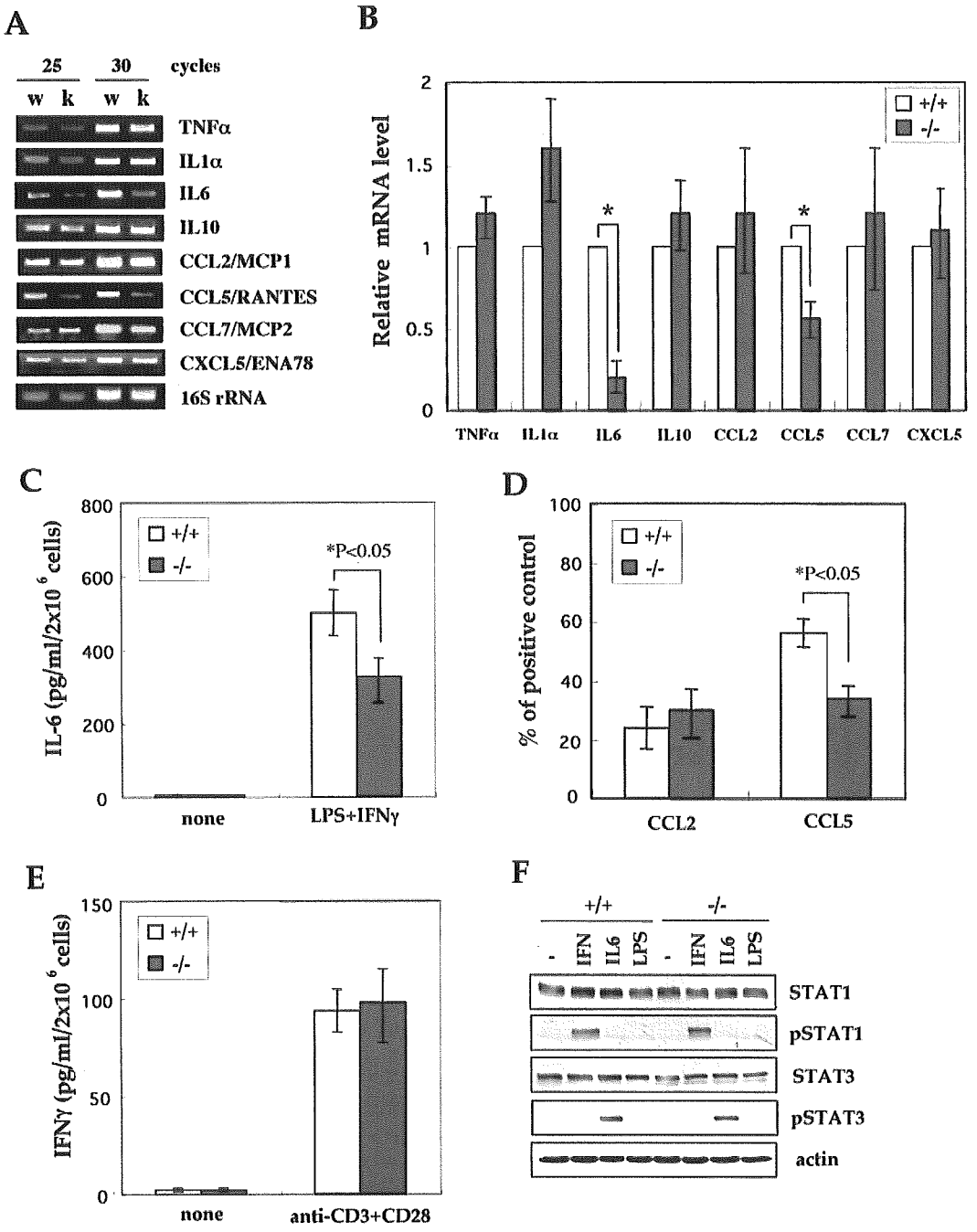

FIG. 4. **Production of cytokines and chemokines from spleen cells.** *A*, total spleen cells were incubated with LPS and IFN-γ for 24 h. Reverse transcription-PCR analysis was performed using total RNA isolated from the spleen in wild-type (*w*) or HSF1-null (*k*) mice. Representative data are shown. *B*, quantification of expression levels of cytokines and chemokines examined in *A*. The means ± S.D. of three experiments are shown. *C*, cells were isolated from spleen in wild-type (*open bars*) and HSF1-null (*closed bars*) mice and were incubated for 24 h in the presence (*LPS+IFNγ*) or absence (*none*) of LPS and IFN-γ. Levels of IL-6 in culture medium were determined by ELISA. *D*, cells were isolated from spleen and were incubated for 24 h in the presence of LPS and IFN-γ. Levels of CCL2 and CCL5 in culture medium were estimated using the cytokine array. *E*, spleen cells were incubated for 24 h in the presence (*anti-CD3+CD28*) or absence (*none*) of anti-CD3 and anti-CD28 antibodies. IFN-γ levels in culture medium were determined by ELISA. The means ± S.D. from three experiments are shown. *Asterisks* indicate $p < 0.05$. *F*, spleen cells isolated from wild-type (+/+) and HSF1-null (−/−) mice were incubated with IFN-γ, IL-6, or LPS for 15 min. Whole cell extracts were prepared, and Western blot analyses were performed using each specific antibody.

to −659) is highly conserved in human *IL-6* gene, and HSF1 can bind specifically to the HSE2 oligonucleotide (Fig. 5*B*). Furthermore, ChIP analysis revealed that HSF1 binds to the upstream region (−827 to −565) containing the HSE2 sequence *in vivo* in response to the immunization (Fig. 5*C*). The location of the HSF1 binding site is far from a transcription start site compared with locations of binding sites of major regulatory factors NF-κB, NF-IL-6, and serum response factor, which are within −60 to −180 bp (20). HSF1 also bound the upstream region of *Hsp70* gene and enhanced Hsp70 expression (Fig. 5*D*). These results indicate that the immunization with SRBC activates HSF1, which binds to the upstream region of *IL-6* gene *in vivo*.

We also found an HSE consensus sequence at position −529 to −512 within −1,000 bp from the transcription start site of the *CCL5* gene (28). However, ChIP analysis showed no binding of HSF1 to the *CCL5* gene (data not shown). Because CCL5 expression is induced by many cytokines such as tumor necro-

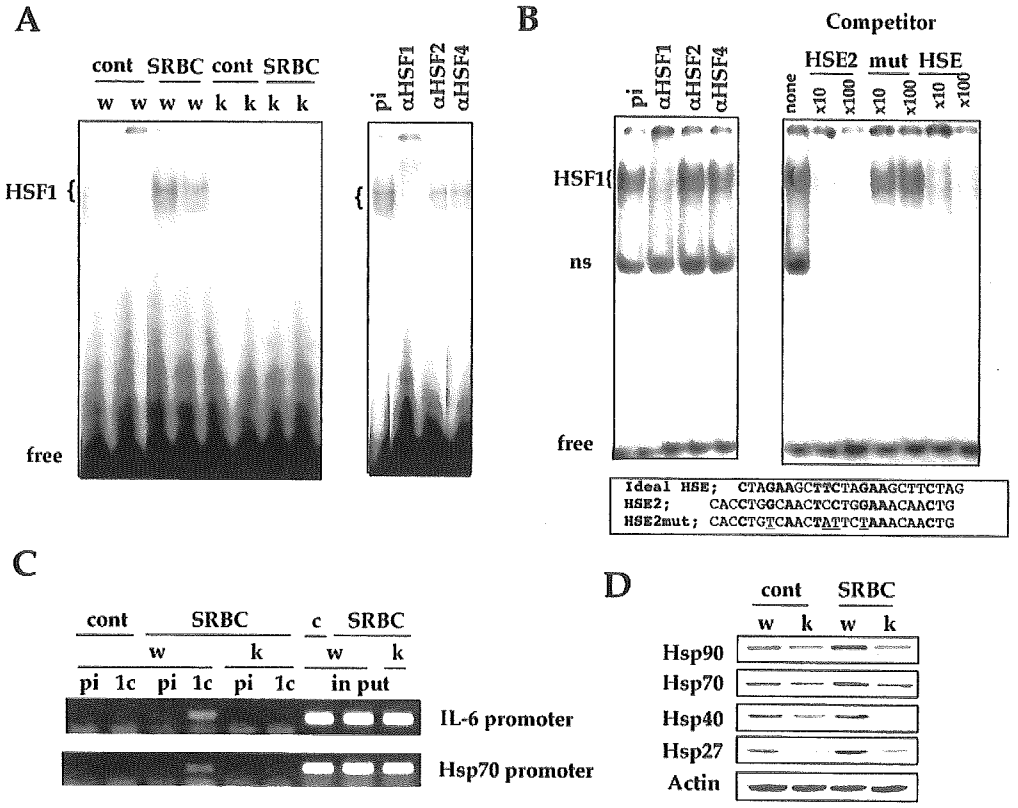

FIG. 5. **HSF1 binds to the *IL-6* gene as well as the *Hsp70* gene in response to the SRBC immunization.** *A*, whole cell extracts were prepared from the untreated spleen (*cont*) in wild-type (*w*) and HSF-null (*k*) mice and the spleen at 6 days after the immunization with SRBC (*SRBC*). A gel shift assay was performed using a ^{32}P-labeled ideal HSE oligonucleotide in the absence (*left panel*) or presence of preimmune serum (*pi*) or each specific antibody (*right panel*). *B*, whole cell extract was prepared from HeLa cells overexpressing hHSF1, and a gel shift assay was performed using a ^{32}P-labeled HSE2 (*HSE2*) oligonucleotide in the presence of preimmune serum or each specific antibody. Specificity of the binding was examined by adding to the binding reaction unlabeled HSE2, mutated (*mut*) HSE2, or ideal HSE oligonucleotides. *free*, free oligonucleotide probe. *ns*, nonspecific binding. Sequences of oligonucleotide probes are shown on the *bottom*. *C*, wild-type and HSF1-null spleen cells were prepared from control mice and mice at 6 days after the immunization with SRBC. Chromatin immunoprecipitation-enriched DNAs using preimmune serum or anti-HSF1 serum (anti-HSF1c; *1c*) as well as input DNAs were prepared, and DNA fragments of the *IL-6* gene (−827 to −565) and *Hsp70* gene (-272 to +47) were amplified by PCR. *D*, Western blot analysis was performed using extracts from the spleen isolated before and 6 days after the immunization with SRBC.

sis factor-α, IL-1, and IFN-γ (29, 30), reduction of CCL5 expression may be an indirect effect.

Reduced Expression of IL-6 and CCL5 in Peritoneal Macrophages in HSF1-null Mice—We further examined cytokine and chemokine expression in peritoneal macrophages. LPS is a potent stimulator of macrophages and induces production of various cytokines, nitric oxide, and superoxide. Macrophages isolated from HSF1-null mice produced much less IL-6 and CCL5 than macrophages isolated from wild-type mice did (Fig. 6, *A* and *B*). Nitric oxide production from HSF1-null macrophages was similar to that from wild-type cells (Fig. 6*C*). These results clearly indicate that production of IL-6 and CCL5 reduces in HSF1-null macrophages. Interestingly, stimulated macrophages isolated from wild-type mice adhered to culture plates, whereas those from HSF1-null mice did not, suggesting dysfunction of macrophages in HSF1-null mice (Fig. 6*D*).

DISCUSSION

In this study, we demonstrated that IgG2a and IgG1 production is impaired in HSF1-null mice in response to the immunization with SRBC. Especially the serum level of IgG2a in HSF1-null mice is significantly lower than its peak level in wild-type mice at any time point after the immunization. Furthermore, proliferation of spleen cells, probably plasma blasts, is inhibited in HSF1-null spleen in response to the immunization. The heat shock response is a fundamental defense mechanism against various stresses such as heat, ultraviolet light, and oxidation. HSF1 regulates this response by inducing Hsps. In addition to the classical role of HSF1, recent reports show that HSF1 is required for developmental processes such as oogenesis, spermatogenesis, and brain formation with unknown mechanisms (5–7). This study extends our understanding of HSF1 function. HSF1 plays a role in activating the acquired immune system in addition to the crucial role in heat shock response.

We further provide possible mechanisms of impaired IgG2a and IgG1 production. Expression of many cytokine and chemokine genes related to the immune response is induced in many cells including lymphocytes, monocytes, and epithelial cells. We found that expression levels of IL-6 and a chemokine CCL5 are markedly lower in stimulated HSF1-null spleen cells than those in wild-type mice. IL-6 is a multifunctional cytokine that regulates the immune response and inflammation (24, 31). During an antibody response dependent on T cell help, IL-6 is secreted by a germinal center cells and promotes expansion of plasma blasts. In IL-6-null mice, control serum IgG level is normal, but antigen-specific IgG response is reduced (31). Like HSF1-null mice, the IgM response is normal in IL-6-null mice. Furthermore, overexpression of IL-6 induces plasmacytosis, which is associated with significant increase of serum IgG1 (32). CCL5/RANTES is a CC chemokine that induces lymphocyte migration and activates the immune response (33). Especially, CCL5 promotes antigen-specific IgG2a and IgG3 produc-

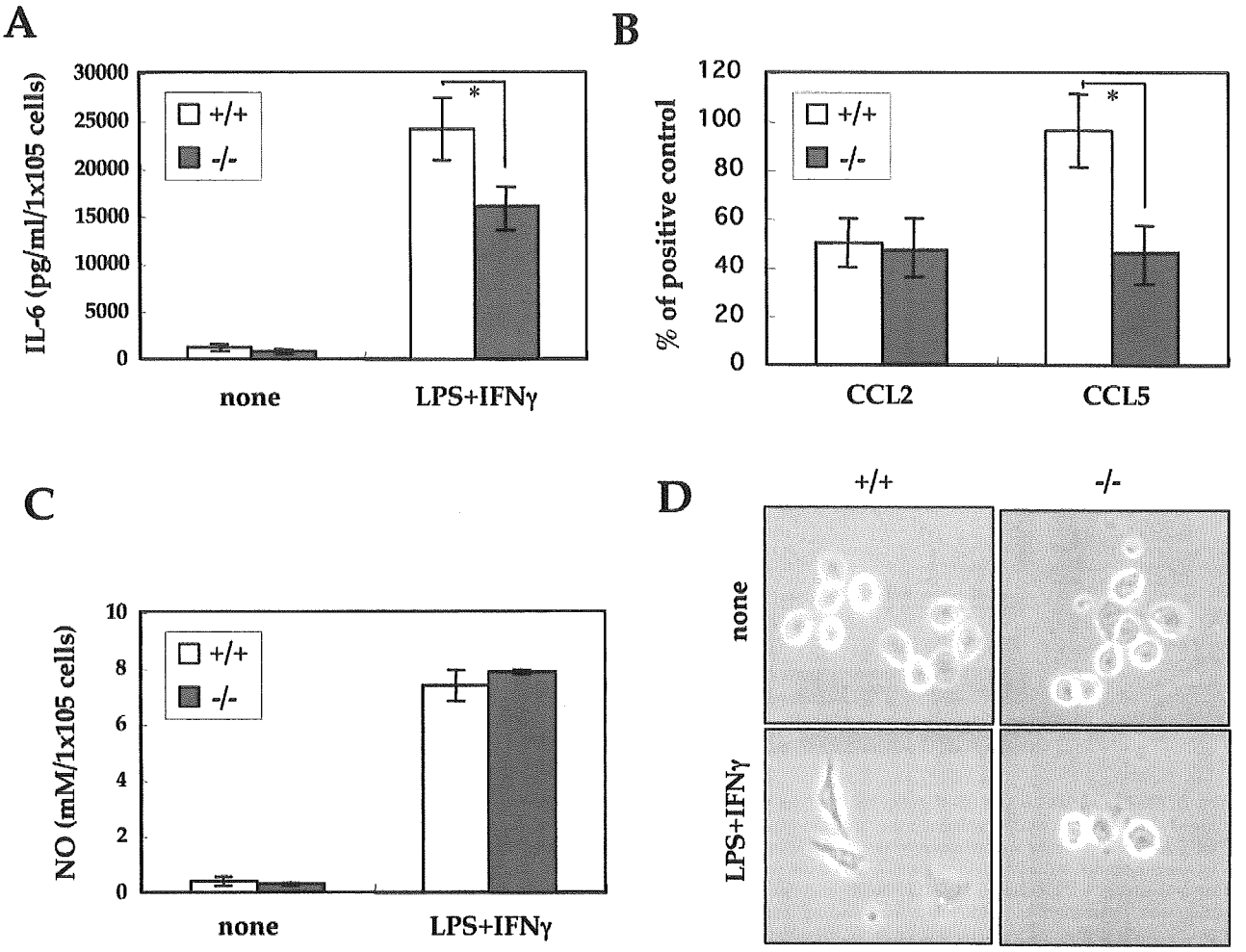

FIG. 6. **Cytokine and chemokine production in peritoneal macrophages.** *A*, IL-6 production in culture medium of macrophages was determined by ELISA after incubation of LPS and IFN-γ for 48 h. *B*, chemokine levels in culture medium of macrophages were determined using the cytokine array. *C*, nitric oxide production in culture medium was determined. The means ± S.D. of three experiments are shown. *D*, peritoneal macrophages were isolated from wild-type (+/+) and HSF-null (−/−) mice and were incubated in the presence (*LPS+IFNγ*) or absence (*none*) of LPS and IFN-γ for 48 h. Morphology was observed using an Axiovert 200 microscope.

tion. These observations suggest that the impaired production of IgG2a and IgG1 may be partly the result of reduced expression of IL-6 and CCL5.

We showed here that HSF1 in the spleen cells is activated in response to immunization with SRBC and induces expression of Hsps in a germinal center where plasma blasts are expanding (Fig. 5 and data not shown). Activation of HSF1 was detected in isolated B cells in the spleen (data not shown). HSF1 activation may be triggered by stimulation of cell growth because Hsp70 and Hsp90 expression is induced when cell growth is stimulated in human resting T cells by the treatment of mitogen (34). In addition to the HSF1 binding to *Hsp70* gene, we showed that HSF1 binds directly to the *IL-6* gene and is required for full induction of IL-6 expression. Interestingly, IL-6 induces expression of Hsp70 and Hsp90 and also activates HSF1 in some cells (35–37). Therefore, activation of HSF1 and induction of IL-6 may mutually affect inflammatory conditions positively.

Acknowledgments—We are grateful to Drs. T. Fujita, M. Sugai, N. Tokuda, and M. Kawano for reagents and discussion.

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bjh research paper

Molecular mimicry by *Helicobacter pylori* CagA protein may be involved in the pathogenesis of *H. pylori*-associated chronic idiopathic thrombocytopenic purpura

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 British Journal of Haematology, **124,** 91–96

Received 4 August 2003; accepted for publication 22 September 2003

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Summary

The eradication of *Helicobacter pylori* often leads to platelet recovery in patients with chronic idiopathic thrombocytopenic purpura (cITP). Although this clinical observation suggests the involvement of *H. pylori*, little is known about the pathogenesis of cITP. We initially examined the effect of *H. pylori* eradication on platelet counts in 20 adult Japanese cITP patients. Then, using platelet eluates as the probe in immunoblot analyses, we examined the role of molecular mimicry in the pathogenesis of cITP. *Helicobacter pylori* infection was detected in 75% (15 of 20) of cITP patients. Eradication was achieved in 13 (87%) of the *H. pylori*-positive patients, seven (54%) of which showed increased platelet counts within the 4 months following treatment. Completely responsive patients also showed significant declines in platelet-associated immunoglobulin G (PAIgG) levels. Platelet eluates from 12 (nine *H. pylori*-positive and three *H. pylori*-negative) patients recognized *H. pylori* cytotoxin-associated gene A (CagA) protein, and in three completely responsive patients, levels of anti-CagA antibody in platelet eluates declined after eradication therapy. Cross-reactivity between PAIgG and *H. pylori* CagA protein suggests that molecular mimicry by CagA plays a key role in the pathogenesis of a subset of cITP patients.

Keywords: chronic idiopathic thrombocytopenic purpura, *Helicobacter pylori*, molecular mimicry, cytotoxin-associated gene A protein.

Idiopathic thrombocytopenic purpura (ITP) is an acquired autoimmune disorder characterized by a low platelet count and mucocutaneous bleeding. The cause is destruction of platelets in the reticuloendothelial system mediated by platelet-bound autoantibodies (Cines & Blanchette, 2002). The targets of the anti-platelet autoantibodies include a variety of platelet proteins, among them glycoproteins IIb/IIIa, Ib/IX, Ia/IIa and IV. Several groups have recently reported that eradication of *Helicobacter pylori* leads to platelet recovery in patients with chronic ITP (cITP) (Gasbarrini *et al*, 1998; Grimaz *et al*, 1999; Tohda & Ohkusa, 2000; Emilia *et al*, 2001; Kohda *et al*, 2002; Veneri *et al*, 2002). Although these clinical observations suggest the involvement of *H. pylori*, little is known about the mechanisms responsible for triggering production of the anti-platelet autoantibodies involved in the pathogenesis of cITP.

The gram-negative bacterium *H. pylori* is the human pathogen responsible for chronic gastritis and peptic ulcers; moreover, infection with this organism also increases the risk of gastric cancer and mucosa-associated lymphoid tissue lymphoma (Suerbaum & Michetti, 2002). During the gastritis,

H. pylori infection induces production of anti-gastric epithelial autoantibodies through a process of molecular mimicry involving the gastric epithelium and one or more *H. pylori* antigens (Negrini *et al*, 1996). Notably, *H. pylori* has also been implicated in the pathogenesis of some autoimmune diseases, such as rheumatoid arthritis, autoimmune thyroiditis and Sjögren's syndrome (Gasbarrini & Franceschi, 1999). In the present study, we therefore examined the effect of *H. pylori* eradication in a group of Japanese cITP patients with the aim of understanding better the role an autoimmune response mediated by molecular mimicry in the pathogenesis of cITP.

Patients and methods

Patients

Twenty adult cITP Japanese patients (five men and 15 women; mean age, 53 years) with cITP were enrolled prospectively. ITP was defined by thrombocytopenia (platelet count $<120 \times 10^9$/l) without megakaryocytic hypoplasia in the bone marrow, and by exclusion of other causes. Patients with secondary autoimmune thrombocytopenia were excluded. Platelet-associated immunoglobulin G (PAIgG) levels were determined using an enzyme-linked immunosorbent assay (normal range: 9·0–25·0 ng/10^7 platelets).

^{13}C-urea breath tests (Otsuka, Tokyo, Japan) were used to diagnose *H. pylori* infection. Eradication of *H. pylori* was assessed 8 weeks after treatment using the same test. The clinical responses to *H. pylori* eradication were evaluated 4 months after treatment: a complete response was defined as an increase in the platelet count to more than 120×10^9/l; a partial response was defined as an increase $>20 \times 10^9$/l above pretreatment platelet counts.

The regimen for *H. pylori* eradication, which entailed administration of clarithromycin (400 mg twice daily), amoxicillin (1500 mg twice daily) and lansoprazole (60 mg twice daily) for 7 d, was administered to both *H. pylori*-positive and -negative patients. During the study period, no other new therapies for ITP were added, although patients who were receiving maintenance therapy for ITP continued to do so with no changes.

This study was approved by the Institutional Review Board of Yamaguchi University Hospital; informed consent was obtained from all participants according to the terms of the Declaration of Helsinki.

Platelet eluates

Platelets were harvested from 40 ml of whole blood in ethylenediaminetetraacetic acid (EDTA), washed four times with phosphate-buffered saline (PBS) containing 2% EDTA and 15% acid-citrate-dextrose, and resuspended in PBS containing 0·2% bovine serum albumin (BSA). PAIgG was eluted from 5×10^7 washed platelets using ether according to the method of von dem Borne *et al* (1980), after which the eluates were stored at −20°C until use.

Cell lysates from H. pylori

Helicobacter pylori (NCTC11637) was cultured in Brucella-broth as described previously (Okamoto *et al*, 2002). To obtain cell lysates, the cells were washed twice with 50 mmol/l PBS and suspended in 10 mmol/l PBS, after which they were incubated with lysozyme for 20 min at room temperature, sonicated, and centrifuged at 8000 × g for 15 min at 4°C. The resultant supernatant was collected as the cell lysate.

Immunoblot analysis

Helicobacter pylori cell lysates were subjected to 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) containing 0·1% Tween 20 (TBS-T) and then incubated for 12 h at 4°C with platelet eluates from 5×10^7 washed platelets in 4 ml of TBS containing 3% BSA. After washing the labelled membranes with TBS-T, they were incubated for 1 h with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) polyclonal antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA).

Immunoprecipitation

Samples of cell lysate containing 1 mg of *H. pylori* protein in 500 μl of PBS were incubated for 2 h at 4°C with 2 μg of goat anti-cytotoxin-associated gene A (CagA) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), after which immunoprecipitation was facilitated with protein G-Sepharose (Amersham Biosciences, Piscataway, NJ, USA). The immune complexes were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analysed by immunoblotting with platelet eluates.

Statistical analysis

Differences between *H. pylori*-positive and *H. pylori*-negative patients with respect to age, gender, disease duration and platelet count were analysed using non-parametric Mann–Whitney *U*-tests, as were differences in PAIgG levels in responding and non-responding patients before treatment. PAIgG levels before and after treatment were compared using the Wilcoxon signed-rank sum test. *P*-values <0·05 were considered significant.

Results

Patient characteristics and outcome

The characteristics and outcomes of all 20 cITP patients studied are shown in Table I. Before treatment, both infected and uninfected patients were PAIgG-positive and showed no

Table I. Characteristics and outcomes of patients.

Patient*	Age (years)	Sex	Contraction period (months)	Previous treatment†		Eradication	Platelet count‡			PAIgG (ng/10^7plts)		Response§
				Therapy	Response§		Before	1 month	4 month	Before	4 month	
P1	48	F	72	PSN, S	NR	Yes	36	183	134	87·9	30·2	CR
P2	64	F	126	–	–	Yes	57	114	127	54·7	60·2	CR
P3	70	F	120	PSN, K	NR	Yes	17	137	211	46·7	23·8	CR
P4	67	M	25	–	–	Yes	76	156	196	29·8	26	CR
P5	46	F	12	PSN	PR	Yes	27	229	276	59·3	22·2	CR
P6	17	F	35	PSN	PR	Yes	66	220	198	53·1	20·3	CR
P7	61	M	37	–	–	Yes	33	78	80	137·7	106·9	PR
P8	54	M	108	–	–	Yes	18	16	13	297·9	411·7	NR
P9	44	F	106	PSN, S	NR	Yes	6	6	2	1006·1	853·1	NR
P10	37	F	24	–	–	Yes	103	95	106	64·4	34·8	NR
P11	62	F	156	PSN	NR	Yes	10	22	26	241	103·6	NR
P12	52	F	108	PSN	NR	Yes	45	45	44	218	125·6	NR
P13	61	F	23	PSN	PR	Yes	49	52	29	71·8	209·7	NR
P14	49	F	180	PSN	NR	No	32	30	28	74·6	54·8	NR
P15	63	M	60	–	–	No	23	30	46	70·8	89·9	PR
N1	28	F	27	PSN, Ig, K	NR		12	15	20	520	142·5	NR
N2	26	F	84	PSN	PR		111	117	83	65·9	114·2	NR
N3	55	M	216	–	–		8	10	8	172·1	212·8	NR
N4	64	F	181	PSN, S	NR		23	22	24	45·6	61·4	NR
N5	57	F	252	PSN	PR		42	43	42	250·5	623·7	NR

*P and N indicate positive and negative for *H. pylori* infection respectively.
†PSN, prednisolone; S, splenectomy; Ig, intravenous immunoglobulin; K, kami-kihito herbal medicine.
‡Platelet counts ($\times 10^9$/l) before, 1 month and 4 months after eradication.
§CR, complete response; PR, partial response; NR, no response.

significant differences with respect to age, gender, disease duration or platelet count. The prevalence of *H. pylori* infection was 75% (15 of 20), and bacterial eradication was achieved in 13 (87%) of the *H. pylori*-positive patients. Four months after treatment, 7 (54%) of the patients in whom *H. pylori* had been eradicated showed increased platelet counts. Of those, six responded completely and one showed a partial response. In addition, one patient (P15) in whom eradication was not achieved also showed a partial response. Despite our failure to eradicate *H. pylori* in that patient, the urea breath test levels decreased after treatment. All patients who responded completely showed recovery of platelet counts within 1 month after eradication. By contrast, none of the five *H. pylori*-negative patients showed improvement in their platelet counts, despite receiving the same treatment regimen.

The PAIgG levels also declined significantly in responding patients following *H. pylori* eradication; no significant decline was observed in non-responding patients (Table II). Notably, the pretreatment levels of PAIgG were significantly lower in responding patients than in non-responding ones ($P = 0{\cdot}016$, Table II).

Table II. Comparison of PAIgG levels.

	PAIgG (mean ± SEM, ng/10^7 platelets)		
	Before treatment	After treatment	*P*-value
Responding patients ($n = 6$)	67·0 ± 13·5	41·4 ± 12·1	0·04
Non-responding patients ($n = 14$)	252·3 ± 79·1	245·7 ± 73·8	0·87

H. pylori immunoblot analysis

The presence of antibodies recognizing both platelets and *H. pylori* antigens was evaluated by subjecting platelet eluates from 18 cITP patients to Western blot analysis. Because of severe thrombocytopenia or platelet aggregation, sufficient platelets for analysis could not be obtained from two patients (P9 and P11). Platelet eluates from 12 (nine *H. pylori*-positive and three *H. pylori*-negative) patients recognized one or more *H. pylori* proteins, and all 12 eluates recognized a single 140 kDa *H. pylori* protein (Fig 1A). Contamination of the platelet eluates by serum IgG was excluded, as none of the eluates from three *H. pylori*-positive non-thrombocytopenic volunteers recognized any *H. pylori* proteins (Fig 1B).

The CagA antigen is a highly antigenic *H. pylori* protein. Its molecular weight,140 kDa (Fig 1C, lane 1), suggested that it might be the protein recognized by the platelet eluates from

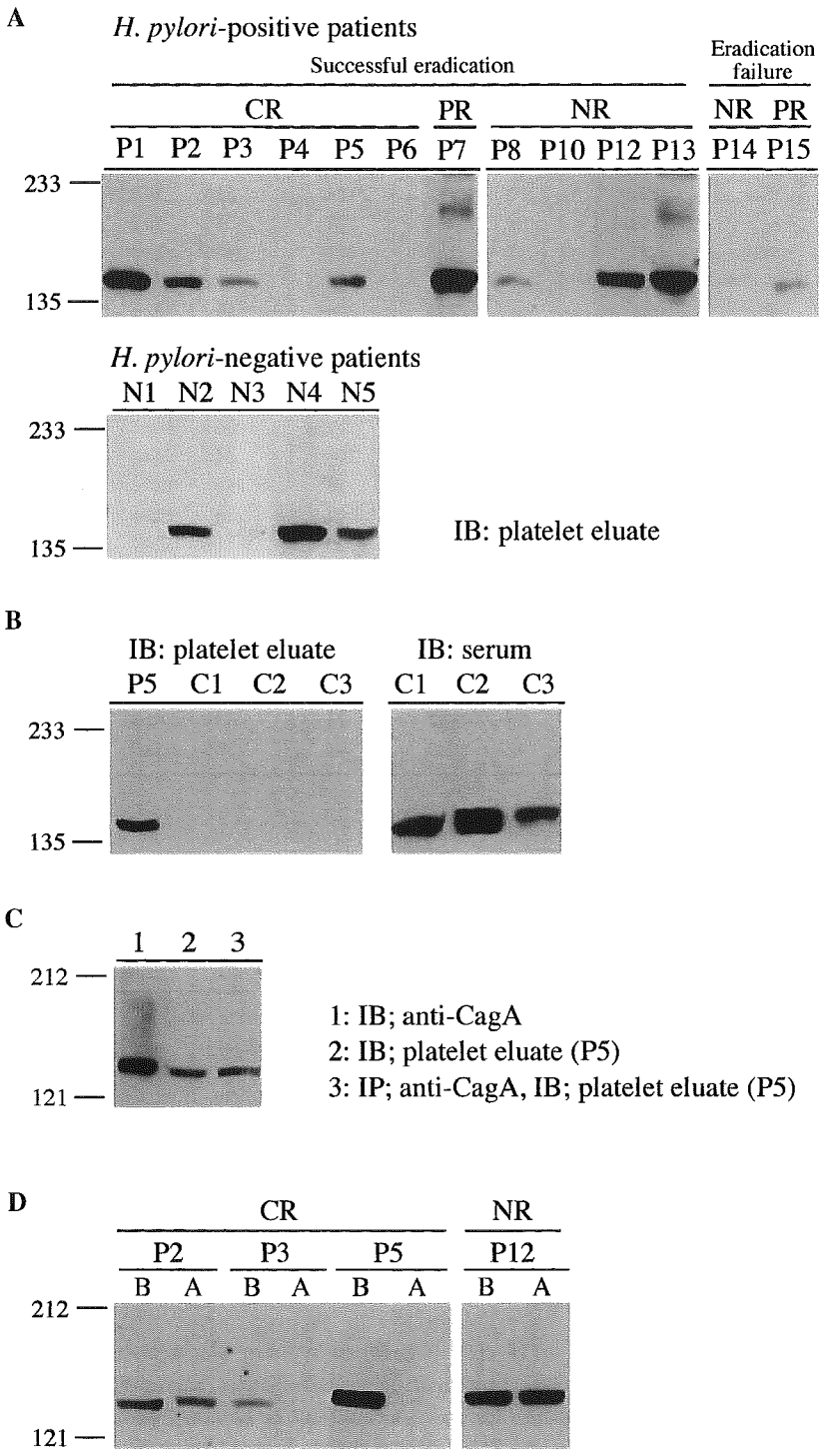

Fig 1. Immunoblot and immunoprecipitation assays. (A) *Helicobacter pylori* proteins were separated by SDS-PAGE and immunoblotted with platelet eluates (PAIgG) from *H. pylori*-positive (P1-P15) and *H. pylori*-negative (N1-N5) cITP patients. (B) *H. pylori* proteins were also immunoblotted with platelet eluates and serum from *H. pylori*-positive non-thrombocytopenic volunteers (C1-C3). P5 is shown as a positive control. (C) *H. pylori* proteins were immunoblotted with anti-CagA antibody (lane 1) or platelet eluate from a *H. pylori*-positive cITP patient (P5) (lane 2). In lane 3, total *H. pylori* lysate was subjected to immunoprecipitation with anti-CagA antibody and then immunoblotted with platelet eluate from P5. (D) Comparison of the levels of anti-CagA antibody present in platelet eluates before and after *H. pylori* eradication. *H. pylori* proteins were immunoblotted with platelet eluates from completely responsive patients (P2, P3, and P5) and one unresponsive patient in whom eradication was successful (P12). B and A, before and after eradication respectively: CR, complete response; PR, partial response; NR, no response; IB, immunoblot; IP, immunoprecipitation. Numbers on the left indicate molecular weights in kDa. For all immunoblot analyses, eluates from 5×10^7 platelets were used as primary antibodies.

cITP patients (e.g., P5) (Fig 1C, lanes 1, 2). This idea was substantiated when the CagA protein that was immunoprecipitated using a specific anti-CagA antibody was recognized by platelet eluate (Fig 1C, lane 3). Furthermore, levels of anti-CagA antibody decreased in platelet eluates from three patients who responded completely to eradication therapy (P2, P3 and P5). By contrast, no reduction in anti-CagA antibody was observed in the eluate from a non-responding patient (P12), although the *H. pylori* was successfully eradicated (Fig 1D).

Discussion

Several clinical studies have demonstrated a beneficial effect of *H. pylori* eradication on platelet recovery in cITP patients

(Gasbarrini *et al*, 1998; Emilia *et al*, 2001; Kohda *et al*, 2002; Veneri *et al*, 2002) and a similarly good response rate (54%) was achieved in the present study. Moreover, by treating *H. pylori*-negative patients with the same regimen of antibiotics, we showed that the drugs themselves exerted no direct pharmacological effects leading to improved platelet counts, which confirmed that the efficacy of the treatment was eradication-dependent and therefore limited to *H. pylori*-positive cITP patients. Thus, to relieve the autoimmunity seen in patients with *H. pylori*-associated cITP, it is essential to eliminate the persistent infection.

There are a variety of infectious organisms that express molecular mimic antigens involved in the pathogenesis of autoimmune diseases (Wucherpfennig, 2001) – e.g. rheumatic fever, Guillain-Barré syndrome (Yuki *et al*, 1993) and immune thrombocytopenia (Bettaieb *et al*, 1992; Bettaieb *et al*, 1996). Among these, *H. pylori* induces production of anti-gastric epithelium autoantibodies (Claeys *et al*, 1998); moreover, monoclonal antibodies against *H. pylori* reportedly cross-react with several other human tissues, including salivary gland, renal tubular epithelium and duodenal epithelium (Ko *et al.*, 1997). We have shown here that PAIgG from several cITP patients recognized *H. pylori* CagA protein and that cross-reactive antibody levels decreased following *H. pylori* eradication in patients that showed a complete response. This is consistent with the idea that *H. pylori* infection exerts a causative effect on the autoimmunity responsible for cITP via molecular mimicry. In addition, the finding that platelet eluates from three of five *H. pylori*-negative cITP patients also recognized CagA suggests that the anti-CagA antibody present in the eluate is the anti-platelet autoantibody produced in cITP, and is not an anti-*H. pylori* antibody produced during normal immune responses to bacterial infection.

Molecular mimicry is one way to break immunological tolerance and initiate the production of autoantibodies. Normally, autoreactive B cells cannot produce autoantibodies because they receive no help from autoreactive $CD4^+$ T cells, which are functionally deleted. However, if a cross-reacting non-self antigen is encountered, the B cells can present peptides from this molecule to non-self reactive $CD4^+$ T cells, thereby driving them to produce autoantibodies (Roitt *et al*, 1998). We suggest that *H. pylori* CagA may be such a cross-reactive antigen. Anti-platelet autoreactive B cells recognize CagA and can present it to *H. pylori*-reactive $CD4^+$ T cells under conditions of persistent infection. B cells may also produce anti-platelet autoantibodies without the help of autoreactive $CD4^+$ T cells in a subset of cITP patients. Enzyme-linked immunoSPOT assays have recently been used to evaluate these autoreactive anti-platelet B cells (Kuwana *et al*, 2002). Although we could use this approach in the present study, it may be useful in future studies for further analysis of the pathogenesis of *H. pylori*-associated cITP.

The good platelet recovery achieved after eradication of *H. pylori* in the present study is consistent with earlier studies from Japan and Italy (Gasbarrini *et al*, 1998; Emilia *et al*, 2001; Kohda *et al*, 2002; Veneri *et al*, 2002). On the other hand, a Spanish group reported lower response rates (13%), although the prevalence of *H. pylori* infection does not differ among ethnic groups (Jarque *et al*, 2001). This probably reflects the fact that some *H. pylori* strains do not harbour the CagA gene, and that CagA-positivity varies depending upon the geographic location (Perez-Perez *et al*, 1997; Mobley *et al*, 2001). In Japan, most *H. pylori* strains do harbour the CagA gene (Maeda *et al*, 1997), which probably accounts for the efficacy of eradication therapy in the treatment of cITP there. Recently, a French group reported no reactivity between platelet eluates and *H. pylori* proteins in ITP (Michel *et al*, 2002). We think the apparent absence of cross-reactivity might be because of the small number of patients examined in that study or the low incidence of CagA-positivity in France.

Unfortunately, because the amounts of PAIgG available from individual patients were very limited, we were unable to determine the platelet antigens that PAIgG recognizes in common with CagA protein. In addition, cross-reactivity between platelet antigens and *H. pylori* antigens other than CagA may also be involved in the pathogenesis of *H. pylori*-associated cITP, as the platelet eluates from two completely responsive patients (P4 and P6) did not recognize CagA protein.

In summary, we have demonstrated cross-reactivity between PAIgG and *H. pylori* CagA protein and suggest that molecular mimicry by CagA of an unknown platelet antigen is crucially involved in the pathogenesis of a subset of cITP cases. Further investigation should enable identification of the platelet antigen that shares an epitope with CagA, as well as clarification of the host susceptibility factors via which autoimmunity is induced.

Acknowledgments

We thank Drs Hideo Yanai, Takeshi Okamoto and Mitsuo Kimoto (Yamaguchi University School of Medicine) for helpful suggestions, for providing control samples, and for help with the bacterial culture. We also thank Drs Keiji Ogura, Douglas Berg (Washington University), and Kiwamu Okita (Yamaguchi University School of Medicine) for valuable discussions.

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Functional phenotype of phosphoinositide 3–kinase p85α–null platelets characterized by an impaired response to GP VI stimulation

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Phosphoinositide 3–kinases (PI3Ks), a family of lipid kinases comprising 3 classes with multiple isoforms, have been shown to participate in different phases of platelet signaling. To investigate the roles that enzymes play in platelet function in vivo and determine which isoforms are important for particular signaling events, we analyzed platelet function of gene knockout mice deficient in the p85α regulatory subunit of heterodimeric class IA PI3K. The kinase activity of p85α−/− platelets was only 5% of the activity of platelets from wild-type littermates. Platelet aggregation induced by adenosine diphosphate (ADP), thrombin, U46619, phorbol 12-myristate 13-acetate (PMA), or botrocetin was not defective in p85α−/− mice, compared with wild-type animals. In contrast, aggregation induced by collagen and collagen-related peptide (CRP) was partially but readily impaired in p85α−/− mice. Both P-selectin expression and fibrinogen binding in response to CRP were also decreased to a similar extent in p85α−/− platelets. Platelets from p85α−/− mice appeared to spread poorly over a CRP-coated surface with intact filopodial protrusions. Significant attenuation of CRP-induced tyrosine phosphorylation in known PI3K effectors such as Btk, Tec, PKB/Akt, and phospholipase Cγ2 were observed with p85α−/− platelets, whereas no alteration was noted in upstream molecules of Syk, LAT, and SLP-76. Considered as a whole, these results provide the first genetic evidence that PI3K p85α plays a significant role in platelet function, almost exclusively in the glycoprotein (GP) VI/Fc receptor γ chain complex–mediated signaling pathway. (Blood. 2003;102:541-548)

Introduction

Phosphoinositide 3–kinases (PI3Ks) constitute a family of lipid kinases that are ubiquitously expressed in many cell types. These enzymes play a key role in the regulation of a variety of cellular processes: proliferation, survival, glucose metabolism, cytoskeletal remodeling, and vesicular trafficking.[1] PI3Ks phosphorylate an inositol ring of membrane-embedded inositol phospholipid at the 3′ position and generate D3 phosphoinositides (PtdIns-3-P; PtdIns-3,4-P2; PtdIns-3,5-P2; and PtdIns-3,4,5-P3), which then function as potent second messengers to relay signals by recruiting downstream molecules to the vicinity of the cellular membrane.[2,3] The most favored targets of the enzyme via its phosphoinositide products are the pleckstrin homology (PH) domain–containing effector molecules. These include Tec family tyrosine kinases, serine/threonine kinases such as Akt/protein kinase B (PKB), guanosine diphosphate/guanosine triphosphate (GDP/GTP) exchange factor (GEF) families such as Vav, and phospholipase (PLC)γ subtypes.[1-3] Of the 3 classes of PI3K (classes I-III), class IA and IB enzymes have been extensively studied in platelets[4-6] and are positioned downstream of membrane receptor stimulation and preferentially catalyze phosphorylation of PtdIns-4,5-P2 in vivo. The class IA subclass enzyme is a heterodimer comprising p110 catalytic and regulatory subunits, and to date 3 p110 catalytic subunits (α, β, and δ) and 3 regulatory subunits (85α, 85β, and 55γ) derived from different genes have been reported.[1] In addition, the 85α protein possesses 2 alternatively spliced variants, p55α and p50α.[7] Regulatory subunits act as adaptor molecules to activate the p110 catalytic subunit via interactions between their Src homology 2 (SH2) domains and specific phosphorylated tyrosine residues (Y-X-X-M motif) of upstream signaling molecules. In addition, class IA enzymes are known to be activated by G proteins.[8,9] A class IB subclass enzyme, PI3Kγ, is also abundant in platelets; it comprises a heterodimer complex with a p110γ catalytic subunit and a unique p101 regulatory subunit. The enzyme has been shown to be specific for G protein–coupled receptor (GPCR) activation through G protein βγ subunits.[4,10] Class IA PI3K utilizes a series of nonreceptor tyrosine kinases and associated adaptor molecules when it is activated downstream of adhesion receptor engagement to transduce signals. In fact, the collagen-induced platelet

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Submitted November 25, 2002; accepted March 10, 2003. Prepublished online as *Blood* First Edition Paper, March 20, 2003; DOI 10.1182/blood-2002-11-3327.

Supported in part by grants from Keio Gijuku Academic Department Funds and Keio University Grant-in-Aid for Encouragement of Young Medical Scientists (N.W.) and by Health Science Research Grants for Pharmaceutical and Medical Safety (Y.I. and M.H.) from the Ministry of Health, Labor and Welfare, Tokyo, Japan.

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activation pathway via the glycoprotein (GP) VI/Fc receptor γ chain (FcRγ) complex evokes significant lipid kinase activity.[11] Upon GP VI cross-linking by collagen, immunoreceptor tyrosine-based activation motifs (ITAMs) displayed on the FcRγ subunit are phosphorylated by the protein tyrosine kinases Lyn and Fyn, leading to binding and activation of the Syk tyrosine kinase. Following assembly with adaptor molecules such as LAT and SLP-76, PI3K is activated via its p85 adaptor subunit.[12,13] With GP VI stimulation, platelet activation induced by aggregated immunoglobulin G (IgG) also involves class IA PI3K as a major signaling element associated with another ITAM-containing receptor, FcγR IIA.[14,15] Class IA enzymes are also implicated in processes downstream of GP Ib/IX/V complex–mediated signaling[16] and upstream of integrin α_{IIb}/β_3 (GP IIb/IIIa) complex activation (inside-out signaling),[4] and in postintegrin cellular responses (outside-in signaling), including conformational changes induced by actin rearrangement.[17,18] Platelet activation stimulated by GPCRs is physiologically important for platelet thrombus formation and is closely associated with PI3K activity.[4] Since PI3Kγ is activated only by G protein βγ subunits, the enzyme may be responsible for causing the observed increase in PI3K lipid products in response to stimulation with thrombin, adenosine diphosphate (ADP), and the thromboxane A_2 analog U46619.[4,19,20] However, class IA PI3K is also activated by GPCRs, and this type of activation may involve either a nonreceptor tyrosine-phosphorylated intermediate or the direct engagement of G protein βγ subunits.[9,21] Although a large body of evidence has clearly demonstrated that PI3K is intimately associated with different phases of platelet activation, the exact roles the enzyme plays in platelet functions in vivo and which isoforms are important for particular signaling events are yet to be determined. Platelets are terminally differentiated anucleate cells, and conclusions from most studies predominantly rely on indirect measurement of PI3K activity using structurally distinct inhibitors of PI3K (wortmannin and LY294002). This means that data analyses may be inherently limited in terms of specificity. To directly address these issues, we analyzed the function of platelets deficient in class IA PI3K p85α proteins in gene knockout mice.

Materials and methods

Materials and antibodies

Adenosine 5′-triphosphate (ATP), apyrase, phorbol 12-myristate 13-acetate (PMA), Arg-Gly-Asp-Ser (RGDS) peptide, acetylsalicylic acid (ASA), prostaglandin E_1 (PGE_1), bovine serum albumin (BSA), and poly-L-lysine were all purchased from Sigma-Aldrich (Tokyo, Japan). Phosphatidylinositol was obtained from Funakoshi (Tokyo, Japan), U46619 and A23187 from Merck KGaA (Darmstadt, Germany), and adenosine diphosphate (ADP) from Biopool (Ventura, CA). Collagen was supplied by Nycomed (Munchen, Germany). Collagen-related peptide (CRP) was prepared as previously described.[22] Botrocetin was a generous gift from Dr Yoshihiro Fujimura (Nara Medical College, Kashiwara, Japan). Human thrombin was provided by Welfide (Osaka, Japan). Alexa Fluor 488–conjugated human fibrinogen was from Molecular Probes (Eugene, OR). Fluorescein isothiocyanate (FITC)–conjugated antimouse P-selectin antibody was purchased from BD Pharmingen (San Diego, CA). Polyclonal anti-Btk antibody was kindly provided by Dr Owen Witte (University of California, Los Angeles). Anti-Syk, PLCγ2, SLP-76, p110α, p110β, p110δ and Akt/PKB polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antiphosphotyrosine monoclonal antibody (clone 4G10) and anti-PI3K $p85^{PAN}$, LAT, and Tec polyclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Anti–phospho-Akt antibody was obtained from New England Biolabs (Beverly, MA). A specific antibody against p85β was prepared as described.[23] Thin-layer chromatography (TLC) plates were purchased from Whatman International (Maidstone, United Kingdom).

Mice

PI3K p85α−/− mice[24,25] were backcrossed to C57BL/6 mice for more than 7 generations before intercrossing heterozygous mice. The targeting strategy allowed selective disruption of p85α expression, while leaving gene products for p55α and p50α isoforms intact.[25] All mice were maintained under strict pathogen-free conditions. All experiments were performed in accordance with Keio University Institutional Guidelines.

Bleeding time

At 2 to 3 months of age, mice were anesthetized using diethylether. An incision was made 1 cm from the tip of the tail, and the emerging blood was blotted onto Whatman 3M paper (Whatman International) every 15 seconds. Bleeding times were defined as the time required for all visible signs of bleeding to stop.[26]

Blood collection and preparation of platelets

Whole murine blood was collected in syringes containing 100 μL acid citrate dextrose (ACD; 120 mM sodium citrate, 110 mM glucose, and 80 mM citric acid) by cardiac puncture under diethylether anesthesia. Blood cell counts were determined using an automated blood cell counter. Blood from 2 to 6 mice (2 to 4 months old, both sexes) was pooled for preparing platelet samples. Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 660*g* for 1 minute at 22°C. The residual blood sample was diluted using 400 μL RCD solution (36 mM citric acid, 5 mM glucose, 5 mM KCl, 103 mM NaCl, and 2 μM PGE_1, pH 6.5) containing 10% (volume/volume) ACD and 0.4 U/mL apyrase, then centrifuged at 660*g* for 1 minute to recover diluted PRP. To prepare washed platelets, PRP was combined with diluted PRP pretreated with 0.2 mM ASA at 37°C for 15 minutes and diluted using an equal volume of RCD solution containing 10% (vol/vol) ACD and 0.4 U/mL apyrase. Washed platelets were then obtained by centrifuging at 1000*g* for 7 minutes. Isolated platelets were resuspended at a final concentration of 5×10^5/μL in modified Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM NaH_2PO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid], and 5 mM glucose, pH 7.3) containing apyrase.

Aggregation studies

Platelet concentrations of PRP were adjusted to 3×10^5/μL using platelet-poor plasma (PPP). A total of 125 μL PRP was placed in siliconized glass tubes and incubated at 37°C for 10 minutes before stimulation. Aggregation was optically monitored using a platelet aggregometer (Hema Tracer TM Model 601; Niko Bioscience, Tokyo, Japan). PPP was used as a reference to indicate 100% aggregation. For studies utilizing thrombin, washed platelets without pretreatment under ASA were suspended in modified Tyrode-HEPES buffer before use.

Flow cytometry

Washed platelets suspended in modified Tyrode-HEPES buffer containing 0.4 U/mL apyrase were labeled using FITC-conjugated antimouse P-selectin antibody (with 1 mM RGDS peptide and 1 mM $CaCl_2$) or Alexa Fluor 488–conjugated human fibrinogen (1 mM $CaCl_2$). Following stimulation with the appropriate concentrations of CRP, labeling of murine platelets was performed for 30 minutes. Samples were then analyzed using a FACSCalibur flow cytometer (Nippon Becton Dickinson, Tokyo, Japan).

Morphologic analysis of adherent platelets under electron microscopy

Control discoid platelets were obtained as follows: platelets in modified Tyrode-HEPES buffer containing 2 mM $MgCl_2$ were fixed in 1% glutaraldehyde, placed on poly-L-lysine–coated coverslips, and allowed to adhere to the surface for 60 minutes. Collagen and CRP were diluted to 10 μg/mL and

6 μg/mL, respectively, with modified Tyrode-HEPES buffer and immobilized on coverslips in culture dishes overnight at 4°C. Coverslips were washed 3 times with modified Tyrode-HEPES buffer and incubated with 2% BSA for 2 hours at room temperature for blocking. Platelets suspended in modified Tyrode-HEPES buffer containing 2 mM $MgCl_2$ (1×10^5 platelets/μL, 100 μL) were brought into contact with collagen- or CRP-coated coverslips in culture dishes and platelets were allowed to adhere at 37°C for 10 minutes. After rinsing 3 times to remove nonadherent platelets, coverslips were further incubated at 37°C for 20, 50, and 80 minutes. Adherent platelets were fixed using 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 minutes. Fixed platelets adhering to coverslips were washed 5 times in 0.1 M phosphate buffer (pH 7.4), postfixed using 1% osmium tetroxide in the same buffer for 15 minutes, dehydrated in a graded ethanol series, and then dried using a Hitachi ES-2020 freeze dryer (Hitachi, Tokyo, Japan) and *t*-butyl alcohol. Specimens were coated with an amorphous and continuous layer of sublimated osmium tetroxide (approximately 10 nm) using an NL-OPC80 osmium plasma coater (Nippon Laser & Electronics Lab, Nagoya, Japan). Slides were then examined under a Hitachi S-4500 field emission scanning electron microscope (Hitachi) at an accelerating voltage of 10 kV. Lengths of filopodia, defined as protrusions from the platelet body less than 130 nm in width, were measured using NIH Image software (National Institutes of Health; http://rsb.info.nih.gov/nih-image/). The area of the CRP-coated slides covered by platelets was analyzed using the same software.

Immunoprecipitation and Western blotting

Platelets in modified Tyrode-HEPES buffer containing 0.4 U/mL apyrase, 1 mM RGDS peptide, and 1 mM EGTA (ethylene glycol tetraacetic acid) were stimulated using the appropriate concentrations of collagen or CRP. Reactions were terminated with the addition of an equal volume of ice-cold lysis buffer (2% Nonidet P40 [NP-40], 20 mM Tris [tris(hydroxymethyl)aminomethane], 300 mM NaCl, 10 mM EDTA [ethylenediaminetetraacetic acid], 2 mM Na_3VO_4, 10 mg/mL aprotinin, 1 mg/mL pepstatin, 10 mg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.3). Debris was removed by centrifugation at 20 000*g* for 10 minutes. Platelet lysates were incubated with antibodies for various proteins and protein A–sepharose beads (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) for 2 hours at 4°C under continuous rotation. The beads were washed extensively in 2-fold diluted lysis buffer. Precipitated proteins were extracted in Laemmli sample buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Separated proteins were transferred to Hybond-ECL membrane (Amersham Pharmacia), which was then blocked in 10% BSA in TBS-T (20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20) and hybridized sequentially using primary antibodies and horseradish peroxidase–conjugated secondary antibody (Amersham Pharmacia). Bound antibodies were detected using an enhanced chemiluminescence (ECL) Western blotting kit (Amersham Pharmacia).

PI3K assay

Platelets were lysed and immunoprecipitated using anti-$p85^{PAN}$ antibody (Upstate Biotechnology), which reacts with all 3 p85α isoforms (p85α, p55α, and p50α) in addition to p85β and p55γ subunits. Beads were washed 3 times in PI3K buffer (25 mM Tris, 0.5 mM EGTA, and 100 mM NaCl, pH 7.4) and suspended in PI3K buffer containing 200 μg/mL phosphatidylinositol. Following preincubation at 37°C for 10 minutes, the reaction was initiated with the addition of 2.5 μL start solution (200 mM $MgCl_2$, 200 μM ATP, 3 μCi [0.111 MBq] γ-^{32}P-ATP; Amersham Pharmacia). After incubation for 10 minutes, the reaction was terminated with the addition of 100 μL stop solution (chloroform, methanol, and 11.6 N HCl in the ratio of 50:100:1). Labeled phosphoinositides were extracted using chloroform, washed 3 times in 100 μL wash solution (mixture of 50 μL methanol and 50 μL 1 N HCl), separated by thin-layer chromatography, and analyzed using a BAS 2000 bioimaging analyzer (Fuji, Tokyo, Japan).

Results

The p85α protein is the major class IA PI3K isoform expressed in platelets

To explore the relative contributions of the various isoforms of class IA PI3K, we first examined the expression of various isoforms in murine platelets by Western blotting using anti-$p85^{PAN}$ antiserum, which has been shown to recognize all variants of p85α (p85α, p55α, and p50α), p85β, and p55γ.[23-25] Among the variety of hematopoietic tissues examined, expression levels of p85α isoform far exceeded those of p55α and p50α in platelets, spleen, thymus, and fetal liver, whereas bone marrow expressed similar amounts of all 3 isoforms (Figure 1A). In platelets from p85α−/− mice, selective disruption of the p85α isoform did not result in significant up-regulation of p55α, p50α, and p85β proteins to compensate for the lack of p85α protein (Figure 1B).[24,25] In addition, expression patterns of p85α, p55α, and p50α isoforms in p85α+/− platelets did not significantly differ from those of p85α+/+ platelets (Figure 1B). The p85β protein was detected in p85α−/− platelets only when the film was overexposed (data not shown). When expression of the p85β protein in platelets was examined using a specific antibody, the levels of p85β in p85α−/− and p85β+/+ platelets were found to be not significantly different (Figure 1C). It was also found that the $p85^{PAN}$ antiserum equally and efficiently captured p85β proteins in platelet lysates from either p85α+/+ or

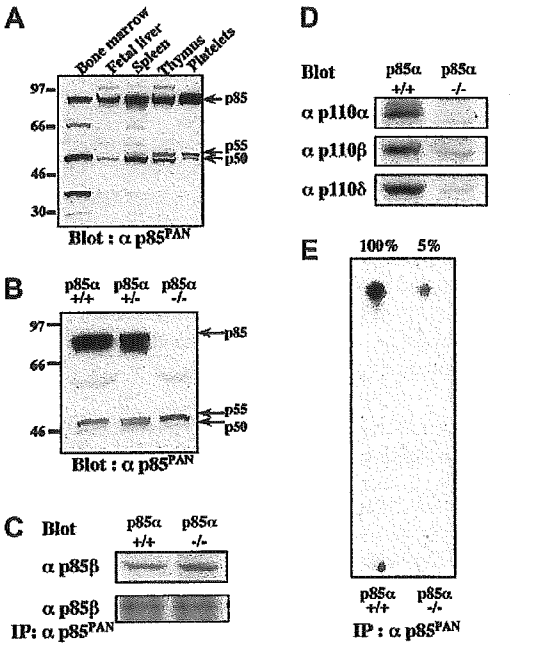

Figure 1. Differential expression patterns of regulatory and catalytic subunits of PI3K class IA and their kinase activities in platelets from p85α−/− and wild-type mice. (A-B) Expression patterns of regulatory subunits of PI3K class IA in wild-type hematopoietic organs (A) and in platelets from wild-type p85α+/+, p85α+/−, and p85α−/− mice (B), shown by Western blot with $\alpha p85^{PAN}$. (C-D) The amount of p85β protein in platelet lysate and its immunoprecipitate with anti-$p85^{PAN}$ antiserum (C) and the amount of p110 catalytic subunits (p110α, p110β, and p110δ) in platelet lysate from p85α+/+ and p85α−/− mice (D), shown by Western blot with p85β-specific antiserum and anti-p110 polyclonal antibodies, respectively. Whole-cell lysate (50 μg per lane) or its immunoprecipitate was resolved using SDS-PAGE. (E) PI3K activity of wild-type and p85α−/− platelets measured using anti-$p85^{PAN}$ antiserum and resolved by thin-layer chromatography. The wild-type response was set to 100% and the activity of p85α−/− platelets was estimated to be 5% of the wild-type response.