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幹細胞を利用した分化誘導培養による人工血液の開発に関する研究　分担研究報告書

# マウス幹細胞から造血・免疫系細胞への分化誘導技術の開発
## —細胞の多分化能の維持機構の解明—

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研究要旨

Pax5遺伝子欠損マウス由来ProB細胞を、1ヶ月程度 *in vitro* で培養したところ、多分化能を失いT細胞への分化能が完全に失われてしまうことが明らかとなった。我々はこの長期の *in vitro* 培養中にT細胞への分化能力を維持させる作用をもつサイトカインを探索する目的で、培養後のT細胞の再構築を指標として各種サイトカインのスクリーニングを行った。その結果、培養液中にLIFを添加した場合にのみ、*in vitro* での長期培養後もT細胞への分化能力が高く維持されていることを見いだした。一旦分化能力を失ったProB細胞に再度LIFを作用させてもT細胞への分化能は回復しなかったことから、LIFは直接T細胞系列への分化を促進するというよりは、未分化細胞におけるT細胞系列への分化能力を維持させる作用があるものと考えられた。LIF添加培養によってProB細胞中でGATA遺伝子の発現上昇がみられた。LIF処理したProB細胞はin vitro培養後NK細胞への分化が確認できたが、マクロファージへの分化はみられなかった。

A.研究目的

幹細胞が多系列への分化能を保持しながら自己複製できるメカニズムの解明は、人工血液の開発を目指す再生医療技術の開発において非常に重要である。免疫担当細胞を含めて全ての血球は骨髄中の造血幹細胞から分化するが、この多系列分化能と自己複製能の維持機構はよくわかっていない。本研究ではこの多系列分化能と自己複製能の維持機構をマウスモデルを中心に解明して造血幹細胞の安定培養供給技術や *in vitro* 分化誘導と *in vivo* への移植による免疫細胞、特にTリンパ球の分化誘導・制御技術の開発と人工血液医療への応用を試みる。

B.研究方法

長期 *in vitro* 培養後のPax5 欠損proB細胞のT細胞分化能をみる目的で、Pax5 欠損

proB 細胞を分離採取直後または ST2 間質細胞を、IL7 など添加一ヶ月培養後に致死量以下で放射線照射した RAG2 欠失マウスに投与して３週間後、再構築した胸腺細胞をフローサイトメトリーによって解析した。

LIF による Pax5 欠損 proB 細胞の T 細胞生成のポテンシャルを *in vitro* 長期培養の間維持させることができるかをみる目的で、Pax5 欠損 proB 細胞を ST2 間質細胞、IL7 に加えて表記の種々のサイトカインを添加してそれぞれ一ヶ月 *in vitro* 培養、$10^7$ 細胞を致死量以下で放射線照射した RAG2 欠失マウスに注入して３週間後、再構築したドナー由来の胸腺細胞をフローサイトメトリーによって解析して細胞数を測定した。

さらに、分離採取した Pax5 欠損 proB 細胞を ST2 間質細胞を、IL7 存在下に LIF 添加有り又は無しで１ヶ月 *in vitro* 培養した状態で、或は（ LIF 添加無しで T 細胞分化能を失った状態で）もう１ヶ月 LIF 添加を追加したカクテルで培養後、$10^7$ 細胞を致死量以下で放射線照射した RAG2 欠失マウスに注入して３週間後、再構築したドナー由来の胸腺細胞をフローサイトメトリーによって解析して細胞数を測定した。

LIF による NK 細胞やマクロファージへの分化能の維持をみる目的で、$Pax5^{-/-}$ proB 細胞を LIF 存在下、非存在下で１ヶ月培養し、NK やマクロファージのリニエージを *in vitro* 分化させるプロトコールで培養した。NK 細胞の分化には IL-7 を抜いた後 IL-2 を加えて１５日間培養した。マクロファージの分化には前記の IL-2 のかわりに M-CSF を加えて培養した。

LIF で処理した Pax5 欠損 proB 細胞における遺伝子発現変化をみる目的で、$Pax5^{-/-}$ pro-B 細胞を LIF 添加、非添加で一カ月培養して、 この細胞から全 RNA 抽出した。Notch1, Bmi-1 、CXCR4 mRNA の発現量をリアルタイム RT-PCR 解析で測定した。各検体の b-actin mRNA に対する相対的 mRNA 値を 表示した。GATA3 タンパクの発現は、$Pax5^{-/-}$ proB 細胞を LIF 存在、非存在下で培養し、 その細胞ライゼートを GATA3 特異的抗体を用いたウエスタンブロットで解析した。

LIF で処理した Pax5 欠損 ProB 細胞の DNA マイクロアレイ解析は、$Pax5^{-/-}$ proB 細胞を LIF 存在下、非存在下に *in vitro* で１ヶ月培養後、日立 Ace ジーン(マウス３万遺伝子)を用いて行った。

*in vitro* 培養での造血幹細胞への LIF の影響は、GFP トランスジェニックマウス骨髄から $Sca1^+$ 細胞からソートして LIF の有り又は無しで３ 日間培養した。培養した造血幹細胞は、 致死量以下で放射線照射した $RAG^{-/-}$マウスに注入し、３６日後に再構築した胸腺細胞と脾細胞をフローサイトメトリーで解析した。

C.研究結果

Pax5 はＢ細胞系列への分化に必須のマスター転写因子の一つであるが、同時にＢ細胞以外の様々なリニエージの分化を抑制するリプレッサーとして働いていることもよく知られている（図１）。実際、Pax5 遺伝子をノックアウトしたマウスから得られたプロＢ細胞は、プロＢ細胞でありながらＢ細胞以外の様々な血球系細胞へと分化する能力を保持していることが示されている。したがって、こ

のプロB細胞は造血幹細胞に極めて近い性質を持つ培養細胞であると言ってよく、人工血液研究モデルの材料としても適していると考えられる。最近では Pax5 の上流因子である E2A 遺伝子を欠損したプロB細胞も同様の分化多能を持つことが報告されており、多分化能を持つ幹細胞様の培養細胞が注目されている。実際、我々は Pax5 ノックアウトプロB細胞を RAG2 ノックアウトマウスに移入し、3週後にフローサイトメトリー解析を行ったところ、野生型と同様の胸腺が再構築できることを確認している。ところが、この細胞を *in vitro* で培養し続けると、急速にT細胞への分化能力を失い、一ヶ月間の培養後には胸腺を再構築する能力が完全に失うことが明らかとなった（図2）。

そこで、*in vitro* の培養系にサイトカイン等を加えることによりT細胞への分化能が維持できるのではないかと考え、様々なサイトカインを加えて一ヶ月間培養した後に胸腺の再構成実験を行なった。その結果、意外なことにLIFを加えただけでProB細胞のT細胞への分化能が長期間維持できることが明らかとなった。 LIF は ES 細胞を未分化状態に保つのに必須のサイトカインであることはよく知られているが、LIF が造血系の細胞の分化維持に関わっているという報告はない。次に LIF の効果が多分化能を維持させるのか、あるいはT細胞系列へと分化を積極的に推進させるのかどうかについて検討した。LIF 無しで一ヶ月培養して一旦分化能を失ってしまった細胞に LIF を添加してもT細胞分化能は回復しなかったことから、LIF はT細胞への分化を積極的に推進するというよりは、T細胞への分化能を維持する作用を持つものであると考えられた。

次に、T細胞以外のリニエージの分化に対する LIF の作用の検討を行った。*in vitro* 分化誘導系によってプロB細胞から NK 細胞へと分化を誘導することができるが、この NK 細胞への分化はT細胞分化能と同じく LIF 無しで喪失し、LIF 添加によって維持された。いっぽう、マクロファージへの分化能は LIF 添加によって逆に失われてしまうことが明らかとなった。したがって、LIF はプロB細胞の多様なリニエージへの分化能を全て維持させるというよりは、T細胞や NK 細胞への分化を特異的に維持することが考えられた。

次のステップは LIF がT細胞系列への分化を維持する分子メカニズムを解明することである。LIF を添加することにより、プロB細胞にどのような変化が起こるかをまず検討した。T細胞リニエージへのコミットメントに関与することが知られているいくつかの遺伝子についてその発現が変化するかどうかの検討を行った。T細胞系列への分化を制御していることが知られている Notch1 の発現は、LIF 添加によりかえって減少していた。しかしながら、同じくT細胞分化に必須の転写因子である GATA3 の発現は LIF 添加により増大していた。また、造血幹細胞の未分化性の維持に関与することが知られている Bmi1 や Hoxb4 の発現は増大しておらず、未分化性の維持に関与する分子メカニズムはまだ解明できていない。そこで、LIF の添加培養によってどのような遺伝子の発現が変化するのかをマイクロアレイを用いて網羅的な検討を行った。まだ解析途中ではあるが、これらのうちのいくつかの遺伝子に注目して解析を進めていく予定である。

最後に、LIFがPax5ノックアウトプロB細胞という特殊な細胞だけでなく、骨髄中の造血幹細胞の分化能維持にも関与するかどうかを、GFPトランスジェニックマウスの造血幹細胞を用いて検討した。GFPマウスより得たSca1陽性の骨髄細胞を造血幹細胞のソースとして用い、*in vitro*でLIFと共に6日間培養した後、放射線照射したRAG2欠損マウスに移入した。まだ解析したマウスの総数は少ないが、LIFを添加培養した造血幹細胞はT細胞へと分化する数が増大していた。これらの結果から、LIFは造血幹細胞においてもT細胞系列への分化を促進する作用があることが示された。

D.考察

得られた結果を要約すると、Pax5ノックアウトプロB細胞において培養系にLIFを添加することによりT細胞への分化能を*in vitro*で長期間維持できることを初めて明らかにした。またこの分化能の維持は全てのリニエージにおいて有効なのではなく、T細胞およびNK細胞への分化を維持する作用があることが明らかとなった。さらに、Pax5プロB細胞のみならず、骨髄中の造血幹細胞のT細胞系列への分化もLIFによって維持増強されたことから、*in vitro*における造血系細胞の未分化能の維持、T細胞系列への分化の制御にLIFが利用できる可能性が示唆された。造血幹細胞の未分化性と自己複製能の維持は未解明な部分が多いが、Wnt受容体からNotch1に至るシグナル伝達経路やN-cadherin, b-catenin を介するシグナルの関与が主要なものと考えられてきた（図9）。我々の成果は、LIFによるLIFR/gp130を介するSTAT3活性化、GATA3の発現上昇が、造血幹細胞の未分化性と自己複製能の維持に重要な役割をしていることを示唆するものである。今後、LIFからGATA3に至る経路とWnt等の他のシグナル伝達経路とのクロストークの関与も含めて解析していくことの必要性が示唆された。

E.結論

LIF共存下で培養することにより、Pax5欠損プロB細胞はT細胞への分化能力を*in vitro*で長期間維持することができ、LIF添加により Pax5 欠損プロB細胞においてGATA3の発現が増加していた。LIFはPax5欠損プロB細胞のT細胞への分化能のみならず、NK細胞への分化能も維持させたが、逆にマクロファージへの分化能は喪失させた。LIFには造血幹細胞の多分化能を維持、亢進させる作用があることが示唆された。

F.健康危険情報

特にない

G.研究発表

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H.知的財産権の出願・登録情報

【特許の取得】
申請中
・ 発明者 白井睦訓、鈴木春巳他　権利者 山口大学「新規T細胞機能遺伝子探索技術の開発とそれを利用した新規T細胞分化遺伝子」
申請準備中
・ 発明者 白井睦訓、鈴木春巳他　権利者 山口大学「サイトカインを利用した新規造血・免疫系幹細胞の分化技術と人工血液医療への応用」

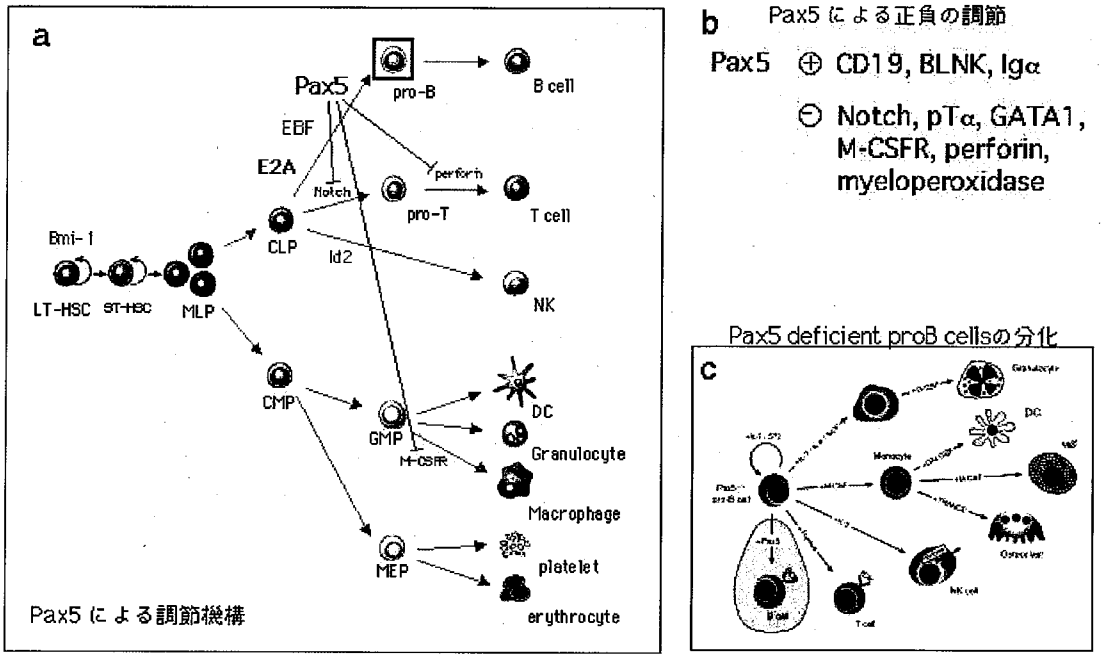


図１　Pax5 の役割（a,b)とPax5$^{-/-}$ proB cells　（造血幹細胞様培養細胞：Hematopoietic stem cell like cells）の分化（c)

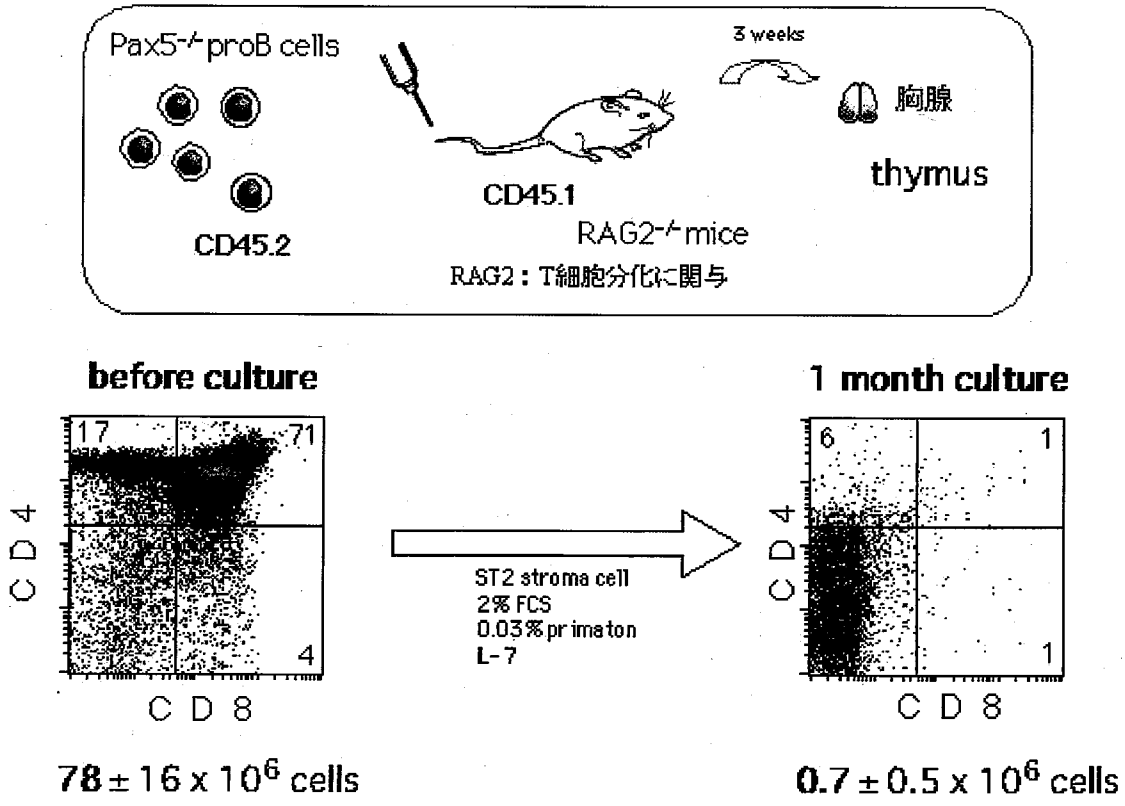


図２　Pax5欠損プロB細胞株はin vitro長期培養でT細胞分化能を失う

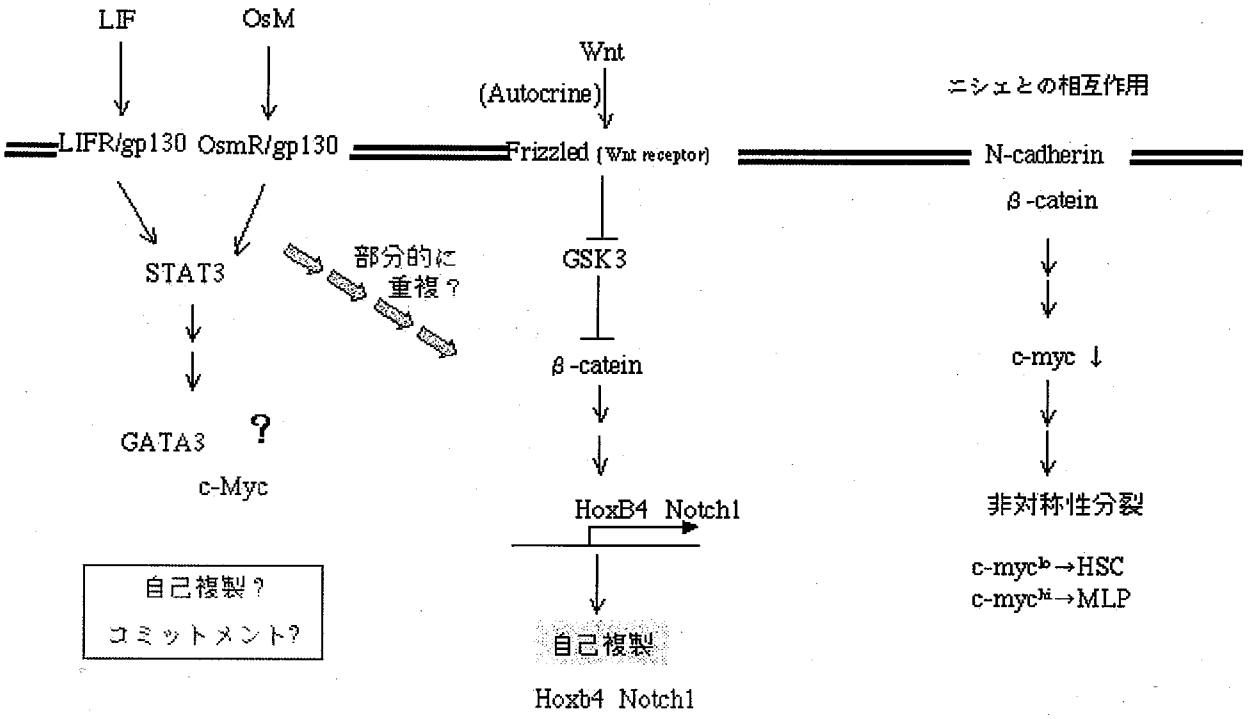


図９　造血幹細胞の未分化性と自己複製能の維持の制御シグナル

# 研究成果の刊行に関する一覧表

# 研究成果の刊行に関する一覧表

【 雑誌 】

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

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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

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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 GCG 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 $1 \times 10^8$ 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 ($1 \times 10^5$/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 ($2 \times 10^6$/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 ($2 \times 10^6$/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 ($1 \times 10^6$ 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′; mIlsp70F, 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

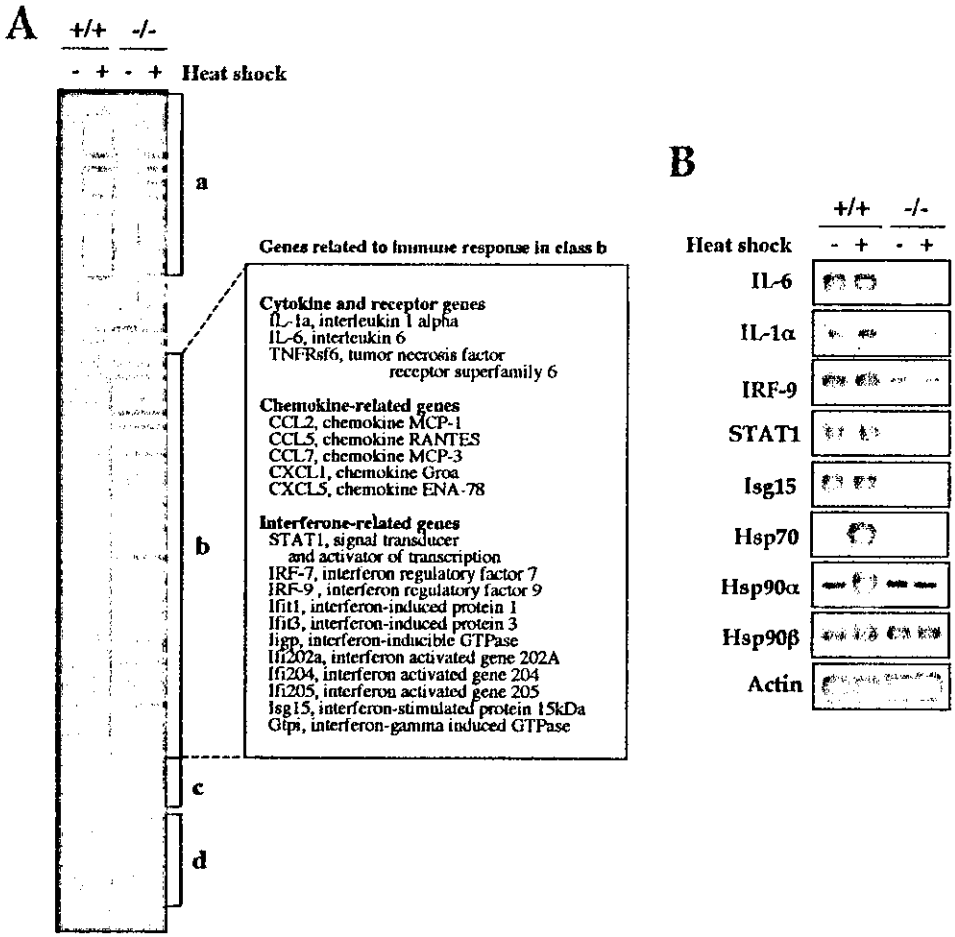


FIG. 1. **Changes in mRNA levels in wild-type and HSF1-null MEF cells.** *A*, total RNAs were isolated from wild-type (+/+) and HSF-null (−/−) MEF cells maintained at 37 °C (−) or heat-shocked at 42 °C for 1 h (+). Genes undergoing more than 3-fold change are cluster analyzed based on microarray analysis. 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-

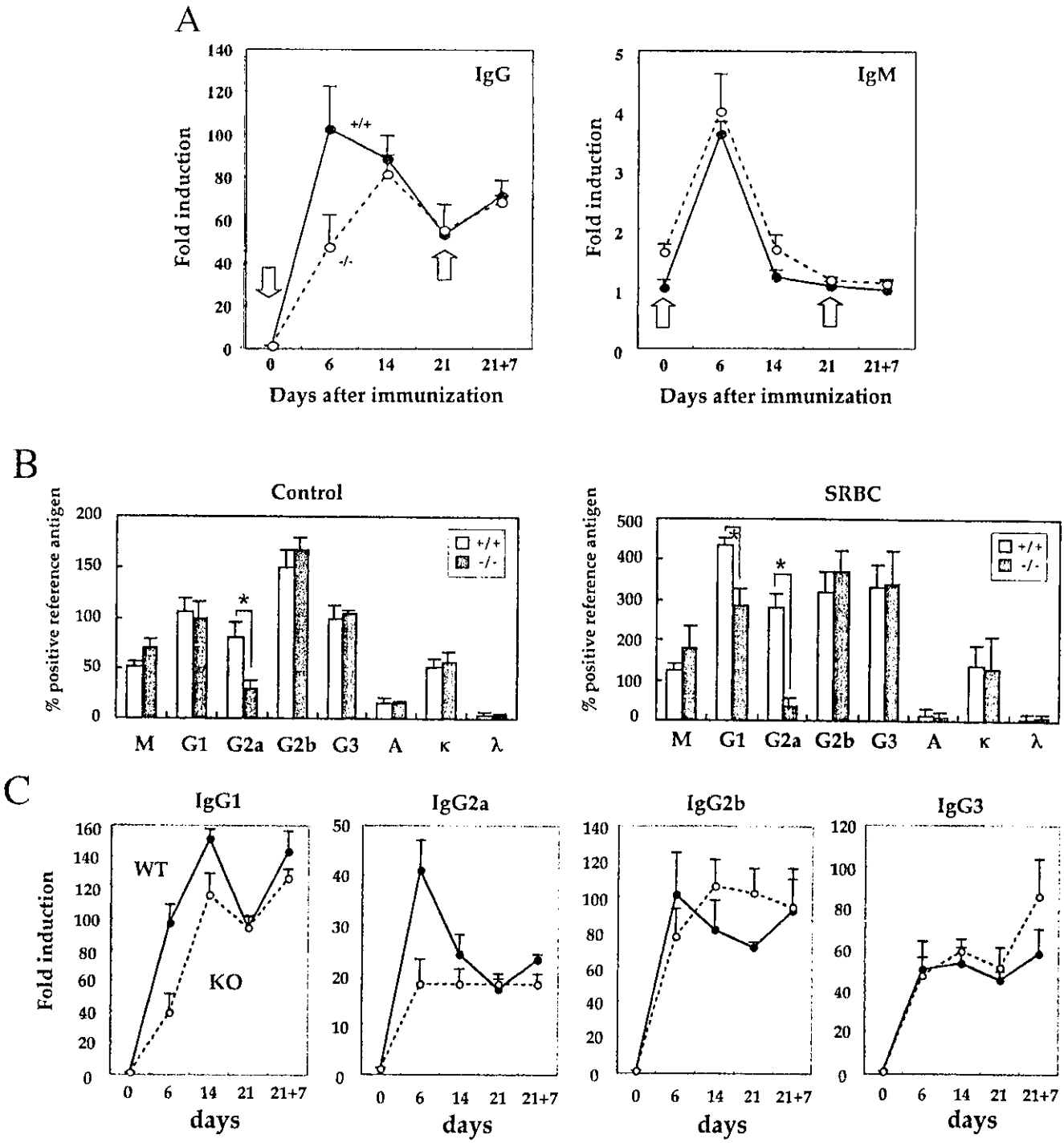


FIG. 2. **Serum immunoglobulin levels in response to SRBC immunization in wild-type and HSF1-null mice.** *A*, 5 wild-type (*closed circles*) and five HSF1-null mice (*open circles*) were immunized at day 0 and boosted at day 21 (*open arrows*) and were bled at the indicated time points. Levels of SRBC-specific 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

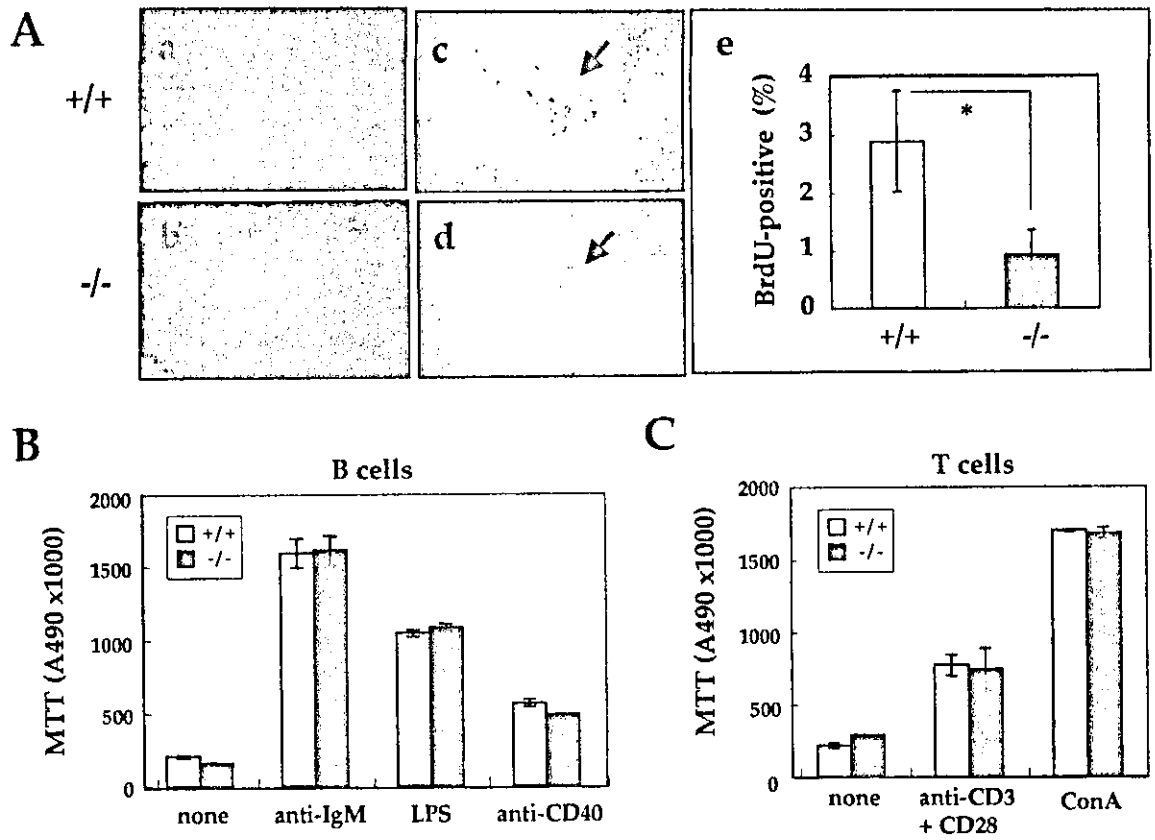


FIG. 3. **Proliferation of spleen cells *in vivo* and *in vitro*.** *A*, after BrdUrd injection, the spleen was dissected, and cryosections were stained with hematoxylin (*a* and *b*). Serial sections were stained with an anti-BrdUrd antibody (*c* and *d*). Clusters of BrdUrd-positive cells are indicated 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. 3C). 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. 1A) 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. 4C). 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. 1A). 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

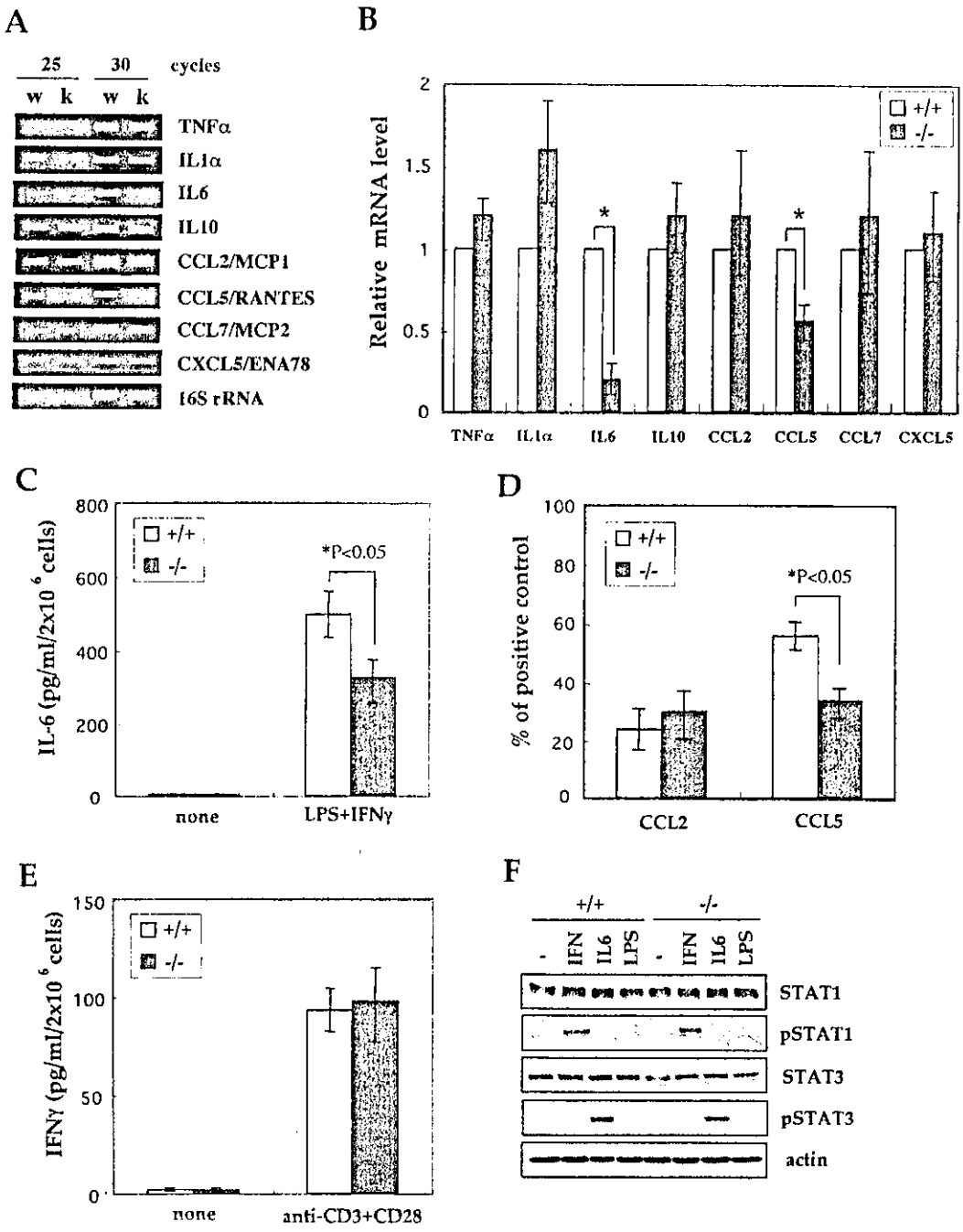


FIG. 4. **Production of cytokines and chemokines from spleen cells.** *A*, total spleen cells were incubated with LPS and IFN-γ for 24 h. Reverse transcription-PCR analysis was performed using total RNA isolated from the spleen in wild-type (*w*) or HSF1-null (*k*) mice. Representative data are shown. *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 IISF1 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-

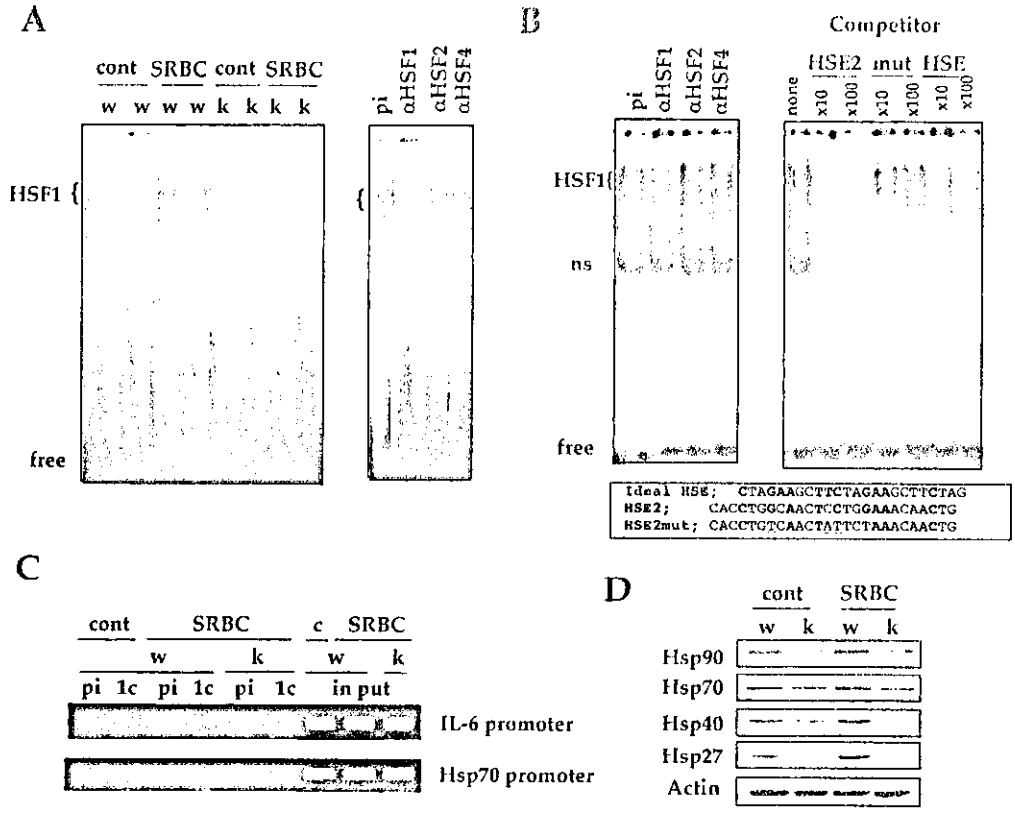


FIG. 5. **HSF1 binds to the *IL-6* gene as well as the *Hsp70* gene in response to the SRBC immunization.** *A*, whole cell extracts were prepared from the untreated spleen (*cont*) in wild-type (*w*) and HSF-null (*k*) mice and the spleen at 6 days after the immunization with SRBC (*SRBC*). A gel shift 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-

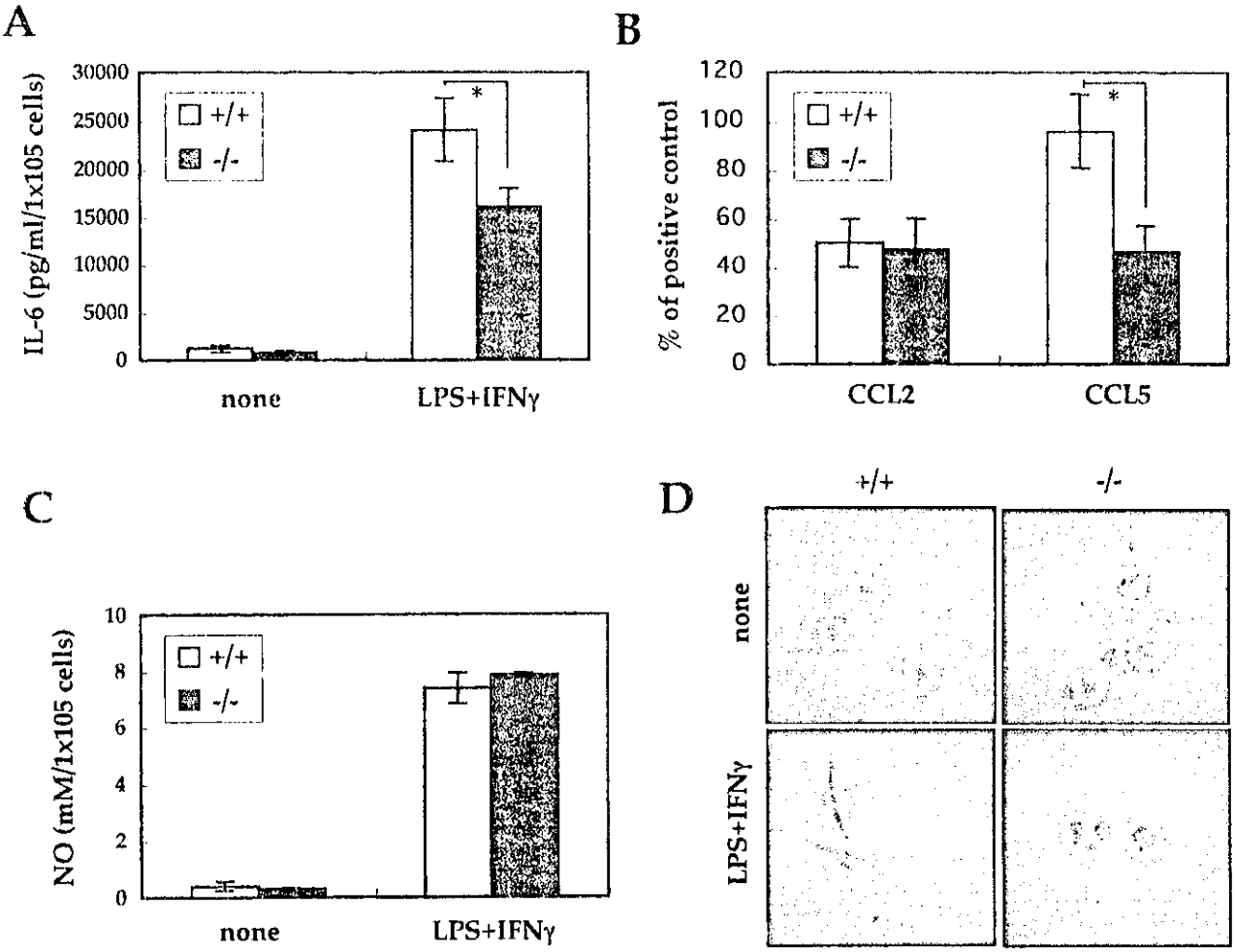


FIG. 6. **Cytokine and chemokine production in peritoneal macrophages.** *A*, IL-6 production in culture medium of macrophages was determined by ELISA after incubation of LPS and IFN-γ for 48 h. *B*, chemokine levels in culture medium of macrophages were determined using the cytokine array. *C*, nitric 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.

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