The innate immune system senses various molecular motifs within pathogens known as pathogen-associated molecular patterns (PAMPs) through germline-encoded pattern-recognition receptors (PRRs). TLRs are membrane-bound PRRs that recognize various PAMPs on the cell surface or within endolysosomes and induce the expression of inflammatory cytokines and type I IFN (1–4). RIG-I–like receptors (RLRs), RIG-I and MDA5, which are composed of an RNA helicase domain and caspase recruitment domains (CARDs), are cytoplasmic PRRs that sense RNA viruses to induce antiviral innate immune responses (1, 4–6). Cells express additional cytoplasmic PRRs that recognize dsDNA derived from viruses, bacteria, and damaged host cells. Absent in melanoma 2 (AIM2) was identified as a dsDNA sensor that triggers caspase-1–dependent IL-1β production (7–13). This pathway is required for host defense against Francisella tularensis infection (12, 13). However, a cytosolic DNA sensor responsible for the induction of type I IFN production has not yet been discovered. The signaling pathways through RLRs, DNA sensors, and several TLRs culminate in the activation of the transcription factors NF-κB and IRF3 (14). NLRC5 overexpression promoted IL-1β production via caspase-1, suggesting that NLRC5 constitutes an inflammasome. However, there was no reduction of IL-1β in NLRC5-deficient cells in response to known inflammasome activators, suggesting that NLRC5 controls IL-1β production through an unidentified pathway. These findings indicate that NLRC5 is dispensable for cytokine induction in virus and bacterial infections under physiologic conditions. The Journal of Immunology, 2011, 186: 000–000.

Accepted for publication June 24, 2010. Accepted for publication November 6, 2010. This work was supported in part by National Institutes of Health Grant PO1 AI070167 and by a grant-in-aid for Specially Promoted Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. H.K. was supported in part by Postdoctoral Fellowship P08123 from the Japan Society for Promotion of Science and Kishimoto Foundation Fellowships from the World Premier International Immunology Frontier Research Center, Osaka University, Osaka, Japan. Address correspondence and reprint requests to Prof. Shizuo Akira and Dr. Taro Kawai, Laboratory of Host Defense, World Premier International Immunology Frontier Research Center, Osaka University, Osaka, Japan. E-mail addresses: sakira@biken.osaka-u.ac.jp and tkawai@biken.osaka-u.ac.jp

The online version of this article contains supplemental material.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1002094

Published December 8, 2010, doi:10.4049/jimmunol.1002094

NLRC5 Deficiency Does Not Influence Cytokine Induction by Virus and Bacteria Infections

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Nucleotide-binding domain and leucine rich repeat containing gene family receptors (NLRs) are cytosolic proteins that respond to a variety of pathogen and host components to induce inflammatory cytokines. NLRC5 is a recently identified member of the NLR family that has been implicated in positive and negative regulation of antiviral innate immune responses. To clarify whether NLRC5 controls antiviral innate immunity in vivo, we generated NLRC5-deficient mice. Macrophages and dendritic cells derived from NLRC5-deficient mice induced relatively normal levels of IFN-β, IL-6, and TNF-α after treatment with RNA viruses, DNA viruses, and bacteria. The serum cytokine levels after polyinosinic-polycytidylic acid infection were also comparable between NLRC5-deficient and wild-type (WT) mice (14). On the other hand, NLRP3, NLRC4, and NAIP5 constitute an inflammasome. However, there was no reduction of IL-1β in NLRC5-deficient cells in response to known inflammasome activators, suggesting that NLRC5 controls IL-1β production through an unidentified pathway. These findings indicate that NLRC5 is dispensable for cytokine induction in virus and bacterial infections under physiologic conditions. The Journal of Immunology, 2011, 186: 000–000.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AIM2, absent in melanoma 2; BMDM, bone marrow-derived macrophage; CARD, caspase recruitment domain; DC, dendritic cell; GMDC, GM-CSF-induced bone marrow dendritic cell; IKK, IκB kinase; IRF, IFN regulatory factor; LRR, leucine-rich repeat; mon, multiplicity of infection; MSU, monosodium urate; NDV, Newcastle disease virus; NLR, nucleotide-binding domain and leucine-rich repeat containing gene family receptor; PAMP, pathogen-associated molecular pattern; PEC, peritoneal exudate cell; poly (dA:dT), poly(deoxyadenylic-deoxycytidylic) acid; poly-IC, polyinosinic-polycytidylic acid; PRR, pattern-recognition receptor; RLR, RIG-I–like receptor; WT, wild type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1002094
responses (23, 24). However, the in vivo function of NLRC5 in antiviral immune responses remains unclear. In this study, we have generated NLRC5-deficient mice and analyzed their phenotypes in terms of antiviral innate immune responses.

Materials and Methods

Generation of NLRC5-deficient mice

The NLRC5 gene was isolated from genomic DNA extracted from embryonic stem cells (GSI-I) by PCR. The targeting vector was constructed by replacing a 1.5-kb fragment encoding the NLRC5 open reading frame (exon 4) with a neomycin-resistance gene cassette, and an HSV-thymidine kinase gene driven by the PGK promoter was inserted into the genomic fragment for negative selection. After the targeting vector was electroporated into embryonic stem cells, G418 and ganciclovir doubly-resistant colonies were selected and screened by PCR and further confirmed by Southern blot analysis. Homologous recombinant embryonic stem cells were micro-injected into C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain NLRC5 mice and NLRC5+/+ mice. Mice were bred and maintained in a specific-pathogen-free facility at the Research Institute for Microbial Diseases in accordance with the specifications of the Association for Assessment and Accreditation of Laboratory Animal Care. All mouse protocols were approved by the Osaka University Animal Care and Use Committee. All mice were killed at 5–9 wk of age.

Plasmids

The cDNA fragments of human NLRC5 and NLRC5 full-length (aa 1–655) obtained by RT-PCR (Takara) were cloned into the pFlag-CMV-2 vector (Sigma-Aldrich). The sequences of all the constructs were confirmed using an ABI PRISM Genetic Analyzer (Applied Biosystems). Expression selected and screened by PCR and further confirmed by Southern blot analysis. Homologous recombinant embryonic stem cells were micro-injected into C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain NLRC5−/− and NLRC5+/− mice. Mice were bred and maintained in a specific-pathogen-free facility at the Research Institute for Microbial Diseases in accordance with the specifications of the Association for Assessment and Accreditation of Laboratory Animal Care. All mouse protocols were approved by the Osaka University Animal Care and Use Committee. All mice were killed at 5–9 wk of age.

FIGURE 1. Generation of NLRC5-deficient mice. A, Schematic diagrams of the genomic structure of the murine NLRC5 gene. The targeting vector and the predicted mutated allele are shown. Black boxes denote exons. E, EcoRI sites in the genome. B, Southern blot analysis of progenies from heterozygote intercrosses. Genomic DNA was extracted, digested with EcoRI, electrophoresed, and hybridized with the probe indicated in A. The analysis produced a single 11.0-kb band for WT mice (+/+), a 1.8-kb band for homozygous mutant mice (−/−), and both bands for heterozygous mice (+/−). C, GMDCs were prepared from WT (+/+), NLRC5−/− mice and infected with NDV (moi = 1.0) for the indicated times. Isolated total RNA samples were subjected to RT-PCR analyses for the expressions of NLRC5 and β-actin mRNAs.

FIGURE 2. Responses to TLR ligands in NLRC5-deficient mice. A, GMDCs prepared from WT (+/+) and NLRC5-deficient (−/−) mice were pretreated with or without IFN-γ (10 ng/ml) and stimulated with medium (lanes 1, 6), 1 ng/ml (lanes 2, 7), 10 ng/ml (lanes 3, 8), 100 ng/ml (lanes 4, 9) or 500 ng/ml (lanes 5, 10) LPS for 24 h. The culture supernatants were analyzed for their levels of IL-6 and TNF-α by ELISA. B, GMDCs were stimulated with CpG DNA 1 μM and MALP2 100 ng/ml for 24 h. The culture supernatants were analyzed for their levels of IL-6 by ELISA. C, GMDCs were stimulated with 100 ng/ml LPS for the indicated periods. Total RNA were subjected to quantitative PCR analyses for expression of IL-6, TNF, and Cxcl10. ND, not detected.
plasmids for pro–IL-1β and procaspase-1 were provided by Dr. Jürg Tschopp (University of Lausanne, Switzerland).

**Cells, viruses, bacteria, and reagents**

HEK293 cells were cultured in DMEM supplemented with 10% FCS. Peritoneal exudate cells (PECs) were collected 3 d after i.p. injection of 4% thioglycollate medium. Bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 μM 2-ME, and 10 ng/ml murine GM-CSF (PeproTech) or 40 ng/ml murine macrophage CSF (PeproTech) for 6–8 d or 4 d to allow differentiation to GM-CSF-induced bone marrow dendritic cells (GMDCs) or bone marrow-derived macrophages (BMDMs), respectively. Newcastle disease virus (NDV) and HSV-1 (F-strain) were described previously. *Salmonella typhimurium* (SL1334) (25) or *Listeria monocytogenes* (EGD) were grown in Bacto Brain Heart Infusion medium (BD Biosciences) while *Franciscella tularensis* spp novicida U112 was grown in Bacto Tryptic Soy Broth medium (BD Biosciences) with shaking at 37˚C. After determination of their number by the absorbance at 600 nm, the bacteria were washed twice and infected at each multiplicity of infection (moi) indicated in the figure legends. LPS from *Salmonella minnesota* Re-595, ATP, and calf thymus DNA were purchased from Sigma-Aldrich. CpG ODN 1668, polyinosinic-polycytidylic acid (poly-IC), and poly(deoxyadenylic-eoxythymidylic) acid [poly (dA:dT)] were purchased from Invivogen. MALP2 was purchased from Alexis Biochemicals. For stimulation, poly-IC or poly (dA:dT) was mixed with the chemicals. Transfection, immunoprecipitation, and immunoblotting

**RT-PCR**

Total RNA was isolated using the TRIzol reagent (Invitrogen) and reverse transcribed with RTase (Toyobo) according to the manufacturer’s instructions. PCR was then performed with the following primers: NLRC5, 5’-ATGACGCTGAGAGCATC-3’ and 5’-GGAGGTTCCATGGCAGA-3’; Ifnβ, 5’-CCA-

**FIGURE 3.*** Responses to poly-IC in NLRC5-deficient mice. A, GMDCs prepared from WT (+/+ ) and NLRC5-deficient (−/−) mice were pretreated with or without IFN-γ and stimulated with medium (lanes 1, 6), 1 μg/ml (lanes 2, 7), 10 μg/ml (lanes 3, 8), 50 μg/ml (lanes 4, 9) or 100 μg/ml (lanes 5, 10) poly-IC (pIC) for 24 h. The culture supernatants were analyzed for their levels of IFN-β, IP-10, and IL-6 by ELISA. B, GMDCs were stimulated with 100 μg/ml poly-IC for the indicated periods. Total RNA were subjected to quantitative PCR analyses for expression of IFN-β, Cxcl10, and IL-6. C, WT (n = 5) and NLRC5-deficient (n = 4) mice were injected i.p. with poly-IC. The IFN-β, IL-6, RANTES, and IL-1β levels were measured in serum samples prepared at the indicated times. ND, not detected.
Generation of NLRC5-deficient mice

To understand the physiologic roles of NLRC5, we generated NLRC5-deficient mice using a standard gene targeting method. We designed a targeting vector to disrupt exon 4 harboring the in-frame deletion of the oligomerization domain of NLRC5 (Fig. 1A). The heterozygosity and homozygosity of the obtained mice were verified by Southern blot analysis (Fig. 1B), and nullizygosity was confirmed by RT-PCR analysis using total RNA isolated from expected NLRC5-deficient and wild-type (WT) GMDCs after NDV infection (Fig. 1C). Mutant homozygous mice for the disrupted NLRC5 allele were born at the expected Mendelian ratio, grew healthy until 48 wk of age under specific pathogen-free conditions, and were fertile. No obvious changes in hematopoietic cell development were observed in NLRC5-deficient mice as determined by FACs analysis (Supplemental Fig. 1).

Normal inflammatory cytokine and type I IFN production after stimulation with agonists for TLRs, RLRs, and a DNA sensor

Initially, we examined the responses to bacterial LPS, a TLR4 ligand. We pretreated GMDCs derived from WT and NLRC5-deficient mice with or without IFN-γ and stimulated them with different concentrations of LPS (1–500 ng/ml). The production of IL-6 and TNF-α was comparable between WT and NLRC5-deficient cells at all concentrations examined, as measured by ELISA (Fig. 2A). Moreover, IL-6 production after treatment with CpG DNA (TLR9 ligand) or MALP2 (TLR2 ligand) was also comparable between WT and NLRC5-deficient GMDCs (Fig. 2B). We then stimulated GMDCs with LPS for different periods of time and subjected their total RNA to quantitative PCR analyses for mRNAs encoding IL-6, TNF-α, and IP-10 (Cxcl10). The expressions of these genes were comparable between WT and NLRC5-deficient cells at all time points examined (Fig. 2C). Moreover, results of the regular RT-PCR indicated that WT and NLRC5-deficient GMDCs displayed similar induction of TNF-α, IL-6, and RANTES after LPS treatment (Supplemental Fig. 2). Thioglycollate-elicited PECs derived from WT and NLRC5-deficient mice also displayed comparable production of IL-6 and RANTES after LPS treatment (Supplemental Fig. 3). These results indicate that NLRC5 is dispensable for TLR2-, TLR4-, and TLR9-induced cytokine production.

Next, we addressed responses to poly-IC, which is a dsRNA analog that acts as a ligand for MD2 and TLR3. We pretreated GMDCs with or without IFN-γ and then stimulated them with various concentrations of poly-IC to measure IFN-β, IP-10, and IL-6 by ELISA. The production of these cytokines was normal in NLRC5-deficient GMDCs (Fig. 3A). Furthermore, the induction of IFN-β, Cxcl10, and IL-6 mRNA was also normal in the absence of NLRC5 at the tested time points (Fig. 3B). We next injected poly-IC into mice and measured the serum cytokine levels by ELISA. The levels of IFN-β, IL-6, RANTES, and IL-1β were comparable between WT and NLRC5-deficient mice (Fig. 3C).

Statistical analysis

Statistical significance between groups was determined by two-tailed Student t test and ANOVA test. Differences were considered as significant when p ≤ 0.05, and highly significant when p ≤ 0.01.

RESULTS

Generation of NLRC5-deficient mice

FIGURE 4. Responses to RNA virus, DNA virus, and immunostimulatory DNA in NLRC5-deficient mice. A, GMDCs were prepared from WT (+/+) and NLRC5-deficient (−/−) mice and infected with NDV (NDV-1, moi = 1.0; NDV-5, moi = 5) for 24 h. The culture supernatants were analyzed for their levels of IFN-β, IL-6, TNF-α, Rantes, and IL-1β by ELISA. B, Total RNA prepared from GMDCs infected with NDV (moi = 1.0) for the indicated times were subjected to RT-PCR analysis for the expressions of the indicated genes. C, GMDCs were unstimulated (−) or stimulated with poly (dA:dT) (0.1, 1.0, or 10 μg/ml) for 24 h. The culture supernatants were analyzed for the production of IFN-β and IL-6 by ELISA. D, GMDCs were infected with HSV-1 (moi = 10) for the indicated times, and isolated total RNA samples were subjected to RT-PCR analyses for the mRNA expressions of the indicated genes. Similar results were obtained from two independent experiments. ND, not detected.
Cell lysates were immunoblotted with an Ab against the p10 subunit of caspase-1. With L. monocytogenes stimulation with poly (dA:dT) or calf thymus DNA or infection (Fig. 4), IL-6 were comparable between WT and NLRC5-deficient GMDCs (poly [dA:dT]) and found that the productions of IFN-β mRNAs postinfection with NDV, which is recognized by RIG-I, (Fig. 4) were also comparable between WT and NLRC5-deficient GMDCs (Fig. 4A). Moreover, the induction of IFN-β and IL-6 mRNA after stimulation with poly (dA:dT) or calf thymus DNA or infection with L. monocytogenes, which is known to introduce DNA into the cytosol, was also comparable between WT and NLRC5-deficient GMDCs (Fig. 4B). We investigated further whether NLRC5 has the ability to activate caspase-1. HEK293 cells were transiently cotransfected with expression plasmids encoding pro–IL-1β or procaspase-1 together with control or NLRC5 expression plasmids, followed by measurement of the IL-1β levels in the culture supernatants by ELISA. IL-1β production was increased when pro–IL-1β and caspase-1 were coexpressed with NLRC5 (Fig. 5A). To further confirm that caspase-1 is activated by NLRC5, we examined the cleavage of caspase-1. FLAG–caspase-1 was cleaved in cells expressing NLRC5 ΔLRR, whose overexpression showed higher production of IL-1β than full length NLRC5 (unpublished observations) as determined by immunoblot analysis using an anti-FLAG Ab (Fig. 5B).

Normal induction of IL-1β after stimulation with NLRP3, NLRC4, and AIM2 activators

The finding that NLRC5 overexpression promotes IL-1β secretion prompted us to investigate whether NLRC5 regulates IL-1β production. We first prepared various cell types such as PECs, BMDMs, and GMDCs and titrated the amount of NLRP3 inflammasome activators to determine optimal concentration for IL-1β production. Our results (some data not shown) and previously reported results showed a similar pattern of IL-1β production irrespective of cell types such as PECs, BMDMs, or GMDCs (26, 27). Using optimal concentration of inflammasome activator, PECs were stimulated with monosodium urate (MSU), LPS plus ATP or nigericin, zymosan, or curdlan, which are known to activate NLRP3 inflammasome and induce comparable levels of IL-1β production between WT and NLRC5-deficient BMDMs (Supplemental Fig. 4). Infection with HSV-1, a DNA virus, resulted in comparable levels of TNF-α, IL-6, RANTES, and IFN-β mRNA induction in both WT and NLRC5-deficient GMDCs (Fig. 4D). These results suggest that NLRC5 is dispensable for cytosolic DNA-induced innate immune responses. Activation of caspase-1 and induction of IL-1β production by NLRC5 overexpression

We next evaluated the role of NLRC5 in cytosolic dsDNA-driven innate immune responses. We stimulated GMDCs with B-DNA (poly [dA:dT]) and found that the productions of IFN-β and IL-6 were comparable between WT and NLRC5-deficient GMDCs (Fig. 4C). We then examined the role of NLRC5 in cytosolic dsDNA-driven innate immune responses. We stimulated GMDCs with B-DNA (poly [dA:dT]) and found that the productions of IFN-β and IL-6 were comparable between WT and NLRC5-deficient GMDCs (Fig. 4C). Moreover, the induction of IFN-β and IL-6 mRNA after stimulation with poly (dA:dT) or calf thymus DNA or infection with L. monocytogenes, which is known to introduce DNA into the cytosol, was also comparable between WT and NLRC5-deficient GMDCs (Fig. 4C).
between WT and NLRC5-deficient macrophages (Fig. 6A, Supplemental Fig. 5). Moreover, we found that IL-1β production by curdlan was comparable between WT and NLRC5-deficient dendritic cells (DCs; Supplemental Fig. 6), suggesting that NLRC5 was dispensable for the NLRP3 inflammasome-triggered IL-1β production in macrophages and GMDCs.

Next, we simulated GMDCs with a different amount of an AIM2 inflammasome activator, poly (dA:dT). The production of IL-1β was comparable between WT and NLRC5-deficient GMDCs (Fig. 6B). Furthermore, the production of IL-1β was also comparable between WT and NLRC5-deficient PECs (data not shown). We then infected the cells with Francisella tularensis, which induces IL-1β production through AIM2 inflammasome. The production of IL-1β by PECs infected with F. tularensis was modestly reduced in culture supernatants from NLRC5-deficient cells compared with those from WT cells (Fig. 6C). However, when F. tularensis was infected into LPS-pretreated macrophages, there was no significant difference in IL-1β production between WT and NLRC5-deficient cells (Fig. 6C), suggesting that NLRC5 is not required for AIM2 activator-induced IL-1β production in macrophages and GMDCs. Finally, we examined caspase-1 activation triggered by S. typhimurium, which activates NLRC4 and NLRP3 inflammasomes (28), in NLRC5-deficient macrophages. In both WT and NLRC5-deficient BMDMs, caspase-1 activation was similarly induced as determined by immunoblot analysis using an anti–caspase-1 p10 Ab (Fig. 6D). Furthermore, in vivo poly-IC–induced IL-1β production was comparable in both WT and NLRC5-deficient mice (Fig. 3C). These observations suggest that NLRC5 is not required for IL-1β production in response to various tested stimulators of NLRP3, NLRC4, and AIM2 inflammasomes in macrophages.

Discussion

Recently, it was shown that NLRC5 has a role to control antiviral innate immune responses. Cui et al. (22) indicated that NLRC5 interacts with IKKα and IKKβ to suppress NF-κB activation; they also showed that NLRC5 interacts with RIG-I via its CARD to suppress RLR signaling. In contrast, reports by Kuenzel et al. (23) and Neerincx et al. (24) indicated that NLRC5 increases IFN-β and NF-κB promoter activation and enhances RLR-mediated antiviral innate immune responses. Therefore, it may be possible that NLRC5 positively and negatively controls RLR-mediated signal pathways.

In our reporter analyses, overexpression of NLRC5 in HEK293 cells significantly suppressed NF-κB activation induced by MyD88, TRAF6, and TRAF2, which are downstream molecules for TLR or TNFRs, but did not abrogate IKKβ-mediated NF-κB activation (Supplemental Fig. 7). These findings suggest that NLRC5 negatively regulates NF-κB activation upstream of IKKβ in TLR and TNFR signaling. Notably, we found that NLRC5 forms a complex with TBK1 (Supplemental Fig. 8A, 8B). We performed yeast two-hybrid screening using TBK1 as a bait to identify NLRC5 (Supplemental Fig. 8A). NLRC5 interacted with TBK1 but not IKKα, another IKK-related kinase, in yeast and HEK293 cells (Supplemental Fig. 8A, 8B). NLRC5 overexpression suppressed TBK1-mediated IFN-β promoter activation in a dose-dependent manner (Supplemental Fig. 8C). Our in vitro data implicated that NLRC5 negatively regulates TBK1 and NF-κB activation. Although the reason for the discrepancies between our data on in vitro characterization of NLRC5 and the findings in the previous in vitro studies is unknown, it is possible that differences in the expression plasmids and cell lines used in each study influenced the results.

It is noteworthy that, under in vivo conditions, NLRC5 was not required for the induction of inflammatory cytokines and type I IFN by macrophages and DCs in response to LPS, poly-IC, dsDNA, NDV, HSV-1, and L. monocytogenes. These findings indicate that NLRC5 deficiency does not affect TLR, RLR, and DNA sensor signaling, at least in mice. However, it cannot be ruled out the possibility that NLRC5 plays a critical role in certain cell types or in unidentified signaling pathways.

Our findings that NLRC5 overexpression induced caspase-1–dependent IL-1β production implicated that NLRC5 forms an inflammasome. However, NLRC5 deficiency did not abrogate IL-1β production in response to various stimulations, including LPS plus ATP or nigericin, MSU, curdlan, poly-IC, poly (dA:dT), and F. tularensis infection, which activate NLRP3 or AIM2 inflammasomes in macrophages. Moreover, Salmonella–induced caspase-1 activation, which was NLRC4 and NLRP3 inflammasome dependent, was intact in NLRC5-deficient macrophages (28). Therefore, NLRC5 inflammasomes may be activated by unknown pathogenic or endogenous activators, or they may play roles in other cell types. Future identification of NLRC5 inflammasome activators is required to understand the physiologic role of NLRC5 in innate immune responses.

Acknowledgments

We thank Dr. Y. Kawaguchi, Dr. W. Hardt, and Dr. O. Fujita for providing the HSV-1, S. typhimurium, and F. tularensis, respectively; Dr. Jürg Tschopp for providing expression plasmids; Dr. Y. Torii, Dr. S. Sato, and Dr. T. Satoh for helpful discussions; Y. Fujisawa, M. Kumagai, A. Miyabe, and A. Shibano for technical assistance; and M. Hashimoto and E. Kamada for secretarial assistance.

Disclosures

The authors have no financial conflicts of interest.

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