CUTTING EDGE

Cutting Edge: Role of Toll-Like Receptor 1 in Mediating Immune Response to Microbial Lipoproteins[1]

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The Toll-like receptor (TLR) family acts as pattern recognition receptors for pathogen-specific molecular patterns (PAMPs). TLR2 is essential for the signaling of a variety of PAMPs, including bacterial lipoprotein/lipopeptides, peptidoglycan, and GPI anchors. TLR6 associates with TLR2 and recognizes diacylated mycoplasmal lipopeptide along with TLR2. We report here that TLR1 associates with TLR2 and recognizes the native mycobacterial 19-kDa lipoprotein along with TLR2. Macrophages from TLR1-deficient ($TLR1^{-/-}$) mice showed impaired proinflammatory cytokine production in response to the 19-kDa lipoprotein and a synthetic triacylated lipopeptide. In contrast, $TLR1^{-/-}$ cells responded normally to diacylated lipopeptide. TLR1 interacts with TLR2 and coexpression of TLR1 and TLR2 enhanced the NF-κB activation in response to a synthetic lipopeptide. Furthermore, lipoprotein analogs whose acylation was modified were preferentially recognized by TLR1. Taken together, TLR1 interacts with TLR2 to recognize the lipid configuration of the native mycobacterial lipoprotein as well as several triacylated lipopeptides. ***The Journal of Immunology*****, 2002, 169: 10–14.**

The Toll-like receptor (TLR)[4] family participates in innate immunity by detecting invading pathogens (1–3). So far, 10 members of the human TLR family have been discovered. Most of the known TLRs recognize discrete pathogen-associated molecular patterns (PAMPs) to trigger the activation of similar intracellular signaling pathways, leading to the nuclear translocation of a transcription factor, NF-κB. The signaling ultimately culminates in the production of proinflammatory cytokines to evoke host defense responses and alert acquired immunity. Recent studies disclosed the ligands for various TLRs. TLR2 recognizes a variety of bacterial components, such as peptidoglycan (PGN), bacterial triacylated lipoproteins, mycoplasmal diacylated lipoprotein, and GPI anchors from *Trypanosoma cruzi* (4–10). TLR4 is essential for responses to LPS, a glycolipid specific to Gram-negative bacterial cell walls. TLR5 is reported to recognize flagellin, a protein component of bacterial flagella. Furthermore, nucleotides specific to pathogens and nucleotide analogs are also detected by TLRs: TLR3, TLR7, and TLR9 participate in the recognition of viral dsRNA, imidazoquinolines, and bacterial DNA with unmethylated CpG motif, respectively (1–3, 11).

There is evidence that TLRs can form heterodimers, which furthers defines their ligand specificity. Notably, TLR6 has a unique property to recognize a mycoplasmal lipoprotein cooperatively with TLR2 (12, 13). TLR6-deficient ($TLR6^{-/-}$) mice failed to produce proinflammatory cytokines in response to diacylated mycoplasmal lipopeptides, termed macrophage-activating lipopeptide 2 kDa (MALP-2), whereas they responded normally to a triacylated bacterial lipopeptide. $TLR2^{-/-}$ macrophages did not respond to either of these lipopeptides (13). These observations indicate that TLR6 discriminates a subtle difference in the acylation of lipopeptides derived from microbial pathogens. Furthermore, these findings raised the possibility that TLR2 forms a heterodimer with a different TLR to recognize other PAMPs, in particular triacylated lipopeptides.

TLR1 shows high similarity with TLR6 (14). It was reported that overexpression of TLR1 inhibited the TLR2-mediated responses to phenol-soluble modulin secreted from *Staphylococcus epidermidis* (15). On the other hand, another report showed that TLR1 participates in the recognition of soluble factors released from *Neisseria meningitides* (16). However, the ligand of TLR1 in vivo is yet to be clarified. In the present study, we generated $TLR1^{-/-}$ mice and analyzed the role of TLR1 in the recognition of bacterial lipopeptides.

Materials and Methods

Generation of $TLR1^{-/-}$ mice

A genomic clone containing mouse TLR1 was isolated from the 129Sv murine genomic library (Clontech Laboratories, Palo Alto, CA). A targeting vector was designed to replace a portion of an exon containing aa

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[4] Abbreviations used in this paper: TLR, Toll-like receptor; PGN, peptidoglycan; PAMP, pathogen-associated molecular pattern; MALP-2, macrophage-activating lipopeptide 2 kDa; ES, embryonic stem; BCG, bacillus Calmette Guérin; HA, hemagglutinin; HEK, human embryonic kidney; Pam_3, *N*-palmitoyl-*S*-dipalmitoylglyceryl; *N*-Pam-5-Lau_2, *N*-palmitoyl-*S*-dilaurylglyceryl; Lau_3, *N*-lauryl-*S*-dilaurylglyceryl; Myr_3, *N*-myristyl-*S*-dimyristylglyceryl; CSK_4, Cys-Ser-$(Lys)_4$.

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575–795 of the mouse *Tlr1* gene with a neo cassette flanked by 1.0 kb of the 5′ genomic fragment and 10 kb of the 3′ genomic arm. An HSV-tk cassette flanked the 3′ genomic arm. A targeting vector was linearized with *Sal*I and introduced into E14.1 embryonic stem (ES) cells. We screened 125 of G418- and ganeyclovir-resistant clones for homologous recombination by PCR and confirmed by Southern blot analysis. Three ES clones were correctively targeted and were injected into C57BL/6 blastocysts. The chimeric mice were bred to C57BL/6 females to obtain F_1 offsprings. TLR1$^{-/-}$ mice were obtained by intercrossing heterozygotes. TLR1$^{-/-}$ mice and their wild-type littermates from these intercrosses were used for experiments.

Mice, bacteria, and reagents

TLR2$^{-/-}$ mice were generated by gene targeting as described previously (7). The *Mycobacterium bovis* bacillus Calmette Guérin (BCG) was purchased from Kyowa (Tokyo, Japan). The native *Mycobacterium tuberculosis* 19-kDa lipoprotein was purified as described elsewhere (4). A synthetic *N*-palmitoyl-*S*-dipalmitoylglyceryl (Pam_3) Cys-Ser-$(Lys)_4$ (CSK_4) and MALP-2 were as described previously (5, 8). A synthetic lipoprotein analog, JBT3002, is as described previously (17). Other lipopeptides carrying different N-terminal acyl functions such as *N*-palmitoyl-*S*-dilaurylglyceryl (*N*-Pam-*S*-Lau_2) CSK_4, *N*-lauryl-*S*-dilaurylglyceryl (Lau_3) CSK_4, and *N*-myristyl-*S*-dimyristylglyceryl (Myr_3) CSK_4 were synthesized by the Peptide Institute (Osaka, Japan).

Preparation of peritoneal macrophages and ELISA

Mice were injected i.p. with 2 ml of 4% thioglycolate (Difco, Detroit, MI). Three days later, peritoneal exudate cells were isolated from the peritoneal cavity. Then the cells were cultured for 2 h and adherent cells were used as peritoneal macrophages. Peritoneal macrophages (5×10^4) were cultured in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FCS and were stimulated with indicated bacterial components for 24 h. Concentration of TNF-α (Genzyme Techne, Minneapolis, MN) and IL-6 (R&D Systems, Minneapolis, MN) in culture supernatants were determined by ELISA.

Expression vectors

Human TLR1 tagged with hemagglutinin (HA) at the carboxyl terminus was generated by PCR and ligated into the expression plasmid pEF-BOS. pFLAG-TLR2 and pFLAG-TLR4 were as described previously (13).

Luciferase assay

Human embryonic kidney (HEK) 293 cells were transiently transfected with indicated vectors along with a pELAM luciferase reporter plasmid (18) and a pRL-TK (Promega, Madison, WI) for normalization of transfection efficiency by Lipofectamine 2000 (Invitrogen, San Diego, CA). Twenty-four hours after transfection, the cells were stimulated with 10 ng/ml Pam_3CSK_4 for 8 h. Then the cells were lysed and luciferase activity was measured using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instruction.

Immunoprecipitation and Western blotting

HEK293 cells were transiently transfected with 3 μg of Flag-tagged TLR2, TLR4, or 6 μg of HA-tagged TLR1 as indicated. After 36 h, the cells were lysed in the lysis buffer containing 1.0% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, and a protease inhibitor mixture tablet, Complete (Roche Diagnostics, Indianapolis, IN). The lysates were precleared for 1 h with protein G-Sepharose and immunoprecipitated with 2 μg of anti-Flag M2 Ab or 2 μg of anti-HA 12CA5 Ab and protein G-Sepharose for 12 h. The beads were washed with the lysis buffer four times and immunoprecipitated proteins were eluted in SDS-PAGE sample buffer, separated on SDS-PAGE, and transferred onto polyvinylidene difluoride membrane. HA-tagged TLR1 was detected with anti-HA Ab (Roche Diagnostics) and HRP-labeled anti-mouse Ig Ab. Flag-tagged proteins were identified with HRP-conjugated anti-Flag M2 Ab. Then the Abs were detected by the ECL system (DuPont, Boston, MA)

Results and Discussion

Generation of TLR1$^{-/-}$ mice

To investigate the functional role of TLR1, the mouse *Tlr1* gene was disrupted by homologous recombination in ES cells. A targeting vector was constructed for the deletion of a part of exon containing aa 575–795 of the mouse *Tlr1* gene (Fig. 1*A*). This portion corresponds to a transmembrane and cytoplasmic region of TLR1. Chimeric males were crossed to C57BL/6 and F_1 heterozygous offspring were obtained. Intercrosses of these heterozygotes gave rise to homozygotes deficient in *Tlr1*. The targeted disruption of the *Tlr1* gene was confirmed by Southern blotting of genomic DNA (Fig. 1*B*). Peritoneal macrophages from TLR1$^{-/-}$ mice did not express TLR1 mRNA (Fig. 1*C*). In contrast, the expression of TLR2 mRNA in TLR1$^{-/-}$ macrophages was normal compared with that in wild-type cells. TLR1$^{-/-}$ mice grew healthy, fertile, and did not show any obvious abnormalities for up to 6 mo. Lymphocyte populations in thymocytes and splenocytes were not altered in TLR1$^{-/-}$ mice (data not shown).

Involvement of TLR1 in the recognition of the native mycobacterial 19-kDa lipoprotein

To screen the components recognized by TLR1, we first examined the cytokine production from TLR1$^{-/-}$ macrophages in response to a variety of PAMPs purified from bacteria. These include the native 19-kDa lipoprotein purified from *M. tuberculosis*, LPS from

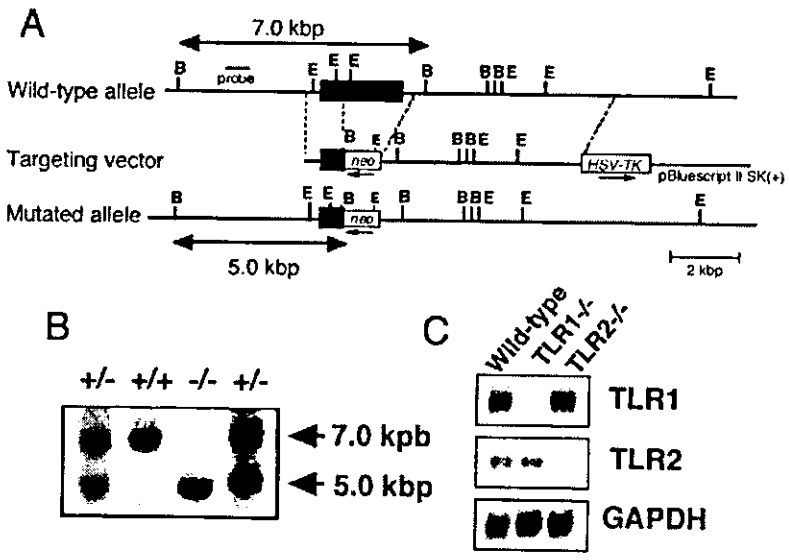

FIGURE 1. Establishment of TLR1-deficient mice. *A*, A mouse TLR1 genomic locus and the targeting vector. A filled box denotes a coding exon. Restriction enzymes: B, *Bam*HI, E, *Eco*RI. *B*, Southern blot analysis of genomic DNA extracted from mouse tails digested with *Bam*HI. DNA was electrophoresed, transferred to nylon membrane, and hybridized with the radiolabeled probe indicated in *A*. *C*, Northern blotting analysis of thioglycolate-elicited peritoneal macrophages. Total RNA (10 μg) was electrophoresed, transferred onto nylon membrane, and hybridized with a cDNA probe for TLR1. The same membrane was rehybridized with that for TLR2 and GAPDH.

Salmonella minnesota Re595, and PGN from *Staphylococcus aureus*. Thioglycolate-elicited peritoneal macrophages from wild-type and TLR1$^{-/-}$ mice were cultured in the presence of these PAMPs for 24 h, and the concentration of TNF-α in culture supernatant was measured. In response to the 19-kDa lipoprotein, wild-type macrophages produced TNF-α in a dose-dependent manner. In contrast, the production of TNF-α from TLR1$^{-/-}$ macrophages was markedly impaired for each concentration of lipoprotein tested (Fig. 2*A*). The production of IL-6 in response to the lipoprotein was also reduced in TLR1$^{-/-}$ macrophages compared with that of wild-type cells (Fig. 2*B*). When stimulated with LPS and PGN, TLR1$^{-/-}$ macrophages produced TNF-α in a dose-dependent manner to almost the same extent as wild-type cells (Fig. 2, *C* and *D*). We next examined whether TLR1 is involved in the recognition of whole mycobacteria. Peritoneal macrophages were cultured with increasing amounts of live *M. bovis* BCG for 24 h, and the concentration of TNF-α in culture supernatant was measured. As shown in Fig. 2*E*, the ability to produce TNF-α in response to BCG was partially impaired in TLR1$^{-/-}$ macrophages. These results indicate that TLR1 is involved in the recognition of the 19-kDa lipoprotein purified from mycobacteria as well as live mycobacteria.

TLR1 enhances synthetic triacyl lipopeptide-mediated responses

Lipoproteins are produced by a variety of pathogens including mycobacteria, Gram-negative bacteria, and *Mycoplasma* species (19). The N-terminal acylated lipopeptide region is responsible for the immunostimulatory activity of bacterial and mycoplasmal lipoproteins. Bacterial and mycoplasmal lipoproteins differ in the degree of acylation of N-terminal cysteine. Lipoproteins of bacteria are triacylated, whereas those of mycoplasma are diacylated (20). Synthetic lipoprotein analogs consisting of a palmitoyled version of *N*-acyl-*S*-diacyl cysteine and *S*-diacyl cysteine mimic the immunostimulatory activity of bacterial and mycoplasmal lipoprotein, respectively (21, 22).

We have previously shown that TLR2 is essential for both tri- and diacylated lipopeptide response and TLR6 specifically recognizes diacylated lipopeptide in conjunction with TLR2 (13). Cytokine production in response to the 19-kDa lipoprotein preparation was abrogated in TLR2$^{-/-}$ macrophages (23). All of these results suggested that TLR1 also cooperates with TLR2 to recognize triacylated lipoprotein. To clarify the chemical structure recognized by TLR1, we stimulated peritoneal macrophages from wild-type and TLR1$^{-/-}$ mice with the synthetic bacterial lipopeptide Pam_3CSK_4, and synthetic mycoplasmal diacylated MALP-2. TLR1$^{-/-}$ macrophages showed significantly impaired TNF-α production in response to Pam_3CSK_4 compared with wild-type cells, whereas TLR1$^{-/-}$ cells responded normally to MALP-2 (Fig. 3, *A* and *B*). These results indicate that TLR1 is involved in the recognition of triacylated bacterial lipoprotein. In addition, TLR1 and TLR6 differentially recognize TLR2 ligands, distinguishing the degree of acylation of the lipopeptide.

To further investigate whether the coexpression of TLR1, TLR2, and TLR6 results in the modulation of NF-κB activity in response to lipopeptide stimulation, HEK293 cells were cotransfected with TLR1, TLR2, and TLR6 expression vectors along with pELAM-luciferase reporter plasmid. Transfected cells were stimulated with 10 ng/ml Pam_3CSK_4 for 8 h, and luciferase activity was measured. As shown in Fig. 3*C*, the expression of TLR2 conferred the NF-κB activation in response to Pam_3CSK_4 stimulation and coexpression of TLR1 significantly enhanced the activation. In contrast, coexpression of TLR6 and TLR2 did not augment the NF-κB activation induced by Pam_3CSK_4 stimulation. These results indicate that TLR1 and TLR2, but not TLR6, are involved in the cooperative recognition of Pam_3CSK_4.

We then examined whether TLR2 and TLR1 interact in mammalian cells. HEK293 cells were cotransfected with Flag-tagged TLR2 or TLR4 and HA-tagged TLR1. Immunoprecipitation of HA-tagged TLR1 resulted in coprecipitation of Flag-tagged TLR2, but not of TLR4 (Fig. 3*D*). Reciprocally, HA-tagged TLR1 also coprecipitated with Flag-tagged TLR2. However, stimulation with Pam_3CSK_4 did not affect the extent of association between TLR1 and TLR2 (data not shown). These results suggest that TLR1 and TLR2 associate in a ligand-independent manner in HEK293 cells.

N-Pam-S-Lau$_2$ lipopeptides were preferentially recognized by TLR1

Although the response to Pam_3CSK_4 was significantly impaired in TLR1$^{-/-}$ mice, we can still observe TLR1-independent cytokine production. Since TLR1 and TLR6 discriminate subtle differences in the lipid moiety of lipopeptides, we hypothesized that there are some other ligands recognized by TLR1 more preferentially and that the configuration of lipid moiety is critical for the involvement of TLR1.

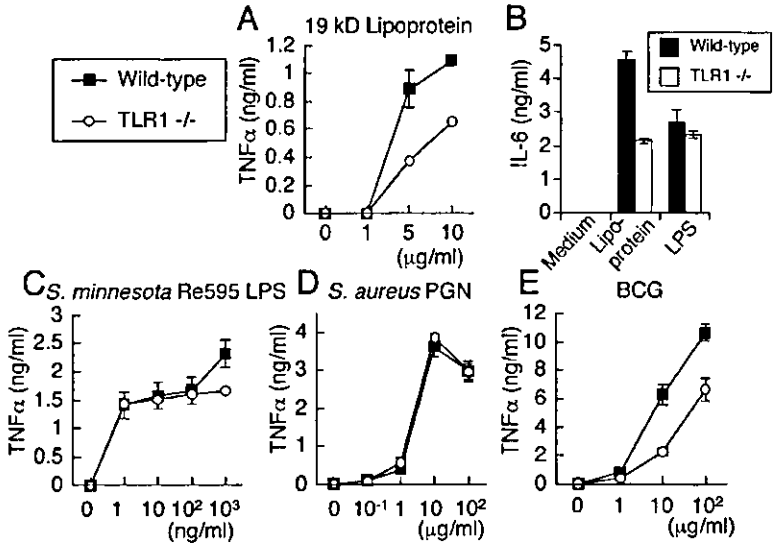

FIGURE 2. Impaired TNF-α production in response to the mycobacterial 19-kDa lipoprotein in TLR1-deficient macrophages. *A*, *C*, *D*, and *E*, Peritoneal macrophages (1×10^5) prepared from wild-type and TLR1$^{-/-}$ mice were stimulated with increasing concentrations of 19-kDa lipoprotein purified from *M. tuberculosis* (*A*), live *M. bovis* BCG (*C*), *S. Minnesota* Re595 LPS (*C*), *S. aureus* PGN (*D*) and live *M. bovis* BCG (*E*) for 24 h. Then TNF-α concentration in the culture supernatant was measured by ELISA. Data are shown as the mean ± SD of triplicate wells and are representative of three independent experiments. *B*, IL-6 concentration was measured in the culture supernatant of wild-type and TLR1$^{-/-}$ macrophages stimulated with 1 μg/ml 19-kDa lipoprotein and 100 ng/ml LPS. Data are shown as the mean ± SD of triplicate wells.

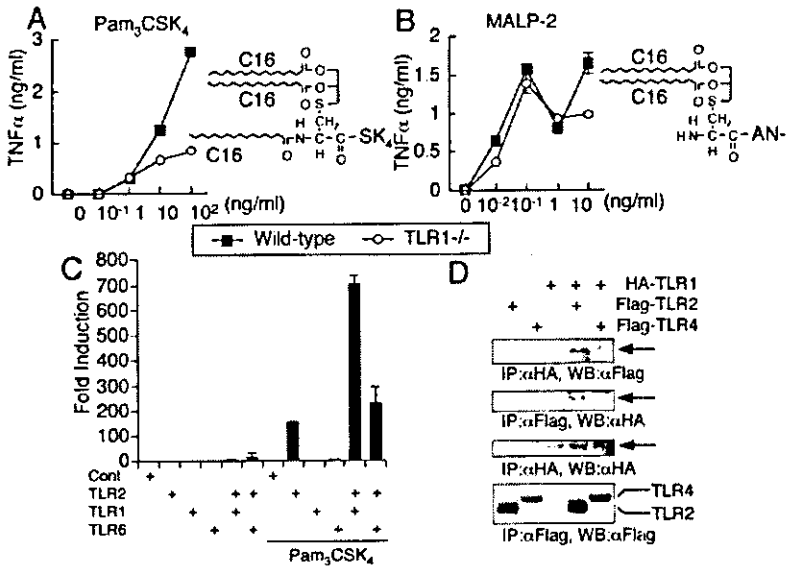

FIGURE 3. Involvement of TLR1 in synthetic bacterial lipopeptide recognition. *A* and *B*, Peritoneal macrophages (1×10^5) from wild-type and $TLR1^{-/-}$ mice were cultured with increasing concentrations of Pam_3CSK_4 (*A*) and synthetic MALP-2 (*B*) for 24 h. Then the concentration of TNF-α was measured. Data are shown as the mean ± SD of triplicate wells and are representative of three independent experiments. *C*, HEK293 cells were transiently cotransfected with control vector, TLR1, TLR2, and TLR6 expression vectors plus pELAM-luc reporter plasmid. After 24 h, the cells were stimulated with 10 ng/ml Pam_3CSK_4 for 8 h, and the cell lysates were assayed for luciferase activity. *D*, HEK293 cells were transiently transfected with the indicated combination of expression vectors for 4.0 μg/ml Flag-TLR2 or Flag-TLR4, and 6.0 μg/ml HA-TLR1. Total amount of plasmid DNA was kept constant with 10 μg by supplementing with empty vector. Thirty-six hours after transfection, the cells were lysed, immunoprecipitated with anti-Flag or anti-HA Ab (IP), and subsequently immunoblotted with anti-Flag or anti-HA Ab (WB) as indicated.

To further screen the specific ligands recognized by TLR1, we synthesized lipopeptides bearing different combinations of fatty acids at their N terminus. These include Myr_3CSK_4, Lau_3CSK_4, *N*-Pam-*S*-Lau_2CSK_4, and a lipoprotein analog used for anticancer therapy, JBT3002 (17) As shown in Fig. 4, they differ in the length of fatty acids substituted on the N-terminal cysteine of the peptides. The lipid moiety of *N*-Pam-*S*-Lau_2CSK_4 and JBT3002 are the same. We stimulated macrophages from wild-type, $TLR1^{-/-}$, and $TLR2^{-/-}$ mice with these compounds and measured TNF-α production. All of these synthetic lipopeptides activated wild-type cells to produce TNF-α in a dose-dependent manner (Fig. 4). Macrophages from $TLR2^{-/-}$ mice did not produce any detectable TNF-α in response to either of these lipopeptides. The ability of $TLR1^{-/-}$ cells to produce TNF-α was also impaired in response to Myr_3CSK_4 and Lau_3CSK_4 (Fig. 4, *A* and *B*). Interestingly, when stimulated with *N*-Pam-*S*-Lau_2CSK_4 and JBT3002, the production TNF-α of was profoundly defective in $TLR1^{-/-}$ cells, indicating that a subtle difference in lipid moiety of lipoprotein is critical for the TLR1 requirement (Fig. 4, *C* and *D*).

In summary, the present study provides evidence that TLR1 is involved in the recognition of triacylated lipoproteins as well as mycobacterial products. TLR1 and TLR2 cooperate to detect Pam_3CSK_4 by interacting in each other, indicating that TLR2 pairs with TLR1 or TLR6 to recognize different PAMPs. However, it is still unknown whether TLR2 forms a heterodimer with other TLR or whether there exists a large receptor complex consisting of TLR1, 2, 6, and others. Further studies will clarify the exact components of the receptor complex. In addition, the response to PGN was not abrogated in either TLR1-or TLR6-deficient mice. It is possible TLRs other than TLR1 and TLR6 pair with TLR2 to recognize PGN or TLR2 alone may be sufficient to detect it. Since TLR10 is highly homologous to both TLR1 and TLR6, it is also a candidate to form a pair with TLR2 to recognize PGN (24). Finally, *N*-palmitoyl-*S*-lauryl lipopeptide and its analog were preferentially recognized by TLR1. Although the structural basis for the TLR1 requirement remains unclear, these compounds are useful to study the specific role of TLR1 in vivo. Investigations pursuing TLR1-specific agonists/antagonists may give us a new strategy to design adjuvants and treatments for disease in which triacylated lipoproteins are involved in pathogenesis.

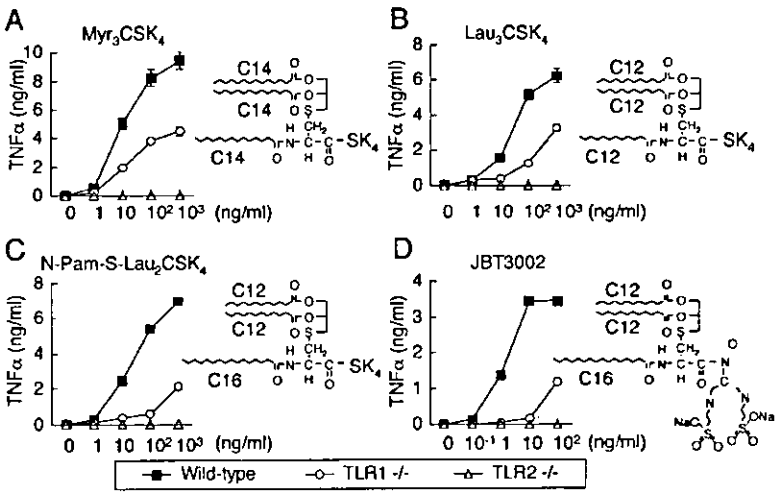

FIGURE 4. Differences in the lipoylation altered TLR1 responsibility. Peritoneal macrophages (1×10^5) from wild-type, $TLR1^{-/-}$, and $TLR2^{-/-}$ mice were stimulated with increasing concentrations of Myr_3CSK_4 (*A*), Lau_3CSK_4 (*B*), *N*-Pam-*S*-Lau_2CSK_4 (*C*), and JBT3002 (*D*) for 24 h. Then the concentration of TNF-α was measured. The results are shown as the mean ± SD of triplicate wells and are representative of three independent experiments.

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THE JAPANESE SOCIETY FOR TUBERCULOSIS

結核菌培養検査で陽性となるまでに時間を要した全身播種結核患者の1例

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要旨：80歳，男性。発熱，咳，食欲不振を主訴に2000年6月に近医を受診した。患者は原因を特定できないまま細菌性肺炎と診断され抗菌薬による治療を受けた。1カ月後，改善を認めないため入院となった。入院後，気管支洗浄液から抗酸菌が検出され当科紹介入院となった。入院時の喀痰塗抹検査にて抗酸菌が陽性でPCRにて結核菌と判明した。抗結核剤の投与（各々1日量；INH 300 mg，RFP 450 mg，EB 1000 mg，PZA 1000 mg）を開始した。しかし，入院後，患者の意識レベルの低下を認めた。頭部MRIでは両側前頭～頭頂部の皮質内に多数の小結節影を認めた。髄液中に抗酸菌は検出されなかったが，結核性髄膜炎を疑い，ステロイドを投与された。髄液所見およびMRI所見にて結核性髄膜炎と診断した。入院後9日目に患者は結核性髄膜炎のため死亡した。喀痰と髄液を小川培地にて培養したところ，いずれもコロニー形成まで14週間を要した。死後の剖検では，肺，肝臓，腎臓，膵臓にびまん性に結核結節の形成を認めた。試験管内において培養増殖速度が遅い結核菌によって発症した全身播種型結核症例を示した。

キーワーズ：粟粒結核，結核性髄膜炎，病原性，培養，抗TBGL抗体

はじめに

抗結核薬の普及とともに，我が国をはじめ先進諸国においては着実に結核患者の減少がもたらされ，死亡率も著明に改善した。しかし，未だに本邦では年間約3000人の結核死を認め，またそれまで減少を続けてきた罹患率は平成9年には増加傾向に転じている。今回われわれは結核菌の全身播種により死亡した剖検例を経験したので，この症例の検査結果および組織所見につき，若干の文献的考察を加え報告する。

症　例

症　例：80歳，男性。

主　訴：発熱。

既往歴：30歳頃より高血圧，糖尿病に罹患し加療されている。79歳時に狭心症と下肢閉塞性動脈硬化症に対しバイパス術を施行された。

家族歴：兄，脳血管障害で死亡。姉，糖尿病。

現病歴：平成12年4月半ば頃より，微熱，咳，食欲不振が認められ，6月中旬に近医を受診した。胸部X線写真にて両側上肺野に粒状影を伴う浸潤影を認め，血液検査でCRPの上昇を認めた。喀痰検査では，結核菌および一般細菌は検出されなかったが肺炎として，外来での抗生物質の内服治療を受けた。しかし臨床症状および胸部X線写真上の所見に改善を認めないため，7月24日同病院に入院となった。入院後の気管支肺胞洗浄液の塗抹検査にて，Gaffky 1号が検出されたため，7月27日当科を紹介され入院となった。

入院時現症：身長175 cm，体重44 kg，意識清明，血圧148/78 mmHgで左右差認めず，脈拍113/分で不整，呼吸数24/分，体温37.9 ℃，眼瞼結膜黄疸および貧血なし，胸部理学的所見は両側上肺野に一致して湿性ラ音を

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認め，収縮期心雑音を聴取した。

入院時検査所見（Table 1）：低蛋白血症，低K血症を認めたほか，血算，生化学検査で異常を認めなかった。CRPは3.3 mg/dlと上昇を認めたが，赤沈の亢進は認めなかった。免疫グロブリン値は正常範囲内であったが抗TBGL抗体は陰性であった。ツ反は陽性であった。

胸部X線写真（Fig. 1）：両側上肺野を中心に，辺縁が不明瞭な融合傾向に富む粒状影と結節影を含む浸潤影を認めた。

胸部CT（Fig. 2）：両側上肺野に大小不同の直径5～10 mmの小結節陰影を認め，また全肺野に無数の粟粒大陰影を認めた。

心電図：V1～4にかけてQSパターンを認めた。

喀痰検査：喀痰塗抹Ziel-Neelsen染色にてGaffky 1号，PCRで*Mycobacterium tuberculosis*と判定された。小川培地での培養では14週目にて陽性となった。一般細菌は陰性だった。

入院後経過：当院入院時の喀痰検査においても*Mycobacterium tuberculosis*を認め結核性肺炎の診断のもとに，抗結核剤の投与（INH 300 mg/日，RFP 450 mg/日，EB 1000 mg/日，PZA 1000 mg/日）を開始した。入院翌日，意識レベルの低下（JSC10）があり項部硬直を認めたが，四肢麻痺はなかった。髄液検査（Table 2）を実施したところ，圧の上昇，単核球優位の細胞数増加，蛋白量増加および糖減少（髄液糖/血糖：41%）を認めた。結核菌塗抹検査・PCR検査では共に陰性であった。頭部MRI（Fig. 3）では，両側前頭部脳表に硬膜下水腫と思われるCSF intensityの液貯留を認め，造影にて左右，特に左大脳半球のシルビウス裂近傍部を中心に脳表に沿って増強効果が著明に認められた。また，両側前頭～頭頂部の皮質内に散在性に直径1～2 mmの結節影を認めた。臨床ならびに検査所見から結核性髄膜炎と診断した。その後，急激に意識障害が進行し経口摂取も不能となったため，胃管より抗結核剤の投与を続行したが，入院9日目に結核性髄膜炎にて死亡した。

剖検所見：臓器は家族の強い希望により，胸部正中切開のみ許可され，腹部臓器の摘出は困難であったが，可及的に摘出した。肉眼的所見；両側全肺野に無数の粟粒大の白色結節を認めた。右肺上葉には充実性の硬い部分を認め，結核の原発巣と考えられた。粟粒大の白色結節は腎臓・肝臓・膵臓にも認められた。心臓は前壁中隔壁に直径1 cmの線維化部分を認め陳旧性の心筋梗塞を示唆させた。

顕微鏡学的所見；肺（Fig. 4），肝，腎，膵組織全体に無数の粟粒大～小豆大の結核結節が認められ，その中心は凝固壊死に陥りその周囲に類上皮細胞，さらにその周

Table 1 Laboratory data on admission

Hematology			Serological test		
WBC	2900	/μl	IgG	1193	mg/dl (820～1740)
seg	63	%	IgA	158	mg/dl (90～400)
band	18	%	IgM	151	mg/dl (31)
lymph	15	%	CRP	3.3	mg/dl
mono	3	%	ESR	5	mm/hr
RBC	446×10⁴	/μl			
Hb	13.0	g/dl	Blood gas analysis (O2 2L/min nasal)		
Plt	15.3×10⁴	/μl	pH	7.472	
			PaO_2	97.5	mmHg
Blood Chemistry			$PaCO_2$	38.1	mmHg
T.P	5.2	g/dl			
Alb	3.0	g/dl	PPD (mm) 12×6/25×12		
T.Bil	1.0	mg/dl			
GOT	20	IU/l	sputa examination		
GPT	12	IU/l	General bacteria normal flore		
LDH	465	IU/l	Acid-fast bacilli	smear	Gaffky 1
Alp	239	IU/l		culture	positive at 16 weeks
ChE	120	IU/l		PCR	MT*
BUN	11	mg/dl	Cytology	class I	
Cr	0.5	mg/dl			
Na	133	mEq/l	Anti-HIV antibody	negative	
K	2.9	mEq/l			
Cl	95	mEq/l	Anti-TBGL antibody	0.9	U/ml (cut off index<2.0 U/ml)
FBS	130	mg/dl			
GlyHbAlc	6.60	%	β-D-glucan	3	pg/ml

**Mycobacterium tuberculosis*

囲にリンパ球の浸潤を認めた。加えて右肺尖部の石灰化（均一紫色の索状物質）や胸膜の肥厚・縦隔リンパ節の炭粉沈着の所見は，陳旧性肺結核の存在を強く裏付ける所見であった。

髄液中の菌の証明：剖検時に採取した髄液から結核菌を証明（Fig. 5）したが喀痰と同様培養陽性まで14週間を要した。

考　察

症例は，当院入院後，急激な経過で悪化し死亡に至り，剖検の結果，多臓器におよぶ粟粒結核症と診断された。

われわれの施設では結核菌検査で塗抹あるいはPCR

Table 2 Cerebrospinal fluid data

appearance	yellow, clear
pressure	180 mmH_2O
cells	35:5 (mono:poly) /μl
protein	259 mg/dl
glucose	62 mg/dl (FBS;151 mg/dl)
Cl	107 mEq/l
Pandy	2+
Tryptophan	positive
Fibrin	−
General bacteria	normal flore
Acid-fast bacilli	
smear	negative
PCR	negative
Cytology	class I

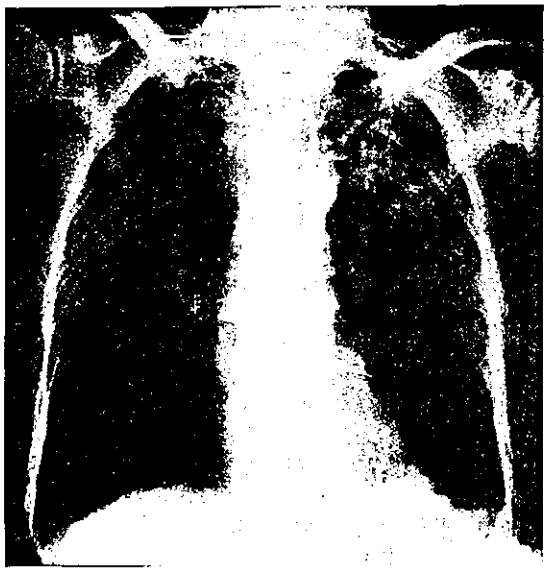

Fig. 1 X-ray film of chest, showing unclear edged small nodular shadow on bilateral upper lobes

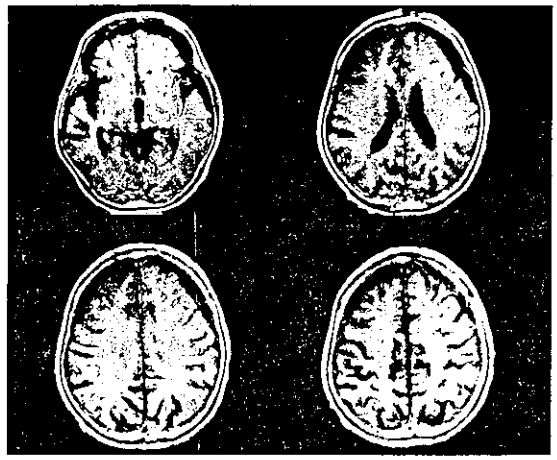

Fig. 3 MRI of the head, showing round enhancing lesions on cortex of bilateral anterior and temporal area

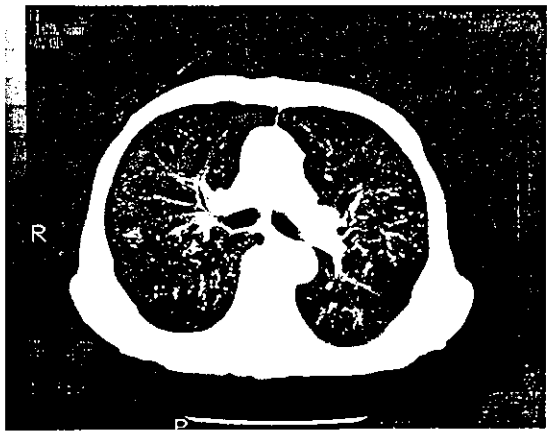

Fig. 2 CT scan of the chest, showing numerous and disseminated nodules in whole lung

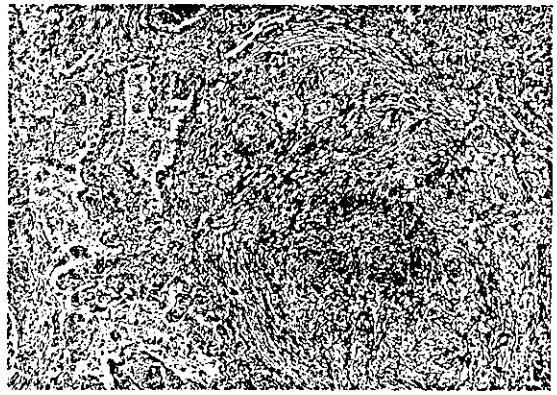

Fig. 4 Specimen from lung granuloma, showing a granuloma with giant cells (Hematoxylin and Eosin. ×100)

Fig. 5 MTB in CSF, obtained after centrifugation at 3000 rpm for 15 min (Auramine-Rhodamine. × 400)

が陽性になった場合は早期に培養を中断せず約半年間は小川培地にて培養（初代分離）を続けて菌の増殖の有無を確かめている。ほとんどのケースでは培養開始後2～4週間の時期に陽性となるが本症例から検出された菌は，可視的コロニー形成まで14週間を要し非常に発育の遅い菌であった。当院入院後の喀痰採取時期は抗結核剤開始前であり，試験管内での結核菌増殖速度に抗結核剤の影響は関与していない。剖検時に採取した髄液検体も慎重に観察した結果，培養開始後14週目に陽性となった。宿主内での結核菌の増殖速度の測定は困難であるが，本症例においては，発症後急速に進行し，2カ月で死亡し，全身に播種性の肉芽腫を生じた点より，生体内での菌の増殖速度は極めて速かったと思われる。しかしながら試験管内での増殖速度が遅いことが今回の症例における菌の特徴であった。その理由としては，喀痰塗抹のGaffky号数が低く，増殖開始時の細菌数が少ないことも否定はできない。しかし髄液由来菌においても培養速度は低く，原因菌の特徴と思われる。一般的には *in vivo* での増殖速度は，*in vitro* 内に比し様々な影響を受ける[1]。代表的な例としてアメリカ南部で大流行した結核菌臨床株CDC1551[2]を別の臨床株HN60，HN878ならびに実験室株H37Rv，Erdmanと，増殖速度および宿主側の免疫応答について比較した実験[3]では，感染初期（1～14日）の増殖速度はCDC1551，HN60，HN878，H37Rvにおいてほぼ差が無かったものの，その後はCDC1551の増殖速度が他の菌株に比較して明らかに遅くなり，CDC1551感染マウス群が他の感染マウス群に比べ明らかに長い生存率を示した。その理由としてCDC1551が他の菌株に比較し宿主側の免疫応答を，より速く強力に導き出すことが明らかとなった。感染当初，増殖速度の速い菌が宿主側の免疫応答の作用によって増殖速度が遅くなったと考えられる。

本症例の生体内での抗結核免疫能として，細胞性免疫の指標であるツベルクリン反応が陽性であり，また，各臓器の組織学的所見でも類上皮細胞肉芽腫の形成が多数認められ，細胞性免疫の大きな異常はなかったと推察される。液性免疫として，結核菌の壁成分であるcord factorをその抗原成分の一部に含む抗TBGL抗体を測定した。液性免疫の指標である免疫グロブリン（IgG，IgA，IgM）値は正常範囲内であったが，抗TBGL抗体は認められなかったことより結核菌に対する液性免疫の部分的な低下を示唆している。本患者が，糖尿病に罹患し，さらに高齢であったことによる可能性もある。

一方で，宿主内での結核菌の増殖に関連する因子としてerp遺伝子が報告されている[4]。erp遺伝子はmycobacterial proteinであるexported repetitive protein（ERP）をencodeしている遺伝子として名づけられたが，ERPはファゴゾームの再構築に関与しており，erp遺伝子が変異しているBCG菌をマウスに感染させた場合，菌は速やかに排除されその増殖速度は非常に緩やかである。また，本症例では剖検の結果，多臓器に結核結節の存在が認められたが，肺外結核をもたらす結核菌側の要因として，最近Petheらは結核菌から発現されるheparin-binding hemagglutinin（HBHA）因子を報告した[5]。通常，結核菌はmacrophageによって取り込まれphagosome内で生存し続けるがHBHAの存在下では上皮細胞（特にⅡ型肺胞上皮細胞）内への結核菌の接着・侵入を許し，肺外結核症の発症に深く関与しているとされている。今後，本症例の結核菌においてもerp遺伝子やHBHA因子などの遺伝子学的検討もし，*in vivo* と *in vitro* の増殖速度の乖離の原因を明らかにしていく必要があると考えられる。

今回の症例は剖検の結果，多臓器に肉芽腫の形成が認められ粟粒結核症と診断されたが，その菌の特徴として培養で肉眼的に可視的コロニーを形成するまでに長期間を要した興味深い1例として報告した。

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Case Report

A CASE OF DISSEMINATED TUBERCULOSIS REQUIRING EXTENDED PERIOD FOR THE IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* ON CULTURE

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Abstract A 80-year-old male visited an outpatient department of a nearby hospital complaining of fever, cough, and poor appetite on June 2000. The patient was diagnosed as bacterial pneumonia and was treated with antibiotics although specific cause could not be identified. After one month, he was hospitalized due to lack of improvement. After admission, acid-fast bacilli (AFB) was found from the bronchial washing. The patient was then transferred to our hospital. Upon admission, sputum smear examination was positive for AFB and MTB was confirmed by PCR. Therapy was initiated with INH 300 mg, RFP 450 mg, EB 1000 mg, and PZA 1000 mg, orally daily. However, on the day following the admission, he became unconscious. Brain MRI showed several small granulomas on the cortex of the bilateral anterior and temporal brain. Although AFB was not detected from the cerebrospinal fluid, tuberculous meningitis was suspected and steroid was given. Nine days after admission, the patient died due to tuberculous meningitis. The isolation of MTB had been attempted on Ogawa culture medium using patient's sputum and liquor, and it took 14 weeks to find colony growth both from sputum and liquor. In the autopsy, numerous granulomas were detected in his lung, liver, kidney, and pancreas. These findings indicate that disseminated growth of MTB occurred *in vivo* in spite of very slow growth of MTB *in vitro*.

Key words: Miliary tuberculosis, Tuberculous meningitis, Virulence, Culture, Anti TBGL (tuberculous glycolipid) antibody

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B Cells Capturing Antigen Conjugated with CpG Oligodeoxynucleotides Induce Th1 Cells by Elaborating IL-12[1]

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APCs initiate T cell-mediated immune responses against foreign Ags. Dendritic cells are professional APCs that play unique roles, including Ag-nonspecific capture, priming of naive T cells, and Th1 induction, whereas B cells generally lack these functions. In this study we uncovered novel aspects of murine B cells as APCs using CpG oligodeoxynucleotides (CpG) conjugated with an Ag. B cells served as efficient APCs independently of surface Igs. This characteristic was underlaid by the CpG-mediated Ag uptake and presentation, which were functional only when CpG were covalently conjugated to Ag. The B cells cultured with CpG-conjugated Ag not only enhanced IFN-γ formation by Th1 cells, but also induced Th1 differentiation from unprimed T cells. These effects paralleled with the increase in the expression of CD40, CD86, and class II molecules on B cells and the coordinated production of IL-12 by the cells. To our knowledge this is the first report revealing that B cells share with dendritic cells common intrinsic characteristics, such as the Ag-nonspecific capture and presentation, and the induction of Th1 differentiation from unprimed T cells. *The Journal of Immunology,* **2002, 169: 787–794.**

Antigen-presenting cells play a critical role in initiating T cell-mediated immune responses. Dendritic cells (DCs)[3] are characterized by the ability to capture a large diversity of Ags in an Ag-nonspecific manner and to present them in a highly immunogenic form (1–4). These features highlight DCs as professional APCs capable of priming naive T cells (5, 6).

B cells also serve as APCs (7–9). Resting B cells are poor at presenting Ag (10–12) or are tolerogenic to T cells (13, 14), and turn into effective APCs with the increased expression of costimulatory molecules after the activation (15–17). However, Ags specifically bound to surface Ig (sIg) on B cells are presented to T cells 10^3- to 10^4-fold more efficiently than those entering the cells in an sIg-independent manner (10, 18, 19). B cells are efficient APCs for Ag-primed T cells (13, 20) and are likely to induce Th2-dominant responses (21–27). It has long been debated whether B cells can prime naive T cells (28–32). Recent in vitro experiments using Ig transgenic (tg) mice demonstrated that B cells have the capacity of activating naive T cells for proliferation (33, 34) and the development of Th2 cells (35) or unpolarized effector T cells (36). However, little is known about the ability of B cells to prime naive T cells for differentiation toward Th1 cells.

The nature of DNAs as immune stimulators has recently been attracting much attention. Initially, CpG oligodeoxynucleotides (CpG) were found to trigger B cells to proliferate and differentiate into Ig-secreting cells (37). CpG were also found to activate monocytes, macrophages, and DCs to produce IL-12, which facilitates the development of Th1 cells (38–45). We reported that the ability of Ag to induce the differentiation of Ag-specific Th1 cells was greatly enhanced when CpG were covalently conjugated to the Ag (46, 47). The underlying mechanisms included the augmented capture of the CpG-tagged Ag by DCs in a CpG-guided manner and the expression of costimulatory molecules and IL-12 by the Ag-pulsed DCs (47). While the binding of CpG to B cells has recently been extensively studied (48), the functional relevance of CpG binding has not been addressed.

In this report we examined the role of CpG in the CpG-Ag conjugate in Ag capture and T cell stimulation by B cells. We observed that the CpG-Ag conjugates were efficiently captured by B cells regardless of the Ag specificity of sIg. The CpG-activated B cells could, in turn, present the Ag and induce the differentiation of Th1 cells from unprimed tg T cells by elaborating IL-12.

Materials and Methods

Animals

BALB/c mice were bred in our animal facility and were used at 7–12 wk of age. BALB/c mice tg for TCR specific for $OVA_{323-339}$ and I-A^d were supplied by Dr. S. Habu (Tokai University, Kanagawa, Japan) (49).

Ags, CpG, and direct conjugation to Ags

OVA (Sigma, St. Louis, MO), BSA (Sigma-Aldrich), and keyhole limpet hemocyanin (KLH; Calbiochem, La Jolla, CA) were conjugated with 2,4,6-trinitrobenzen sulfonate (Wako Pure Chemical, Osaka, Japan). The degree of substitution was 10 trinitrophenyl (TNP) residues/100-kDa Ag. The CpG (1826) used throughout this study consisted of 20 bases containing two CpG motifs (TCCATGACGTTCCTGACGTT) (50) and were fully phosphorothioated (underlining indicates a CpG motif). The control non-CpG oligodeoxynucleotides (ODNs; 1745) were identical, except that the CpG motifs were rearranged (TCCATGAGCTTCCTGAGTCT) (50). Phosphorothioate ODNs were synthesized by Nihon Gene Research Laboratories (Sendai, Japan) or Takara Shuzo (Osaka, Japan). The LPS content of ODN was <6 pg LPS/mg DNA, as measured by a *Limulus* HS-J Single Test (Wako Pure Chemical). The phosphorothioate ODNs were conjugated to OVA, TNP-OVA, TNP-BSA, and R-PE (PE; Molecular Probes, Eugene,

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[3] Abbreviations used in this paper: DC, dendritic cell; Be1, B effector 1; ODN, oligodeoxynucleotide; CpG, CpG ODN; KLH, keyhole limpet hemocyanin; MMC, mitomycin C; PE, R-PE; SA, streptavidin; sIg, surface Ig; tg, transgenic; TNP, trinitrophenyl.

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OR) by mixing SH-conjugated ODNs at the 5′ end and maleimide-activated Ag using sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carobylate (Pierce, Rockford, IL). Unconjugated ODNs were removed by extensive dialysis. Aliquots of CpG-OVA conjugate were purified by gel filtration chromatography to minimize the effects of the contaminating aggregates, as described previously (47). The molar and weight ratios of the ODN to Ag are listed in Table I.

Purification of B cells and the B cell line

Spleen or LN cells depleted of RBC by hypotonic treatment were layered onto 50% Percoll (Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 10 min at 2000 rpm. The cells under the 50% Percoll layer were incubated with anti-B220 magnetic microbeads and enriched for $B220^+$ B cells using a MACS magnetic separation system (Miltenyi Biotec, Auburn, CA). B cells used as APCs for the stimulation of Th cells were further purified by depleting them of $CD11c^+$ DCs with anti-CD11c microbeads before enrichment for $B220^+$ cells. Reanalysis of the recovered $B220^+CD11c^-$ cells revealed that $B220^+$ B cells and $CD11c^+$ DCs comprised >98% and <0.5%, respectively (Fig. 1*B*). Low and high buoyant density B cells were prepared by centrifugation over a discontinuous Percoll gradient containing 55–60 and 70% layers. B cells at the medium/55–60 and 60/70% interface were collected separately and used as large and small B cells, respectively. Where indicated, the BCL1 B cell leukemia line (51) (provided by Cell Resource for Biomedical Research, Tohoku University) was used as alternative APCs for the Th1 induction or activation.

Stimulation of naive OVA-specific T cells with CpG and OVA for the Th1 differentiation

$CD4^+$ T cells were prepared from spleens of unimmunized OVA-specific TCR tg mice by depleting $CD8^+$ and Ia^+ cells using a panning method (47). $B220^+CD11c^-$ B cells (2.5×10^6) purified from unimmunized BALB/c mouse spleens or the BCL1 B cell line were pulsed with OVA and/or CpG for 3 h. The BCL1 cell line was then fixed with 0.5% paraformaldehyde at 37°C for 15 min. After extensive washing they were cocultured with 2.5×10^6 OVA-specific $CD4^+$ T cells in 2 ml medium in 12-well plates. After 6 days of culture, viable lymphocytes (1×10^5) recovered by Ficoll-Paque (Pharmacia Biotech) density-gradient centrifugation were restimulated with 2×10^5 APCs in the presence or the absence of OVA (100 μg/ml) in quadruplicate in 96-well plates. APCs were prepared by treating spleen cells from unimmunized BALB/c mice with mitomycin C (MMC; 50 μg/ml; Wako Pure Chemical) for 30 min at 37°C. After 2 days of culture, the culture supernatants were assayed for IFN-γ and IL-4. To neutralize IL-12 activity, 10 μg/ml anti-IL-12 (Genzyme, Cambridge, MA) or isotype-matched control mAb (rat IgG2a; BD PharMingen, San Diego, CA) were included in the cultures.

Restimulation of OVA-specific Th1 cells with CpG and OVA

OVA-specific TCR tg Th1 (hereafter referred to as Th1) cells were induced in vitro and enriched for $CD4^+$ cells as described previously (47). In brief, spleen cells from unimmunized OVA-specific TCR tg mice were cultured with OVA (100 μg/ml) and IL-12 (1 ng/ml). After 6 days of culture, viable lymphocytes were enriched for $CD4^+$ T cells by a panning method. $CD4^+$ Th1 cells (1×10^5) were cultured in 96-well plates with 2×10^5 untreated spleen cells or $B220^+CD11c^-$ B cells from BALB/c mice or the BCL1 B cell line as APCs in the presence of OVA and/or CpG in quadruplicate. After 2 days of culture, the culture supernatants were assayed for IFN-γ and IL-4. The enriched T cells failed to produce IFN-γ or IL-4 in response to OVA or CpG-OVA in the absence of additional APCs.

Restimulation of anti-OVA Th1 cells by TNP-primed B cells

BALB/c mice were immunized in the hind footpads with TNP-KLH or KLH emulsified in CFA (Difco, Detroit, MI). After 1 wk, popliteal LN cells were pooled from three mice and purified for $B220^+CD11c^-$ B cells as described above. $CD4^+$ Th1 cells (1×10^5) were cultured with 2×10^5 purified B cells in the presence of OVA conjugated with CpG, TNP, or both in quadruplicate in 96-well plates for 2 days, and the culture supernatants were assayed for IFN-γ.

Table I. *Molar and weight ratios of ODN to Ag*

	Molar Ratio	Weight Ratio
CpG-OVA	6.4	0.86
Non-CpG-OVA	6.1	0.81
CpG-OVA-TNP	7.1	0.96
CpG-BSA-TNP	10.6	0.92
CpG-PE	3.7	0.092
Non-CpG-PE	3.7	0.092

Stimulation of the purified B cells with CpG

The purified $B220^+CD11c^-$ B cells (2×10^5/well) from unimmunized BALB/c mice were cultured with LPS (Sigma-Aldrich), CpG, or control non-CpG ODNs in quadruplicate in 96-well plates. After 2 days of culture, the culture supernatants were assayed for IL-12 by ELISA as described below.

Cytokine assay

The concentrations of IFN-γ and IL-4 in the culture supernatants were determined using ELISA as described previously (52). The concentrations of IL-12 were determined using paired anti-IL-12 mAbs (BioSource International, Camarillo, CA) according to the manufacturer's instructions. Tetramethylbenzidine reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used for color development, and ODs determined at 450 nm were converted to concentrations (nanograms per milliliter) according to a standard curve. Standard recombinant mouse IL-12 was purchased from PeproTech (Rocky Hill, NJ).

Reagents used for flow cytometry

FITC-conjugated anti-B220 mAb, PE-conjugated anti-IL-12 mAb, and allophycocyanin-conjugated streptavidin (SA) were purchased from Immunotech (Westbrook, ME), BD PharMingen, and Biomeda (Foster City, AC), respectively. Biotinylated anti-CD40 and anti-CD86 mAbs were purchased from Caltag Laboratories (Burlingame, CA). Anti-$I\text{-}A^d$ mAb (MK-D6) (53) was partially purified from ascites by ammonium sulfate precipitation and conjugated to biotin (Sigma-Aldrich) in our laboratory.

Analyses of B cells by flow cytometry

The $B220^+$ B cells from unimmunized spleens were incubated with PE alone, a mixture of PE and CpG, or graded doses of PE-CpG conjugates overnight. The cells were stained with FITC-conjugated B220 mAb together with biotinylated anti-CD40, anti-CD86, or anti-$I\text{-}A^d$ mAb. The binding of biotinylated mAbs was detected with allophycocyanin-SA. The correlations between PE staining and the CD40, CD86, or $I\text{-}A^d$ expression on the viable $B220^+$ B cells were analyzed using FACSCalibur (BD Biosciences, Mountain View, CA). Propidium iodide (Sigma-Aldrich)-stained dead cells were excluded from analyses. For staining of intracytoplasmic IL-12, the purified $B220^+$ B cells were cultured with CpG or LPS overnight, with 10 μg/ml brefeldin A (Wako Pure Chemical) added for the final 4 h. After staining with FITC-labeled anti-B220 mAb, the cells were treated with cell permeabilization solution (Immunotech, Minneapolis, MN) and then stained with PE-labeled anti-IL-12 mAb (0.3 μg). Where indicated, a 20-fold excess of unlabeled anti-IL-12 mAb (BD PharMingen) was also added. They were analyzed by flow cytometry.

Statistics

Data from in vitro culture experiments are expressed as the mean ± SEM. Each experiment was repeated at least twice. Student's *t* test was used in the analysis of the results.

Results

Activation of Th1 cells by unprimed B cells and CpG-OVA

We first determined whether unprimed B cells could present Ag to OVA-specific Th1 cells. Spleen cells from unimmunized BALB/c mice (Fig. 1*A*) were enriched for B cells using magnetic beads. The purity of $B220^+$ B cells was >98%, and the contamination by $CD11c^+$ DCs was <0.5% (Fig. 1*B*). When spleen cells were employed for APCs, the Th1 cells predominantly produced IFN-γ in response to OVA. With the purified B cells, however, Th1 cells failed to produce IFN-γ in response to 10–100 μg/ml OVA, verifying the depletion of DCs and the high purity of the B cell fraction (Fig. 1*C*). The lack of cytokine production from the Th1 cells cultured with CpG-OVA in the absence of APCs substantiated the depletion of DCs in the Th1 population (data not shown). The purified B cells failed to present OVA in the copresence of 10 μg/ml CpG, whereas the same B cells cultured with CpG-OVA

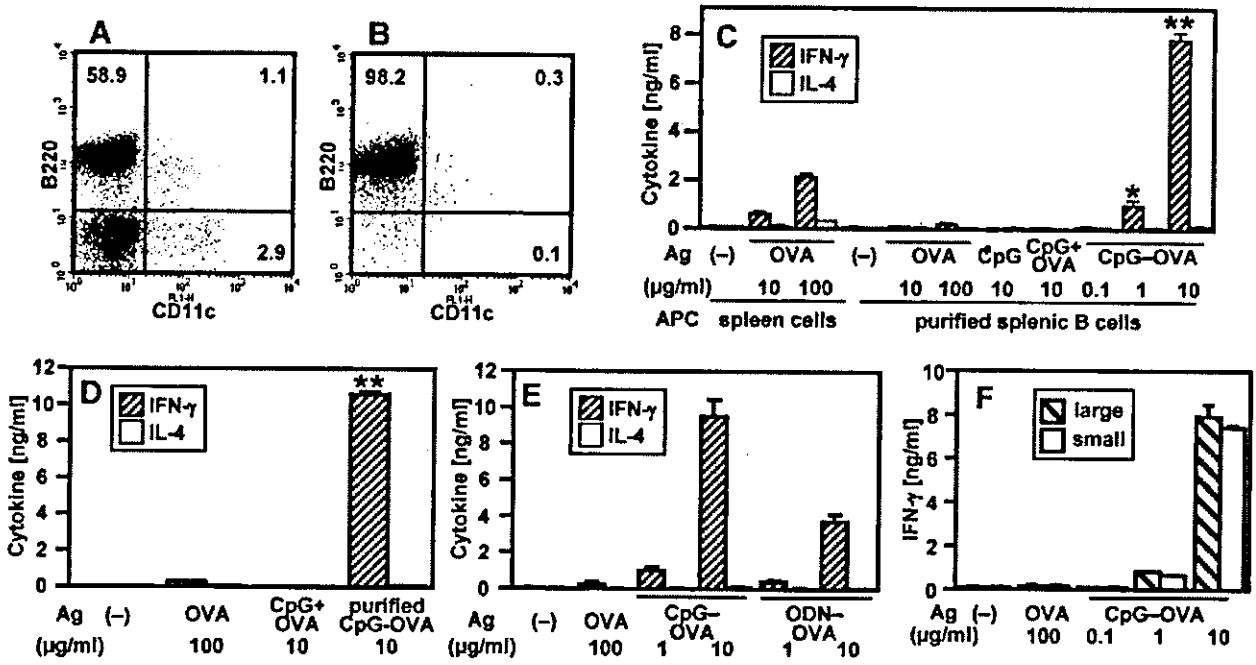

FIGURE 1. Activation of Th1 cells by unprimed B cells and CpG-OVA. *A* and *B*, Spleen cells from unprimed BALB/c mice were stained with mAbs against B220 and CD11c before (*A*) and after (*B*) the purification for $B220^+CD11c^-$ B cells. Contamination of the purified B cell preparation with $CD11c^+$ DC was reproducibly <0.5% (*B*). *C–F*, Th1 cells were induced by culturing spleen cells of OVA-specific TCR tg mice in the presence of OVA (100 μg/ml) and IL-12 (1 ng/ml) for 6 days, and 1×10^5 $CD4^+$ Th1 cells were restimulated with OVA, CpG, a mixture of CpG (10 μg/ml) and OVA (10 μg/ml), graded doses of the CpG-conjugated OVA, purified CpG-OVA, or control ODN-conjugated OVA in the presence of 2×10^5 APCs. The APCs used were spleen cells (*C*), purified $B220^+CD11c^-$ B cells (*C–E*), or fractionated B cells using Percoll density centrifugation (*F*). After 2 days of culture, the culture supernatants were assayed for IFN-γ and IL-4 by ELISA. Flow cytometric results are representative of multiple independent experiments. The in vitro culture experiments were repeated independently two or three times with similar results. *, $p < 0.005$; **, $p < 10^{-4}$ (compared with the CpG + OVA group).

conjugate potently activated Th1 cells for the production of IFN-γ, but not IL-4, in a dose-dependent manner. We also employed purified CpG-OVA that contained minimal amounts of aggregates (47) and found that the monomeric CpG-OVA and CpG-OVA before purification were comparable in their ability to activate Th1 cells (Fig. 1*D*), indicating that the Th1 activation reflects the feature of the monomeric CpG-conjugated OVA, but not the aggregates. Although B cells could also present non-CpG ODN-conjugated OVA to Th1 cells, the IFN-γ levels induced by non-CpG-OVA were significantly lower than those induced by CpG-OVA (Fig. 1*E*). Neither large nor small B cells presented OVA to Th1 cells, whereas both B cell populations presented CpG-OVA to stimulate Th1 cells to comparable levels at the doses tested (Fig. 1*F*).

Differentiation of Th1 cells from naive T cells by stimulation with CpG-OVA and unprimed B cells

We next examined whether purified B cells could induce the differentiation of Ag-specific Th1 cells from naive T cells. $CD4^+$ T cells precultured with MMC-treated spleen cells and OVA developed into effector Th cells that produced comparable levels of IFN-γ and IL-4 upon antigenic stimulation. However, $CD4^+$ T cells precultured with purified B cells in the presence of 100 μg/ml OVA did not produce cytokines upon restimulation with Ag in the presence of APCs (Fig. 2*A*). The B cells failed to present OVA to naive T cells for the development of effector Th cells even after stimulation with a mixture of OVA and CpG. In contrast, the conjugate of the corresponding doses of CpG and OVA induced naive T cells for the development of Th1 cells. Purified monomeric CpG-OVA devoid of aggregates had a Th1-inducing activity comparable to that of CpG-OVA before fractionation, indicating that the Th1 development can be ascribed to the monomeric CpG-OVA conjugates. The non-CpG-OVA conjugate failed to induce the differentiation of naive T cells. It was also found in an additional experiment that IFN-γ production of Th1 cells induced by CpG-OVA increased in a dose-dependent manner (Fig. 2*B*). The results clearly showed that unprimed B cells could present Ag to induce the Th1 differentiation from unprimed T cells if the Ag was in the form of a conjugate with CpG.

Dose-dependent and coordinated increases in Ag uptake and the expression of costimulatory molecules on B cells by CpG-Ag conjugate

We then determined the effects of CpG in the conjugate on Ag uptake by and the expression of costimulatory molecules on B cells. To track the fate of Ag, CpG-conjugated PE was employed. Previous experiments showed that CpG-PE contained no discernible amounts of aggregates, as determined by SDS-PAGE, and that free PE or CpG in the CpG-PE preparation did not affect the function of DC (47). Splenic B cells were incubated overnight with either the conjugate or a mixture of PE and CpG, and PE in $B220^+$ cells and the expression of costimulatory molecules were analyzed by flow cytometry. As shown in Fig. 3, the CpG-PE conjugate and the mixture were comparable in activities for increased expression of CD86, CD40, and class II MHC. However, PE staining in B cells was entirely different between the two; only a minor fraction (1.4–1.8%) of unimmunized B cells phagocytosed PE in the mixture. After incubation with CpG-PE, the proportion of PE-bearing B cells (19.4–100%) and the intensity of PE staining increased in a dose-dependent manner, and the enhanced PE staining paralleled

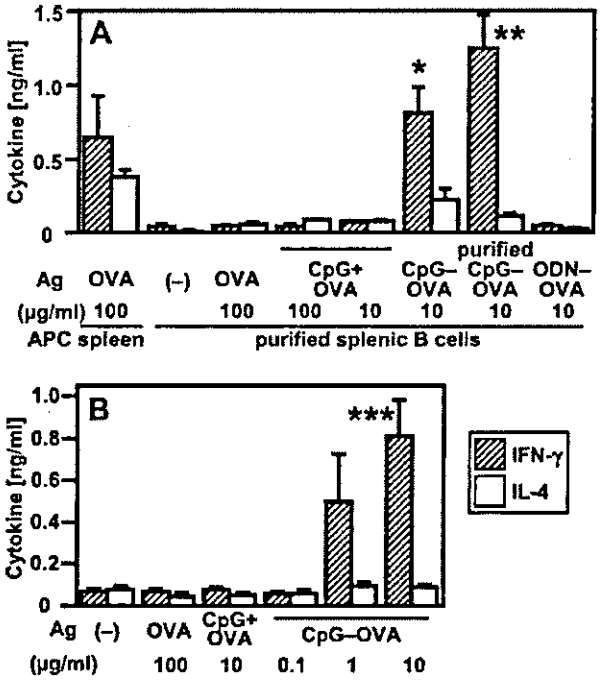

FIGURE 2. Differentiation of Th1 cells from naive T cells by stimulation with CpG-OVA and unprimed B cells. Purified $B220^+CD11c^-$ B cells (*A* and *B*) or spleen cells (*A*) were pulsed with OVA alone or with a mixture or conjugate of OVA and CpG for 3 h. Then, 2.5×10^6 pulsed APCs were cultured with 2.5×10^6 unprimed OVA-specific $CD4^+$ Th cells in 12-well plates. After 6 days of culture, 1×10^5 viable lymphocytes were restimulated with OVA (100 μg/ml) and 2×10^5 MMC-treated spleen cells for 2 days, and culture supernatants were assayed for IFN-γ and IL-4. Spleen cells (*A*), but not purified B cells (*A* and *B*), presented OVA (100 μg/ml) for the development of Th effector cells. Purified CpG-OVA depleted of aggregates exhibited a Th1-inducing ability comparable to that before purification, indicating that the Th1-inducing activity of CpG-OVA can be attributed to the monomeric CpG-OVA (*A*). In *B*, graded doses of CpG-OVA were used to examine a dose-response relationship. The abscissa shows the concentration of OVA in the mixture or conjugate. The concentration of CpG in the CpG plus OVA group was 10 μg/ml. Each experiment was repeated twice independently with similar results. *, $p < 0.05$; **, $p < 0.02$; ***, $p < 10^{-4}$ (compared with the CpG plus OVA group).

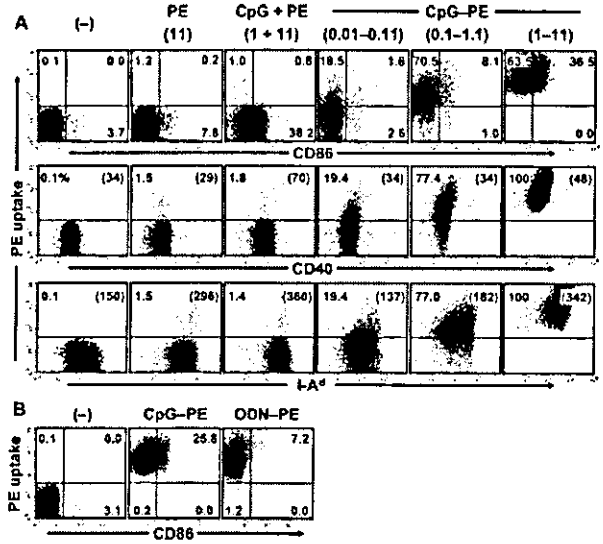

FIGURE 3. Dose-dependent and coordinated increases in the Ag uptake and the expression of costimulatory molecule on B cells by CpG-Ag conjugate. *A*, Aliquots of $B220^+$ B cells from BALB/c mice were incubated overnight with PE (11 μg/ml), a mixture of PE (11 μg/ml) and CpG (1 μg/ml), or graded doses of PE-CpG conjugates. They were stained with FITC-labeled anti-B220 mAb and biotinylated mAb to CD86, CD40, or $I\text{-}A^d$, followed by allophycocyanin-SA. The correlations between PE staining and CD86, CD40, or $I\text{-}A^d$ expression in the gated $B220^+$ B cells are shown. Numbers represent the percentage of cells, and values in parentheses are the mean fluorescence intensity of the CD40 or $I\text{-}A^d$ expression in B cells. Note that the conjugate dose-dependently increased the number of PE-bearing B cells and up-regulation of CD86, CD40, or MHC class II expression. *B*, $B220^+$ B cells were incubated with PE (11 μg/ml) conjugated with CpG- or non-CpG control ODN, and the correlations between CD86 expression and PE staining are shown. One experiment representative of three independently performed experiments is shown.

the increase in the expression of costimulatory molecules. Most notable was CD86 expression. The results show that the CpG-PE conjugate induced concomitant increases in CD86 expression and PE uptake in a dose-dependent fashion. CpG in the mixture with PE induced CD86 expression without an accompanying increase in PE uptake. After activation with CpG-PE conjugate, B cells with higher levels of PE expression exhibited concomitant increases in the expression of CD40 and class II molecules. In additional experiments, the effects of non-CpG control ODN were examined. The control ODN-conjugated PE did not increase the expression of CD86 (Fig. 3*B*).

IL-12 production by CpG-activated B cells

We determined whether IL-12 secreted from B cells facilitated Th1 differentiation by the CpG-OVA conjugate. First, we assessed IL-12 production by CpG-activated B cells. CpG stimulated purified B cells to form IL-12 in a dose-dependent manner (Fig. 4*A*). The CpG-OVA conjugate also induced IL-12 formation to comparable levels as CpG alone at two different doses. Non-CpG ODNs failed to induce IL-12 formation. The unstimulated or LPS-stimulated B cells failed to produce IL-12, as reported previously (54). The results confirmed the exclusion of DCs, which produce IL-12 in response to LPS stimulation (55), in the B cell preparation.

Additional support came from the experiment with neutralizing anti-IL-12 mAb. The purified B cell population, which failed to present OVA to Th cells, presented the CpG-OVA conjugate to induce OVA-specific Th1 cells (Fig. 4*B*). The induction of Th1 differentiation by purified B cells was neutralized by anti-IL-12 mAb, but not by the isotype-matched control. Control non-CpG ODNs failed to induce Th1 differentiation.

More concrete evidence for the formation of IL-12 by B cells was obtained by the staining of intracytoplasmic IL-12 in gated $B220^+$ B cells (Fig. 5). Neither unstimulated $B220^+$ B cells nor LPS-activated B cells were stained with PE-labeled anti-IL-12 mAb, substantiating the lack of nonspecific staining with PE-labeled anti-IL-12 mAb. In contrast, the staining of CpG-activated B cells with PE-labeled anti-IL-12 mAb shifted the staining of the whole population by nearly 4 times, as judged by the increase in the mean fluorescence intensity (from 11.42 to 44.67). The proportion of B cells scored as positive for IL-12 staining increased to 16.5%. IL-12 staining was specific, because pretreatment of CpG-activated B cells with unconjugated anti-IL-12 mAb inhibited binding of PE-labeled anti-IL-12 mAb. Thus, we concluded that CpG-conjugated Ag induced Ag-specific Th1 cell differentiation through the elaboration of IL-12 in B cells.

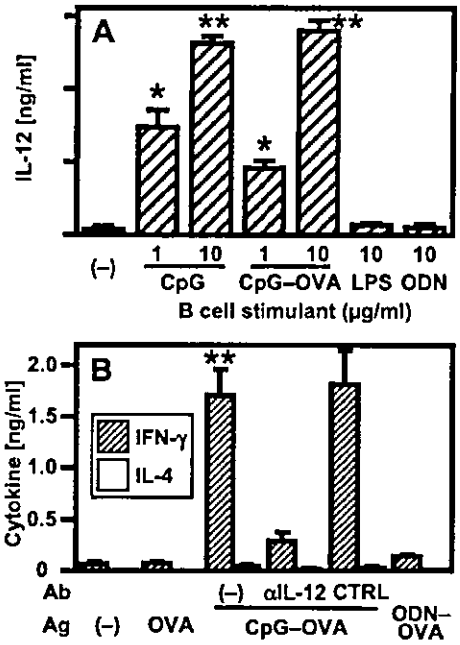

FIGURE 4. CpG-activated B cells elaborate IL-12 and induce the differentiation of Th1 cells. *A*, Purified B220$^+$CD11c$^-$ B cells (2×10^5) were stimulated with LPS, CpG, CpG-OVA, or non-CpG ODN (ODN) in 96-well plates for 2 days, and culture supernatants were assayed for IL-12 by ELISA. CpG and CpG-OVA induced B cells to produce IL-12 to a comparable extent. *B*, Unprimed OVA-specific CD4$^+$ T cells (2.5×10^6) were cocultured with 2.5×10^6 purified B220$^+$CD11c$^-$ B cells pulsed with OVA (100 μg/ml), CpG-OVA (10 μg/ml), or non-CpG ODN-OVA (ODN-OVA; 10 μg/ml) in the presence or the absence of anti-IL-12 (αIL-12) or isotype-matched control (CTRL) mAb (10 μg/ml). After 6 days of culture, 1×10^5 viable lymphocytes were restimulated with OVA (100 μg/ml) and 2×10^5 MMC-treated spleen cells for 2 days, and culture supernatants were assayed for IFN-γ and IL-4. *, $p < 0.01$; **, $p < 10^{-3}$ (compared with the unstimulated group).

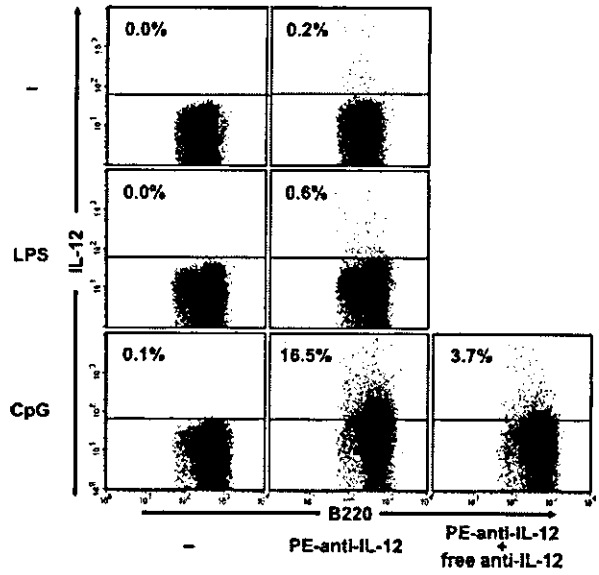

FIGURE 5. Expression of intracytoplasmic IL-12 in CpG-activated B cells. Purified B220$^+$ B cells were cultured with LPS or CpG overnight, with 10 μg/ml brefeldin A added for the final 4 h. After staining with FITC-labeled anti-B220 mAb, the cells were treated with cell permeabilization solution and then stained with PE-labeled anti-IL-12 mAb (0.3 μg). Where indicated, a 20-fold excess of free anti-IL-12 mAb was added at the beginning of the incubation. They were analyzed for intracytoplasmic IL-12 by flow cytometry. The IL-12 expressions in the gated B220$^+$ B cells are shown. Data are representative of four independently performed experiments with similar results.

Synergism between Ag-nonspecific CpG-mediated and Ag-specific sIg-mediated Ag capture by primed B cells

We next examined the effects of sIg-mediated capture of CpG-conjugated Ag on Th1 cell activation. TNP-primed and control B cells were prepared from TNP-KLH- and KLH-primed LN cells, respectively, and cultured with OVA-specific Th1 cells and OVA conjugated with CpG, TNP, or both. Th1 cells produced low levels of IFN-γ in response to TNP- or CpG-conjugated OVA presented by TNP-primed B cells, whereas the same concentration of OVA conjugated with both TNP and CpG stimulated Th1 cells when TNP-primed, but not control, B cells were used as APCs (Fig. 6). In addition, TNP-KLH-primed B cells induced IFN-γ production to a level comparable to that induced by KLH-primed B cells when cultured with Th1 cells and the combination of TNP-BSA-CpG plus OVA-CpG (Fig. 6*A*). These results indicate that the Ag-specific sIg-mediated Ag capture enhanced Ag presentation and Th1 activation by primed B cells in a synergistic manner with Ag-nonspecific CpG-mediated Ag capture.

Activation and induction of Th1 cells by the BCL1 B cell line as APCs

We examined the Ag-presenting ability of the B cell leukemia line to exclude the possible contribution of DCs that might have contaminated the purified B cell population. The BCL1 B cell line activated Th1 cells by presenting CpG-OVA in a dose-dependent manner (Fig. 7*A*), as did the purified B cells shown in Fig. 2*C*. Similarly, the BCL1 B cell line also induced Th1 cells from naive T cells in the presence of CpG-OVA (Fig. 7*B*), as did the purified

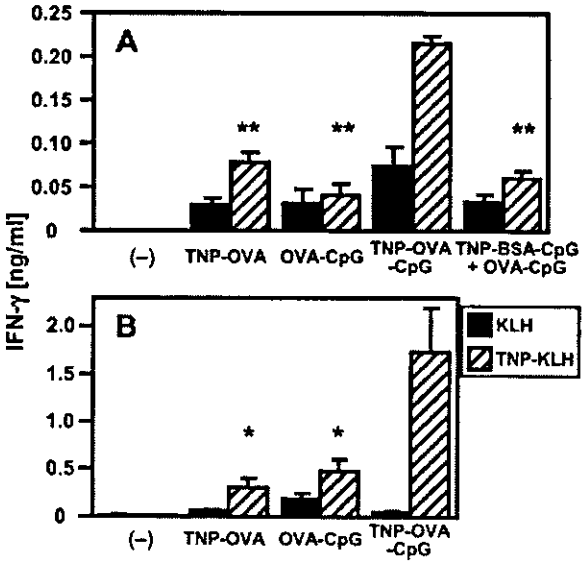

FIGURE 6. Synergism between Ag-nonspecific CpG-mediated and Ag-specific sIg-mediated Ag capture by primed B cells. B220$^+$CD11c$^-$ B cells were purified from the popliteal LN cells primed with TNP-KLH (▨) or KLH (■) in CFA. B cells (2×10^5) were cultured with 1×10^5 CD4$^+$ Th1 cells in the presence of 0.1 μg/ml (*A*) or 1.0 μg/ml (*B*) of the indicated stimulants for 2 days, and culture supernatants were assayed for IFN-γ by ELISA. IL-4 levels were not detected in any culture. Experiments were repeated independently three times with similar results. *, $p < 0.05$; **, $p < 10^{-4}$ (compared with the CpG-OVA-TNP group).

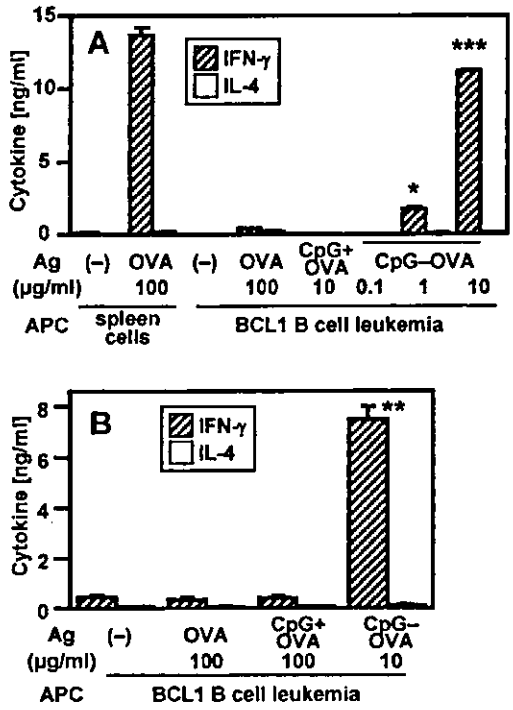

FIGURE 7. The activation and induction of Th1 cells by the BCL1 B cell line as APCs. Experimental protocols in *A* and *B* are the same as those in Figs. 1*C* and 2A, respectively. The BLC1 B cell line, instead of purified $B220^+CD11c^-$ B cells, was used as APCs either without (*A*) or after fixation with (*B*) 0.5% paraformaldehyde. The B cell line works as APCs to stimulate Th1 cells (*A*) or to induce Th1 differentiation from naive T cells (*B*). Each experiment was repeated twice independently with similar results. *, $p < 0.01$; **, $p < 10^{-3}$; ***, $p < 10^{-4}$ (compared with the CpG plus OVA group).

B cells shown in Fig. 2*A*. These results reinforce our contention that purified unprimed B cells, but not contaminating DCs, induce or activate Th1 cells.

Discussion

APCs are initiators of the T cell-mediated immune surveillance system. DCs are well equipped with devices to capture diverse Ags and present antigenic peptides to T cells (1–4). DCs produce IL-12 upon encountering microbes, thereby inducing protective Th1 responses (56–58). B cells also serve as APCs (7–9). Characteristics of B cells include clonally expressed sIgs that promote the capture of Ag (10, 18, 19). B cells tend to induce Th2 responses (21–27) and are unable to induce Th1 cells. Thus, DCs and B cells appear to play distinctive roles in directing immune responses.

In this report we disclosed features of B cells that challenge the ideas described above if Ag is chemically conjugated with CpG. B cells could efficiently capture the conjugate and present antigenic peptides to Th cells regardless of the Ag specificity of sIg (Figs. 1–3), and CpG-stimulated B cells could induce Th1 development by producing a sufficient amount of IL-12 (Figs. 2, 4, and 5). The efficient Ag uptake in an Ag-nonspecific manner and the ability to induce Th1 differentiation from naive T cells had been considered to be unique to DCs. Here, we demonstrate that B cells are also endowed with these characteristics and can work like DCs provided that Ag is linked to CpG.

During the course of our studies of regulatory $CD4^+$ T cells that control Th2 responses, we found that the CpG-OVA conjugate induced Ag-specific Th1 cells and inhibited airway eosinophilia (46). One of underlying mechanisms was the augmented capture of the CpG-tagged Ag by DCs in a CpG-guided manner, because PE conjugated to CpG bound to DCs >100-fold more than PE mixed with CpG (47). In this study we found that the same mechanism for capturing Ag applies to B cells. B cells had been thought to be poor for nonspecific Ag uptake (10, 18, 19). Under physiological conditions, B cells neither efficiently processed Ag (Fig. 3) nor stimulated Th cells (Figs. 1 and 2). The activation of B cells by CpG failed to improve the uptake of Ag (Fig. 3) or the presentation of antigenic peptide to Th cells (Figs. 1 and 2). When CpG were conjugated to Ag, however, B cells could present the Ag and serve as efficient APCs in an Ig-independent manner (Figs. 1–3). The enhanced uptake of CpG-conjugated Ag by B cells is considered to reflect the efficient binding of CpG to surface receptors specific for ODNs (59), which, however, have not been defined yet.

IL-12 was initially identified as a product of human transformed B lines, whereas it had been controversial whether murine B cells produced IL-12 (54, 60). There has been accumulating evidence that B cells as APCs are likely to skew T cell immune responses toward the Th2-dominant phenotype (21–27). In sharp contrast to these earlier studies, we here demonstrate that B cells secreted IL-12 (Figs. 4 and 5) and can play a decisive role in Th1 differentiation from unprimed T cells (Fig. 2). Recently, B effector 1 (Be1) stimulated with Th1 cells was reported to produce IL-12, although IL-12 failed to polarize the naive T cells to differentiate into Th1 cells (61). IL-12 production from Be1 cells was detected upon restimulation following an initial 4-day culture with Th1 cells, whereas naive B cells could secrete IL-12 in response to overnight CpG stimulation. Thus, CpG-activated B cells and Be1 cells appear to represent the distinct activation status of B cells.

The nonspecific Ag uptake that is independent of sIg specificity is mediated by other receptors on B cells. The most notable is CD21 (complement receptor type 2)-mediated endocytosis. The Ag coupled to C3 fragment is taken up in an Ag-nonspecific manner and presented to T cells as efficiently as those bound through sIg (62–64). CD21 ligation failed to up-regulate costimulatory molecules (65, 66), whereas the activation of B cells by CpG enhanced the expression of costimulatory molecules (Fig. 3), which highlights the advantage of CpG as an immunostimulator.

What, then, could the physiological significance of the B cell responses to CpG be? Ag-primed B cells are known to be efficient APCs following Ag capture through sIg (10, 18, 19) and activation (15–17). They are likely to induce Th2-dominant responses (21–27). However, we have found that CpG-activated B cells can initiate Th1 responses (Figs. 1 and 2). Thus, B cells as APCs can modulate the immune outcome by converting Th2-oriented responses to Th1-dominant responses in the presence of CpG. An additional surprising finding is that the Th1 inducibility of Ag-primed B cells was further amplified when the B cells bind to Ag in both CpG- and sIg-mediated manners (Fig. 6). This mechanism might be very advantageous for the induction of defensive Th1 responses against microbial infections. Microbe-specific B cells could bind to bacteria through sIg and CpG when bacteria are tagged with DNA spelled from degraded microbes. This scenario could be plausible, since bacteria express surface receptors specific for DNA (67).

In summary, we showed that B cells share common roles with APCs with DCs, including the Ag-nonspecific capture and the induction of Th1 differentiation from unprimed T cells.

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