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TLR9 Signaling in B Cells Determines Class Switch Recombination to IgG2a

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Although IgG2a is the most potent Ab isotype in the host response to viral and bacterial infections, the regulation of class switch recombination to IgG2a in vivo is not yet well understood. Recognition of pathogen-associated molecular patterns by dendritic cells expressing TLRs, like TLR7, recognizing ssRNA, or TLR9, recognizing DNA rich in nonmethylated CG motifs (CpG), favors induction of Th1 responses. It is generally assumed that these Th1 responses are responsible for the TLR-mediated induction of IgG2a. Using virus-like particles loaded with CpGs, we show here that TLR9 ligands can directly stimulate B cells to undergo isotype switching to IgG2a. Unexpectedly, TLR9 expression in non-B cells did not affect isotype switching in the Ab response against virus-like particles. Thus, TLR9 can regulate isotype switching to IgG2a directly by interacting with B cells rather than indirectly by inducing Th1 responses. The Journal of Immunology, 2007, 178: 2415–2420.

Infection of mice with RNA or DNA viruses induces an antiviral Ab response that is largely restricted to the IgG2a isotype (1). The IgG2a isotype has been shown to be particularly potent in host defense against viral infections (2) due to its specific capacity to activate the complement system (3), bind to Fc receptors expressed on phagocytes (4), and induce Ab-dependent cell-mediated cytotoxicity (5). In humans, IgG1 and IgG4 correspond to IgG2a and IgG1 (6) in the mouse. Because different IgG isotypes markedly differ in their ability to opsonize bacteria and lyse infected target cells, controlled induction of the specific isotypes is of significant interest for the design of effective vaccines.

Class switch recombination of B cells from IgM to IgG is dependent on the cognate interaction of B cells with Th cells (7). Although CD40L-CD40 interaction is necessary to initiate Ab isotype switching (8–14), it is assumed that Th cell-derived cytokines determine whether the B cell switches to IgG1 or IgG2a (15). IFN-γ and IL-4 are key cytokines of Th1 and Th2 cells, respectively, although the latter also typically produce IL-5, IL-10, and IL-13.

By signaling through STAT6, IL-4 drives the switch to IgG1. Indeed, IL-4 or STAT6 deficient mice have a profound defect in IgG1 responses (16–18). IFN-γ has been shown to counteract IL-4 signaling in B cells (19).

However, in vivo, both IFN-γ-dependent (20–24) and IFN-γ-independent (25) IgG2a class switching have been observed, the latter mainly after infection of mice with live viruses and parasites.

Whereas IFN-γ-dependent IgG2a class switching occurs as a consequence of the cognate interaction of B cells with Th1 cells, it is not yet known how IFN-γ-independent IgG2a class switching occurs in vivo.

On a molecular level, T-box transcription factor T-bet seems to play a critical role in IFN-γ-STAT1-mediated IgG2a class switching in B cells (26). Recently, it has been shown that in vitro T-bet expression and up-regulation of IgG2a class switching can be induced by direct stimulation of B cells with CpG DNA (27–29), suggesting that an IFN-γ-independent pathway for class switch recombination to IgG2a might exist which goes via direct TLR signaling in B cells. In addition, it has recently been proposed that B cell responses are generally TLR-dependent (30); however, this has been challenged (31). Moreover, the respective roles of CpG-signaling into B cells vs induction of Th1 responses remain unclear (32).

In the present study, we dissected the importance of Th1/Th2 responses vs direct signals received by B cells for the regulation of IgG isotype switching by TLR ligands like ssRNA and CpG. Using virus-like particles (VLPs) loaded either with RNA, recognized by TLR7 (33), or CpG DNA, recognized by TLR9 (34), we demonstrated that the critical factor for the induction of IgG2a Abs against VLPs was not Th1 or Th2 cytokines. Rather, TLR expression in B cells was pivotal for the balance of IgG1 vs IgG2a Ab titers. Thus, direct stimulation of B cells by TLR ligands can be the driving factor for class switch recombination to IgG2a in vivo.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Harlan. IFN-γ−/− (35), TLR9−/− (34), and μMT (36) mice on a BALB/c background have been described earlier (37). All animals were kept under specific pathogen-free conditions at BioSupport and were used at 8 to 12 wk of age. Experiments were conducted in accordance with protocols approved by the Swiss Federal Veterinary Office.

Production of Qβ VLP (VLP/ssRNA), RNA-free VLP (VLP), and CpG oligomer packaging into VLP (VLP/CpG)

Qβ VLPs were expressed in Escherichia coli using the vector pQβ10 and purified as described previously (38). During the self-assembly of the Qβ coat proteins into VLPs, E. coli-derived mRNA (ssRNA) is packaged into the VLPs; the amount of packaged ssRNA is 300 μg of ssRNA per milligram of Qβ.

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RNA-free VLP can be obtained by digestion of VLP/ssRNA with RNase A. Shortly, 300 µg of RNase A was added to 1 mg of VLP/ssRNA (1 mg/ml in 20 mM HEPES, pH 7.4), the mixture was incubated for 1 h at 37°C in a thermostather (650 rpm), and to ensure complete digestion of RNA, the treatment was repeated three times. The digested VLP was dialyzed overnight against 20 mM HEPES (pH 7.4) with a 300 kDa m.w. cut-off membrane (Spectrum Medical Industries) to remove RNase A. Removal of RNase A was analyzed on a 12% SDS PAGE under reducing conditions. To analyze RNA digestion equal amounts (10 µg) of untreated and treated VLP was loaded on a 1% agarose Tris/acetate/EDTA (TAE)/ethidium bromide (EtBr) gel. Based on the disappearance of the fluorescent signal, success of RNA digestion could be determined.

Packaging of the CpG oligonucleotide 1668 (5’-TCCATGACGTTC CGTGAATAAT-3’, thioester stabilized; Microsynth) into the VLPs was performed as described previously (39). Shortly, the CpG oligonucleotides was added to RNase-treated VLPs to a final concentration of 100 nmol/mg VLP and incubated at 37°C for 3 h. To remove CpG oligonucleotides that were not packaged into the VLPs, the VLPs were dialyzed three times against 20 mM HEPES (pH 7.4) with a 300 kDa m.w. cut-off membrane (Spectrum Medical Industries). To analyze packaging of the CpG oligonucleotides, equal amounts (10 µg) of untreated and treated VLP were loaded on a 1% agarose TAE/EtBr gel. Based on the appearance of the fluorescent signal, success of CpG packaging could be determined. The amount of packaged CpGs was analyzed by Tris borate-EDTA/urea gel electrophoresis. First, the Q8/Cpg VLP was digested with proteinase K overnight at 37°C to remove protein. An equivalent of 0.5 µg of digested VLP was applied on a 10% Tris borate-EDTA/urea gel. As a standard, 5, 10, and 20 pmol of the CpG oligonucleotides packaged into the VLPs were loaded onto the gel. One milligram of packaged Q8 contains 40 nmol of CpG oligonucleotides.

Chemical coupling of OVA peptide to Q8 VLP

Chemical coupling of peptides to Q8 VLPs was described in detail previously (40). OVA peptide was produced in a modified version with an additional cysteine added at the N terminus (NH2-CSSAELKISQAVHAAHAEINAGRCOOH; Peter Henklein, Charité) to allow coupling to Q8 VLPs.

ELISA

ELISA was performed as described previously (40) using HRP-conjugated secondary Abs (HRP goat anti-mouse IgG (H+L), Jackson Immunoresearch; HRP rat anti-mouse IgG1, BD Pharmingen; HRP rat anti-mouse IgG2a, BD Pharmingen; HRP rat anti-mouse IgG2b, Southern Biotech; HRP rat anti-mouse IgG3, Southern Biotech). The dilutions at which secondary Abs were used were standardized as follows: ELISA plates were coated with a defined concentration of mouse IgG1, IgG2a, IgG2b, and IgG3 Abs. Serial dilutions of secondary Abs that were prediluted 1/100 to 1/5000 were done, and titers at which half-maximal OD was reached were defined. For each secondary Ab, the predilution at which half-maximal OD was 8’000 was chosen as working dilution for IgG1, IgG2a, IgG2b, and IgG3 Abs. Serial dilutions of secondary Abs that were prediluted 1/100 to 1/5000 were done, and titers at which half-maximal OD was reached were defined. For each secondary Ab, the predilution at which half-maximal OD was 8’000 was chosen as working dilution for ELISAs which were the following: anti-mouse IgG1, 1/500; anti-mouse IgG2a, 1/2000; anti-mouse IgG2b, 1/5000; anti-mouse IgG3, 1/5000. Secondary Abs were also tested for stability over time by repeating standardization over time.

IFN-γ/L-13 ELISPOT assay

Specific IFN-γ and IL-13 secreting CD4+ T cells were quantified using single-cell suspensions of spleens and inguinal lymph nodes from immunized mice. Briefly, for IFN-γ ELISPOT assay, single-cell suspensions at concentrations of 3 × 106 cells/well in duplicates were stimulated for 2 days with 10 µg/ml VLPs in 96-well round-bottom plates at 37°C, 5% CO2. After 2 days, cells were transferred to multiscreen plates (Millipore Corporation) precoated with 10 µg/ml anti-IFN-γ Ab (BD Pharmingen) and blocked with RPMI 1640 (10% FCS). In the IL-13 ELISPOT assay, single-cell suspensions at concentrations of 3 × 106 cells/well in duplicate were distributed directly to the multiscreen plates precoated with 10 µg/ml anti-IFN-γ Ab (BD Pharmingen) and blocked with RPMI 1640 (10% FCS) and stimulated with 10 µg/ml VLPs. Cells were incubated on multiscreen plates for 24 h at 37°C, 5% CO2. For the detection, cells were removed (PBS, 0.05% Tween 20), and plates were incubated with a biotinylated anti-IFN-γ Ab (BD Pharmingen (4 µg/ml)) or a biotinylated anti-mouse IL-13 Ab (R&D Systems (4 µg/ml)). Subsequently streptavidin-conjugated alkaline phosphatase (Roche) was added, and alkaline phosphatase substrate was finally used to obtain coloration of the spots (AP substrate kit; Bio-Rad).

Purification and adoptive transfer of B cells

Splenocytes were incubated with biotinylated Abs (BD Pharmingen) to CD43 (S7) and Ly-76 (TER-119), and B cells were negatively selected using streptavidin MACS beads (Miltenyi Biotech) and LD MACS separation columns according to the manufacturer’s instructions; purity of B220+ cells was >95%. Cells were subsequently washed with PBS at 4°C; 2 × 107 B cells were resuspended in 200 µl of PBS and injected into the tail vein of a sex-matched recipient. After 2 h, recipients were immunized s.c. with 200 µl of VLP. When DO11.10 transgenic CD4+ T cells were cotransferred, recipients were immunized with 50 µl of VLPs after transfer of B cells.

CFSE labeling and adoptive transfer of transgenic T cells

DO11.10 transgenic CD4+ T cells with a purity of ≥90% were obtained by positive MACS MicroBeads isolation, according to the manufacturer’s instructions (Miltenyi Biotech). Cells were labeled with 2.5 µM CFSE (Molecular Probes) for 7 min at room temperature. The reaction was stopped by addition of FCS to a final concentration of 10%, and cells were subsequently washed with PBS at 4°C; 5 × 106 labeled T cells were resuspended in 200 µl of PBS and injected into the tail vein of a sex-matched recipient. After 14 h, recipients were s.c. immunized with 50 µl of VLP.

Intracellular cytokine staining and flow cytometry

To analyze cell proliferation and cytokine release of adoptively transferred transgenic OVA-specific T cells, single-cell suspensions were prepared from inguinal lymph nodes of treated mice, lymph node cells were resuspended in RPMI 1640 plus 10% FCS and restimulated with 10−7 M PMA (Sigma-Aldrich) and 1 µg/ml ionomycin (Sigma-Aldrich) in vitro for 4 h. The cultures were supplemented with 10 µg/ml brefeldin A (Sigma-Aldrich) during the last 2 h of incubation. Restimulated cells were then fixed in PBS/2% formaldehyde for 20 min and permeabilized in PBS/0.5% saponin (Sigma-Aldrich) for 40 min on ice. During permeabilization, cells were incubated with allop hydroxycyanin-labeled anti-IFN-γ and PE-labeled anti-DO11.10 Abs (BD Biosciences). Events were acquired on a FACSCalibur and analyzed using CellQuest software (BD Biosciences).

Results

ssRNA and CpGs packaged in VLPs determine IgG isotype pattern

We have previously shown that VLPs derived from the coat protein of the bacteriophage Q8 contain E. coli-derived ssRNA when recombinantly produced in E. coli. This ssRNA can be digested by RNase and replaced by CpGs (39). To test the influence of ssRNA and CpGs on the Ab response, we immunized C57BL/6 mice subcutaneously with VLPs devoid of TLR ligands and VLPs containing ssRNA or CpGs in absence of additional adjuvants. All three forms induced potent IgG responses (Fig. 1A). Thus, as shown earlier, highly repetitive Ags, such as viruses and VLPs are able to induce potent IgG responses in the absence of additional signals (41, 42). In marked contrast, when the IgG subclasses were measured, the VLPs behaved qualitatively different; VLPs devoid of TLR ligands induced a response dominated by IgG1 (Fig. 1C), whereas the VLPs filled with RNA or CpGs induced mostly IgG2a (Fig. 1, B and D). The same observations were made in BALB/c mice (not shown).

IgG isotype pattern is regulated by TLR expression

CpGs are well described ligands of TLR9. To test whether CpGs influence IgG subclasses via TLR9, we immunized TLR9-deficient mice with Q8/CpG. In TLR9-deficient mice, induction of IgG2a was abrogated indicating that CpG-induced TLR9 signaling is required for IgG2a class switching (Fig. 2).

Th1/Th2 dichotomy has only a minor influence on IgG1 vs IgG2a isotype switching

To test the role of IFN-γ in driving IgG2a responses, IFN-γ-deficient mice were immunized with VLPs devoid of TLR ligands or
VLPs loaded with ssRNA or CpGs. Both wild-type and IFN-\(\gamma\)-deficient mice mounted strong IgG2a responses upon immunization with VLPs containing TLR ligands, while VLPs devoid of TLR ligands predominantly induced IgG1 (Fig. 3). Thus, the absence of IFN-\(\gamma\) did not affect the induction of IgG2a.

To further study the link between IgG isotype patterns and Th1/Th2 responses, we assessed the number of Q\(\beta\)-specific Th cells producing IFN-\(\gamma\) or IL-13 in C57BL/6 and BALB/c mice. VLP/ssRNA and VLP/CpG induced more IFN-\(\gamma\)-producing Th cells than IL-13-producing Th cells in both mouse strains. Surprisingly, however, VLPs devoid of TLR ligands only induced slightly higher numbers of IL-13-producing Th cells than IFN-\(\gamma\)-producing Th cells (Fig. 4, A and B). Thus, despite very similar Th cell responses (Fig. 4A), there was a clear difference in the IgG isotype pattern induced by VLPs with or without TLR ligands (Fig. 1).

To independently confirm that a Th1 response could be induced in the absence of TLR stimulation, CFSE-labeled DO11.10 TCR-transgenic T cells were transferred into naïve recipient mice. These mice were then immunized with VLPs devoid of TLR ligands or VLP/CpG, which had been coupled to the MHC class II-restricted OVA peptide that is recognized by the transferred DO11.10 T cells. Proliferation and production of IFN-\(\gamma\) was assessed 3 and 6 days later by intracellular cytokine staining. As observed in the ELISPOT experiments, a robust Th1 response was induced in the absence of a TLR9 ligand (Fig. 4C).

Th is needed for the isotype switching toward IgG

The results shown above established that the cytokines released by Th cells were not a key factor for the switching toward IgG2a. In a next set of experiments, we assessed whether cognate interaction between B and Th cells through CD40-CD40L interaction was needed for isotype switching toward IgG induced by CpG-loaded VLPs. To do this, CD40-deficient mice were immunized with...
FIGURE 4. Th1/Th2 differentiation has a minor influence on IgG isotype pattern. C57BL/6 or BALB/c mice were inoculated with 200 µg of VLP/ssRNA, VLP, or VLP/CpG. After 10 days, spleen and inguinal lymph nodes were collected and restimulated with VLP at a final concentration of 10 µg/ml for 3 days in vitro. Numbers of specific T cells expressing IFN-γ or IL-13 in individual C57BL6 mice (n = 3) (A) or BALB/c mice (n = 3) (B) with mean values are shown. Restimulated cells of naive control mice or unstimulated cells of immunized mice did not induce measurable numbers of IFN-γ or IL-13-producing T cells (data not shown). Ab isotype profile in sera of immunized C57BL/6 or BALB/c mice were similar to those shown in Fig. 1 (data not shown). One of two similar experiments is shown. VLP/CpG. As shown in Fig. 5, CD40-deficient mice failed to efficiently produce IgG Abs upon immunization with VLP/CpG, indicating that TLR ligands may modulate the IgG isotype pattern, however, that CD40-CD40L interaction and Th cells were still essential for isotype switching, confirming data by Pasare and Medzhitov (30).

**TLR expression in B cells rather than dendritic cells (DCs) drives production of specific IgG2a antibodies**

To analyze the influence of TLR9 expression in DC and B cells on Ab isotype switching, we transferred either TLR9-deficient B cells or control B cells into B cell-deficient mice. In B cell-deficient mice reconstituted with TLR9-deficient B cells, all professional APCs express TLR9 whereas all B cells are TLR9 deficient. First, we wanted to confirm that the absence of TLR9 in B cells would not affect the Th1/Th2 balance. To this end, CFSE-labeled DO11.10 TCR-transgenic T cells were transferred into B cell-deficient mice reconstituted with TLR9-deficient B cells. These mice were then immunized with VLP/CpG coupled to the MHC class II-restricted OVA peptide, which is recognized by the transferred T cells. As expected, no difference in the production of IFN-γ could be detected in mice harboring TLR9-deficient or control B cells (Fig. 6A). To assess the role of TLR9 expression in B cells in regulating isotype switching to IgG2a, B cell reconstituted mice were immunized with VLP/CpG and IgG2a was assessed. Although B cell-deficient mice reconstituted with normal B cells mounted strong IgG2a responses, mice reconstituted with TLR9-deficient B cells had a greatly reduced IgG2a response (Fig. 6B). This demonstrates that TLR9 expression in B cells and not other APCs is critical for the regulation IgG isotype patterns.

**Discussion**

Most pathogens induce potent Ab responses. There are several features which account for their high immunogenicity including the high density and repetitiveness of their epitopes (Ag organization) as well as their association with TLR ligands. Here we dissect the two parameters. We identified Ag organization as an important feature of pathogens in regulating the overall strength of the IgG response in the past (41, 42). However, TLR ligands further increase the magnitude of the IgG response and in particular determine the IgG isotype pattern. It is generally assumed that TLR ligands drive expression of IgG2a through induction of Th1 responses and IFN-γ. Here we show that this is only partly the case. Rather than stimulating the production of IgG2a Abs indirectly through induction of Th1 responses, the induction of IgG2a class switching is mainly induced via CpG-mediated engagement of TLR9 in B cells. These data help to explain why pathogens generally induce strong IgG2a responses, because most of these pathogens have TLR ligands that are able to directly activate B cells.

In a previous study it was shown that TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in a murine model of systemic lupus erythematosus (SLE). FcγRIIB-/- mice or 56R FcγRIIB-/- mice, two mouse strains which show spontaneous development of IgG2a and IgG2b autoantibodies were crossed with MyD88-/- and TLR9-/- mice and it was shown that MyD88 and TLR9 signaling was required for class switching to pathogenic IgG2a and IgG2b autoantibodies. The development of immune pathology and mortality but not the development of anti-self IgM+ B cells was dramatically reduced in the absence of TLR9 or MyD88 signaling. These findings are consistent with our results. Interestingly, however, this previous study...
FIGURE 6. TLR9 expression in B cells dictates the pattern of IgG isotypes. A, CFSE-labeled OVA-specific CD4+ T cells from DT11.10 transgenic mice were either adoptively transferred into B cell deficient (µMT) mice together with B cells from TLR9−/− mice or TLR9+/+ mice. Mice were immunized 14 h later s.c. with 50 µg of OVA-VLP or OVA-VLP/CpG. Single-cell suspensions were obtained from lymph nodes of recipient mice immunized 6 days previously and stimulated in vitro for 4 h with OVA peptide or 2 h with PMA/ionomycin. Ki67+ CD4+ T cells were analyzed for cell division and intracellular IFN-γ release. One of two similar experiments is shown. B, B cells from TLR9−/− or TLR9+/+ mice were adoptively transferred into B cell-deficient (µMT) mice, and mice were immunized with 50 µg of VLP/CpG 2 h later. Mean VLP-specific Ab titers per group (n = 3) with SDs are shown. Sera of day 11 were analyzed. The difference in the IgG2a titer between the two groups is statistically significant (*, p < 0.05), whereas the difference in the IgG1 titer is not statistically different (p > 0.05). Also the sera of the mice of experiment Fig. 6A were analyzed, and the Ab isotype pattern was the same as shown in Fig. 6B. One of two similar experiments is shown.

reported that generation of non-autoimmune IgG2a and 2b Abs by vaccination were not impaired in TLR9-deficient mice, which might seem inconsistent with our data (43). A possible explanation for the difference may be the different nature of the Ags used and the different questions asked. VLPs induce an IgG response that is dominated by IgG1 unless a TLR ligand is added that deviates the IgG response toward IgG2a. However, even without a TLR ligand some isotype switching toward IgG2a occurs. We have not addressed the question whether this IgG2a switching is reduced in TLR9-deficient mice. However, Ehlers et al. (43) by immunizing mice with NP-CGG or NP-Ficoll have compared the Ab isotype pattern against these Ags in TLR9−/− and TLR9+/+ mice and found no difference.

In another study, it was shown that activation of autoreactive B cells specific for host IgG is mediated by IgG2a-chromatin immune complexes and requires the synergistic engagement of the Ag receptor and a member of the MyD88-dependent TLR family (44). Further studies have shown that the role of the BCR was mainly delivery of the chromatin ligand to TLR9 and revealed hypomethylated CpG motifs, which are part of mammalian chromatin, as the critical ligand for TLR9 and the initiation of systemic autoimmune disease (45). Similarly it was shown that RNA-associated autoantigens activate autoreactive B cells by BCR/TLR7 activation (46). In summary, these studies identify TLR signaling as an essential component for induction of isotype-switched autoantibodies. In marked contrast, using CpG-loaded VLPs, we found a key role for TLR9 signaling for the isotype pattern (IgG1 vs IgG2a) but not for the IgG response per se. Furthermore, we demonstrate that TLR9 expression in B cells rather than DCs is pivotal. A similar pathway has recently been described for LPS-mediated induction of IgG1 and IgG2a class switching in mice via direct engagement of TLR4 in B cells (30).

Our findings offer a rationale for selective induction of this isotype by combined administration of TLR ligands linked to B cell Ags. This is particularly interesting as the only accepted adjuvant in humans is alum, which induces Th2 responses. By combined administration of TLR ligands with B cell Ags, Ag-specific IgG2a can be induced irrespective of the type of Th response.

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Disclosures

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