Toll-Like Receptor 3 Ligand and Retinoic Acid Enhance Germinal Center Formation and Increase the Tetanus Toxoid Vaccine Response

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Immunizations with T-cell-dependent antigens induce the formation of germinal centers (GC), unique lymphoid microenvironments in which antigen-activated B cells undergo class switching, affinity maturation, and differentiation into memory B cells. Poly(I:C) (PIC), a double-stranded RNA, and retinoic acid (RA), a metabolite of vitamin A which induces cell differentiation, have been shown to augment both primary and memory anti-tetanus toxoid (anti-TT) IgG responses. However, their influence on the GC reaction is unknown. In the present study, 6-week-old C57BL/6 mice were immunized with TT and cotreated with PIC, RA, or both. The splenic GC reaction was evaluated using immunofluorescence staining 10 days after TT priming. Each treatment enhanced the TT-induced GC formation (number of GC/follicle and GC area) about two- to threefold, which correlated with the titers of plasma anti-TT immunoglobulin G (IgG). Isotype switching to IgG1 was dramatically stimulated, with the greatest increase in IgG1-positive GC B cells induced by RA-PIC ($P < 0.001$). Moreover, PIC alone and RA-PIC robustly promoted the formation of the follicular dendritic cell (FDC) network in the GC light zone. PIC and RA-PIC also increased IgG1-positive B cells in the periarterial lymphatic sheath regions, where most IgG1-positive cells were plasma cells (CD138/syndecan-1 positive), suggesting that plasma cell generation was also enhanced in non-GC regions. The stimulation of several processes, including antigen-induced GC formation, isotype switching, FDC network formation within GC, and plasma cell differentiation by RA and/or PIC, suggests that this nutritional-immunological combination could be an effective means of promoting a robust vaccine response.

Successful vaccination with protein antigens depends on the differentiation and maturation of antigen-activated B cells into class-switched, long-lived memory B cells. The germinal center (GC) reaction is crucial to these processes. Upon infection or immunization, some antigen-activated B cells that have encountered cognate antigen-activated T helper (Th) cells differentiate directly into antibody-secreting plasma cells and produce antibodies that provide a level of initial protection. However, other antigen-activated B cells migrate into the secondary follicles of lymphoid organs, including spleen and lymph nodes, and participate in the GC reaction, which comprises the formation a dynamic antigen-induced microenvironment in which B cells can differentiate and mature, undergoing processes of class switch recombination and somatic hypermutation (12, 40, 52, 54). Typically, GCs exhibit polarization into two zonal regions, termed the dark zone and the light zone. In the dark zone, newly stimulated B cells proliferate rapidly and undergo somatic mutation. The progeny of these B cells, centrocytes, migrate into the light zone, where follicular dendritic cells (FDC) (1), together with antigen-activated Th cells, provide essential signals for B-cell survival, class switch recombination, affinity maturation, and differentiation into long-lived plasma cells or memory B cells (5, 10, 32, 54). Hence, the formation of the GC structure and the cellular and molecular processes that occur within GCs are essential for the generation of B cells expressing antibodies of the immunoglobulin G (IgG), IgA, or IgE class, with high-affinity antigen-combining sites, as well as for the production of memory B cells that, on reactivation, will produce a rapid, high-output response capable of neutralizing the pathogen and protecting the infected host from developing life-threatening disease (32).

The Toll-like receptor (TLR) family is a group of pattern recognition receptors that are mostly distributed on monocytes and dendritic cells (7, 56). Various TLR members bind conserved microbial structures, initiating reactions that are essential for innate immunity and immunoregulatory for adaptive immunity (39). Thus, TLR ligands are now considered important adjuvant targets for vaccine development (21, 45). Poly(I:C) (PIC), a synthetic double-stranded polyribonucleotide composed of polyribosinosinic acid paired with polyribocytidyl acid, is a mimic of double-stranded RNA viruses (59). PIC binds to TLR3/MDA5 and activates downstream signaling pathways (21). TLR3 is especially implicated in sensing viral infections and in activating innate immunity (31, 58). PIC has long been known for its ability to induce type I and type II interferons (25), stimulate the cytotoxic activity of NK cells (4, 41, 59), and increase antiviral and antitumor reactions (26, 50, 51). Moreover, PIC is a potent activator of adaptive immunity through induction of dendritic cell maturation, regulation of Th1/Th2 responses, and promotion of antigen-specific antibody responses (4, 59), and TLR ligands have been suggested to promote GC formation (42).

Retinoic acid (RA), an active metabolite of vitamin A (ret-
is well known as a potent agent of cell differentiation, including for B cells (9, 11, 18, 33, 36) and other immunoregulatory cells. Vitamin A, RA, and related retinoids have been shown to be necessary for maintaining antigen-specific antibody responses (47). Vitamin A deficiency causes low and dysregulated primary and memory antibody responses against several T-cell-dependent (TD) and polysaccharide antigens (47, 57), including common vaccines, such as tetanus and diphtheria toxoids (46). These impairments are reversible, since providing vitamin A or RA to vitamin A-deficient animals resulted in significant rescue of the anti-tetanus antibody response (16, 24). In humans, supplementation with vitamin A is recognized as an effective means of reducing morbidity and mortality among young children, a population at risk for vitamin A deficiency (53), and vitamin A supplementation has shown therapeutic benefits in infectious diseases, such as measles and diarrhea (53, 57, 60). In animals with normal vitamin A status, RA can act as a potent immunological adjuvant. Recently, we have reviewed studies showing that RA augments the production of an antigen-specific B-cell response in vivo (48). RA has gained attention due to its multiple effects on innate and adaptive immunity, including its ability to modulate cytokine production (27), promote the development of Th2 cells (20), induce gut-homing T cells (22) and T regulatory cells (6), regulate Th17 cells (37), stimulate B-cell maturation (11, 62), and increase primary and memory antibody responses (14, 16, 27). The potentiation of TD antigen-specific antibody production after treatment with RA could be due to a direct involvement in B-cell activation, as RA enhanced the production of IgM and IgG induced by both TD and T-cell-independent antigen in human peripheral blood mononuclear cells (61) and stimulated the plasmacytic maturation of isolated B cells in vitro (11). RA also elevated IgG and IgG1 production triggered by anti-μ or anti-CD40 in vitro (11, 36), stimulated gut-homing B cells (35), and increased the activation of the B-cell memory pool present in human peripheral blood lymphocytes (18). Thus, retinoids may have pleiotropic effects on the development of immune responses after infection and vaccination. However, to our knowledge, the ability of retinoids to modulate GC formation has not been investigated.

On the basis of our previous reports that the combination of RA and PIC, when administered to normal adult or neonatal mice at the time of tetanus toxoid (TT) priming, strongly increased the primary anti-TT IgG response, the number of anti-TT IgG-secreting plasma cells (27, 28), and the anti-TT IgG memory response elicited at a later time by TT alone (27, 28), we hypothesized that PIC and RA influence the initial antigen-induced GC reaction. Therefore, the present study was designed to assess immunocytochemical changes in the spleen and their correlation with plasma antibody titers to test whether PIC and RA, given either alone or together, promote the GC response to TT, a clinically important TD antigen.

**MATERIALS AND METHODS**

**Animals and experimental design.** Animal protocols were approved by the Institutional Animal Use and Care Committee of The Pennsylvania State University. Six-week-old C57BL/6 female mice (Charles River Laboratories, Wilmington, MA) were divided into four groups (the control, RA, PIC, and RA-PIC groups) and fed a nutritionally complete diet (LabDiet 5001, which contains 22 IU vitamin A/g diet; Purina Mills, St. Louis, MO) throughout the experimental period. Mice were immunized with 10 μg of TT (Connaught Laboratories, Swiftwater, PA) by intraperitoneal injection and cotreated with all-trans RA (Sigma, St. Louis, MO) orally at a dose of 37.5 μg/mouse and/or with 2 μg of PIC (stabilized with poly-l-lysine and carboxymethylcellulose and administered intra-peritoneally) on the first day of the experiment, counted as day 0, as previously described (27, 28). From day 1 to day 6 after immunization, the mice were fed the same dose of RA or oil as a control each day. Spleen and blood samples were collected at 10 days postimmunization because this time corresponds to a well-developed state of GC formation (8, 52). In preliminary experiments (data not shown), we confirmed that 10 days postimmunization was an appropriate time to examine the GC response to TT and plasma anti-TT titers.

**Plasma anti-TT antibody enzyme-linked immunosorbent assay.** Plasma anti-TT IgG and anti-TT IgG1 were quantified as previously described (14, 27). Briefly, plasma was serially diluted to ensure that measurements were in a linear dose-response range. A standard of serially diluted pooled immune serum was included on every plate, and titers of antibody were calculated based on this standard curve. One titer unit was defined as the dilution fold that produced 50% of the maximal optical density for the standard sample.

**Preparation of tissue section and immunofluorescence staining of GCs.** Spleen tissue was embedded in a cryostat compound (Tissue-Tek OCT; Sakura, Torrance, CA) and snap-frozen in isopentane precooled by dry ice. The frozen blocks were then stored at −80°C until use. Frozen tissue blocks were cut into 6-μm-thick sections and mounted onto Superfrost glass slides (Fisher Scientific, Houston, TX), with four sections per slide. The sections were dried at room temperature for 1 h and stored at −80°C until use.

Before being stained, tissue sections were fixed with ice-cold acetone for 10 min, followed by rinsing with phosphate-buffered saline twice. The sections were then blocked with 1% bovine serum albumin–phosphate-buffered saline buffer for 1 h. The endogenous avidin and biotin binding was blocked using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) in accordance with the manufacturer’s instructions. After being blocked, the sections were incubated with an antibody mixture in a moist chamber at room temperature for 2 h. For enumeration of GCs, the sections were costained with biotin-potassium agglutinin (PNA) (1:1,000; Vector Laboratories, Burlingame, CA) to detect GCs and with fluorescein isothiocyanate (FITC)-labeled anti-IgD (5 μg/ml; Biocytogenics, San Diego, CA) to identify B-cell follicles (52, 55). To evaluate IgG isotype switching within GCs, the sections were counterstained with biotin-PNA and Alexa 647-labeled anti-IgG1 (1:1,000; Molecular Probes, Eugene, OR). The sections were then washed and incubated with streptavidin-labeled Alexa 568 (1:1,000; Molecular Probes, Eugene, OR) for 45 min. After the wash, coverslips were applied on the sections with antifade mounting medium. The numbers of B-cell follicles, GCs, and IgG1-positive GCs were counted under an Oxford BX60 fluorescence microscope (Olympus, Center Valley, PA). The ratio of GCs to B-cell follicles and IgG1-positive GCs to total GCs were calculated to normalize the differences in spleen size. To measure the size of the GC, a minimum of 20 GCs in each section were photographed with a digital camera, and the area of each GC was measured using Image-Pro software (Media Cybernetics, Bethesda, MD). These measurements are recognized as necessarily semiquantitative; however, all measurements were made under the same conditions so that comparisons between samples could be made.

**Immunofluorescence staining of FDC networks.** The FDC network in GCs was detected using sequential staining. Briefly, the spleen sections were fixed and blocked as described above. The sections were then stained with biotin-conjugated PNA, followed by incubation with streptavidin-Alexa 568 for 45 min. After being washed, the sections were incubated with appropriately diluted (1:20) anti-mouse FDC-M1 (BD Pharmingen, San Diego, CA) in a moist chamber at 4°C overnight. The next day, the tissue sections were washed and then incubated with Alexa 488-anti-rat IgG (1:1,000; Molecular Probes) for another 45 min. The numbers of FDC networks and GCs were counted under a fluorescence microscope. The ratio of FDC networks to GCs was calculated to normalize for possible differences in spleen size. To measure the area of FDC networks, a minimum of 20 FDC networks in each spleen section were photographed with a digital camera, and the area of each FDC network was circumscribed and then measured using Image-Pro software as described above.

**Statistical analysis.** Data are reported as mean ± standard error (SE). Two-way analysis of variance (ANOVA) was used to determine the main effects of RA and PIC and the interaction of RA with PIC, while group differences were determined by Fisher’s protected least-significant-difference test, using Super-Anova software (Abacus Software, Berkeley CA). When group variances were unequal, as for antibody titers, data were subjected to log10 transformation before statistical analysis. Correlations were assessed by linear regression analysis. A P value < 0.05 was considered statistically significant.
RESULTS

RA and PIC enhanced the primary anti-TT IgG response. The plasma titers of anti-TT IgG were measured on day 10 after priming with TT and applying the treatments as illustrated in Fig. 1A. Compared to those of vehicle-treated control mice, the anti-TT IgG titers of mice treated with RA alone, PIC alone, or both combined were significantly increased ($P < 0.05$ between all treatments). However, the combination of RA and PIC was more potent than either agent alone, increasing anti-TT IgG about 60-fold (Fig. 1B). These results confirmed our previous observations of the elevation of the primary anti-TT IgG response induced by RA, PIC, and RA and PIC combined (27) and provided antibody data for correlation with the immunohistochemical results obtained from spleen sections of the same animals.

RA and PIC promote TT-induced GC formation. To assess GC formation, spleen sections were stained with PNA and anti-IgD to detect GCs and B-cell follicles, respectively (Fig. 2A). In control mice, TT induced a weak GC reaction, evidenced as a few small, PNA-positive GCs, with an average about 0.004 mm$^2$ in area (Fig. 2C), treatments with RA alone increased the area of the GC about two- to threefold, and PIC alone as well as RA-PIC enlarged GCs slightly more, about fourfold (Fig. 2A and C). By two-way ANOVA, RA was a positive factor for the GC-to-B-cell-follicle ratio, and PIC was a positive regulator for both this ratio and the sizes of GCs within follicles (Fig. 2B and C). To rule out a nonspecific induction of the GC response, we also investigated the effect of RA-PIC on GC formation in naïve mice. GC formation in spleens of naïve mice was not visible, in contrast to the clearly observable GCs in TT-immunized, vehicle-treated mice (Fig. 2A). Although treatment with RA-PIC slightly induced GC formation, the sizes and number of GCs were not as remarkable as those observed in TT-immunized mice. As a control for PNA specificity, some of the slides were incubated with 0.2 M $\beta$-galactose, which competes with galactosyl residues on glycoproteins for binding to PNA (52). Under these conditions, no PNA-positive GCs could be seen (data not shown). Therefore, these data indicated that the GC response was strictly induced by TT antigen challenge and that the enhancement of GC formation by RA and PIC, monitored by PNA staining, was also an antigen-dependent response rather than a nonspecific amplification.

Linear regression analysis was used to compare the plasma anti-TT IgG titers to the morphological results shown in Fig. 2B and C. The titers of anti-TT IgG were strongly correlated with both the GC-to-B-cell-follicle ratio ($R^2 = 0.69; P < 0.01$) (Fig. 2D) and the size of the GC ($R^2 = 0.51; P < 0.05$) (Fig. 2E). Therefore, RA and PIC promoted the TT-induced GC response, which may have directly contributed to the enhanced anti-TT IgG response measured in plasma.

RA and PIC increase IgG1-positive cells within GC regions. Antibody isotype switching is one of major events that occur in GCs (12). Because RA and PIC have been shown to significantly regulate anti-TT IgG isotypes (27) and IgG1 is the major IgG isotype induced by TT, we wanted to determine if RA and PIC augment the frequency of IgG1-positive cells within GCs. For this analysis, spleen sections were again stained with biotinylated PNA and Alexa 568-streptavidin to detect the GCs, and IgG1 was detected using an FITC-labeled antibody specific to this Ig isotype (Fig. 3A and secondary antibody control shown in Fig. 3B). In control mice immunized with TT, IgG1-positive cells were only weakly detectable (Fig. 3A). RA alone slightly increased the frequency of IgG1-positive cells within GCs, while it increased the ratio of IgG1-positive GCs to total GCs by about 30% (Fig. 3A and C). PIC alone dramatically enhanced both the fluorescence intensity and the frequency of IgG1-positive cells in GCs, elevating the percentage of IgG1-positive GCs about twofold (Fig. 3A and C). The combination of RA and PIC resulted in the highest level of IgG1 expression in GCs and increased the percentage of IgG1-positive GCs about threefold compared with the level for the control group (Fig. 3A and C). Therefore, these results provided visual evidence that the combination of RA and PIC was more effective than either agent alone in enhancing class switching to IgG1 within GCs.

Linear regression analysis indicated that plasma titers of anti-TT IgG1 were strongly correlated ($R^2 = 0.84; P < 0.001$) with the percentage of IgG1-positive GCs (Fig. 3D). These results...
suggest that RA and/or PIC enhanced the anti-TT IgG1 response through an upregulation of IgG1 switching within GCs.

**RA and PIC elevate IgG1-positive plasma cells in the PALS region.** The periarteriolar lymphoid sheath (PALS) is a T-cell area in the spleen, around arterioles, where naïve B cells are exposed to antigen and Th cells and become activated (55). Upon activation, TD antigen-specific B cells either migrate into B-cell follicles and participate in the GC response to differentiate into long-lived plasma cells or may remain in the PALS region and differentiate into short-lived plasma cells (2). In our studies, we noticed that mice immunized with TT and treated with RA and/or PIC showed a higher frequency of IgG1-positive cells located in this region (Fig. 4A). In mice treated with PIC and RA-PIC, the intensity and frequency of IgG1 expression in the PALS region were both dramatically increased (Fig. 4B). To further characterize those IgG1-positive cells in PALS region, we costained the spleen tissue of RA-PIC-treated mice to detect IgG1 and CD138/syndecan-1, a surface marker of plasmacytic cells (2). The results showed that most IgG1-positive cells in the PALS region were also expressing CD138, consistent with the interpretation that they were antibody-secreting cells. Previously, we showed that anti-TT antibody-secreting cells in plasma, measured by an enzyme-linked immunospot assay, correlated well with plasma anti-TT IgG titers (27). Together with these morphological results, the data further suggest that RA and PIC not only strengthen the TT-induced GC reaction but also promote IgG isotype switching and plasma cell generation, in part in the non-GC regions.
RA and PIC promote FDC network formation. Stromal cells in the GC play a crucial role in the GC response (13), and FDC are particularly important for the positive selection of high-affinity B cells (1, 38). Since treatments with RA and/or PIC significantly increased TT-induced GC formation and anti-TT IgG production, we next wanted to test if they affect the formation of FDC networks. Networks of FDC can be visualized by staining with anti-mouse FDC-M1, a marker of FDC. The results clearly showed that the FDC networks were located on one side of the GC (Fig. 5A, RA and PIC), which is consistent with previous reports that FDC networks mostly occupy the light zone of the polarized GC (12, 29, 55). Although RA and/or PIC did not affect the number of FDC networks (data not shown), these treatments significantly enlarged the area of readily visualized FDC networks. In the control group immunized with TT, the FDC networks were small and dim, suggesting the presence of only a few FDC in the GC (Fig. 5A). After treatment of RA, the FDC network formation was slightly expanded, and the average area of the FDC network was increased about 30 to 40%, although this difference was not statistically significant (Fig. 5A and B). Remarkably, administration of PIC and RA-PIC robustly enhanced the formation of the FDC network in GCs, as shown by more-intense staining, and increased the average size of the FDC network about threefold ($P < 0.05$) (Fig. 5A and B).

By linear regression analysis, the titers of anti-TT IgG were strongly correlated with the area of the FDC networks within GCs (Fig. 5C). Therefore, these results suggested that the expansion of FDC networks in GCs, mainly influenced by PIC, could directly contribute to the enhanced GC response as well as the increase in anti-TT antibody response in these groups of mice.

**DISCUSSION**

The combination of RA and interferons has been applied as an effective strategy for cancer chemoprevention and chemotherapy (34, 43). While the ability of vitamin A to reduce childhood mortality and mortality has had a strong impact on public health (53, 57, 60), only recently have basic cellular and molecular mechanisms come to light (6, 11, 18, 22, 27, 35–37, 61). Previously, we reported that the combination of RA and PIC, a TLR3 ligand, significantly enhanced primary and memory anti-TT antibody responses in both adult (27) and neonatal (28) animals. These results suggested that the combination of RA and PIC might act in concert through the cell differentiation-promoting effects of RA and the strong ability of PIC to promote innate immunity, particularly through the type I interferon axis. In the present studies, we hypothesized that RA and PIC could promote the GC formation to a clinically relevant antigen, TT. We realized that TT would activate only a small proportion of T cells and B cells. But because we had previously observed strong promotion of the in vivo antibody response to TT by RA and PIC (15, 27, 28), and due to the...
public health significance of TT vaccination for disease prevention in both infants and adults (44), we believed that it was important to examine the regulation GC formation to a natural vaccine antigen.

Receptors of the TLR family have been shown to play crucial roles in adaptive immunity, such as the Th1/Th2 cytokine response and dendritic cell maturation. However, their role in the response of B cells has not yet been elucidated. Recent studies reported that human B cells, including naïve B cells, GC B cells, and memory B cells, all expressed TLRs 1, 2, 7, and 9 (30). In vitro treatment with TLR agonists, including Pam3CSK4 (TLR1/2), MALP-2 (TLR2/6), R848 (TLR7/8), and CpG (TLR9), could directly stimulate human B cells and promote B-cell-receptor-triggered B-cell proliferation (18, 30, 49). These findings suggest that the ligation of TLRs could provide direct signals to induce B-cell activation. Although B cells do not constitutively express TLR3 (19), PIC can induce the expression of TLR3, as well as costimulatory molecules such as CD80 and CD86, on mouse B cells (17). Moreover, PIC promoted immature anti-snRNP-specific B cells to differentiate into plasma cells and form GCs (17). In contrast, in another study, PIC enhanced human B-cell activation by promoting dendritic cell maturation rather than by direct stimulation (49). Despite the need for additional research to resolve this inconsistency, the present study demonstrated that PIC has the potential to significantly regulate the formation of FDC networks. Hence, PIC could facilitate the developmental program of B cells in GCs by directly modulating the microenvironment for B-cell activation and differentiation.

The potential mechanisms by which RA and PIC enhance antigen-specific antibody response are still under investigation. The combination of RA and PIC has been shown to augment the maturation of antigen-presenting cells (monocytes and dendritic cells), which could consequently facilitate the antigen presentation and downstream T-cell activation (27, 28). Moreover, RA and PIC differentially regulated type 1/type 2 cytokines at both mRNA and protein levels (15, 27, 28), which was strongly correlated with the plasma titers of anti-TT IgG isotypes. Since T-cell help is limiting in the primary antibody response to protein antigen (29), the induction of both type 1/type2 (Th1/Th2) cytokines by PIC could augment T-cell help at the early stages of the vaccine response (27). The GCs play essential roles in the development of TD antibody responses, serving as a home for Ig isotype switching as well as the differentiation of plasma cells and memory B cells before release into the circulation (12, 32, 52). In the present study, we have shown that RA and/or PIC can significantly promote the TT-induced GC reaction, which could be another important mechanism contributing to the enhanced anti-TT antibody response by these agents.

RA and PIC could regulate the GC reaction through at least the following three aspects: GC formation, isotype switching, and FDC network formation. We first observed that RA, PIC, and RA-PIC increased the size and number of GCs per follicle. The enhancement of GC formation depended on antigenic challenge, because RA-PIC did not induce the formation of PNA-positive GCs in naïve mice. Although RA-PIC synergistically augmented anti-TT antibody responses, the combination did not further elevate the size or number of GCs in comparison with either agent alone. This inconsistency suggests that the size and the number of GCs may not completely represent the GC reaction. To further investigate the effect of RA and PIC, each alone and both combined, on the GC response, we assessed the Ig isotype switching, one of the major events and outcomes of the GC response, by staining for IgG1 expression. Previous studies showed that RA and PIC elevate the plasma titer of anti-TT IgG1 in adult as well as neonatal mice (27, 28). RA was also shown to significantly
increase the synthesis of IgG and IgG1 triggered by ligation of the B-cell receptor or by anti-CD40 in human and murine B-cell cultures (11, 36). In the present study, both RA and PIC alone increased the frequency of IgG1-positive cells in the GC, while RA and PIC combined resulted in the highest level of IgG1-positive cells within the GC. The correlation between plasma anti-TT IgG1 levels and IgG1-positive GCs implies that an upregulation of IgG isotype switching should be one of the major aspects of the GC reaction contributing to the enhancement of anti-TT antibody production by RA and PIC. The elevated IgG isotype switching within GCs after treatment with RA and/or PIC could be partially due to the effects of RA and PIC on type 1 and type 2 cytokine production. RA and PIC were shown to significantly increase type 2 cytokines, such as interleukin-4 (IL-4), IL-5, and IL-10, in spleen tissue (27), which could contribute to the increased IgG1-positive cells in the GC. Although only IgG1 was tested in the current study, our previous study of the antibody response showed that all isotypes of IgG were increased by PIC alone and PIC combined with RA (27).

The formation of networks of FDC, the stromal cells resident in the GC, is critical in furthering the GC response (1). The major function of FDC is to present the intact antigen-antibody complex on the cell surface of their dendritic processes, allowing for intimate contact with GC B cells which, when their B-cell receptors are of appropriately high affinity, are prevented from undergoing apoptosis and are positively selected for survival and further differentiation (1, 5, 38). FDC, together with specialized antigen-specific Th cells within GCs, support both B-cell survival and B-cell proliferation, facilitating GC formation (38). In the present study, RA alone did not increase the area of the FDC network, which appeared relatively faint. In contrast, PIC alone and combined with RA remarkably promoted the formation of the FDC network, seen as relatively bright, polarized caps in some GC sections (Fig. 5A, RA/PIC). The size of the FDC networks was positively correlated with the titers of anti-TT IgG in plasma. Thus, the ability of PIC to enhance GC microarchitecture through FDC network formation may be another mechanism contributing to the enhanced anti-TT antibody response in vivo.

Notably, in addition to their effects on enhancing the TT-triggered GC response, RA and/or PIC increased the number of IgG1-positive plasma cells in the PALS region. The PALS region is a T-cell zone in the spleen where naïve B cells are exposed to antigen and initially activated. Upon activation, TD antigen-specific B cells either participate in the GC response and differentiate into long-lived plasma cells or remain in the PALS region and differentiate into short-lived plasma cells (2, 55). The short-lived plasma cells are detectable in the PALS region on day 2 after immunization, peak on days 8 to 10, and then disappear on day 14 (23). Compared with the long-lived plasma cells, these short-lived plasma cells produce IgM and IgG with low affinities and short half-lives and hence contribute mostly to an early adaptive immune response against TD antigen. In the present morphological study, RA, PIC, and RA-PIC significantly increased the generation of plasma cells.
in the PALS region, suggesting that these treatments also enhanced the early and short-lived antibody production. This would be consistent with previous kinetic studies in which the TLR4 antigen lipopolysaccharide stimulated the titer of anti-TT IgG in plasma within 7 days after priming (3), after which titers increased further 10 and 14 days after primary immunization. Thus, RA and PIC could augment both the early and the late phases of plasma cell development, which occur in the PALS region and in the GC region, respectively.

Overall, the present study provides the first evidence that RA and PIC can potentiate the antibody response to TT by modifying several GC-related mechanisms essential for a TD antibody response. These processes may contribute importantly to the promoting adjuvant properties of RA combined with PIC in increasing the in vivo vaccination responses to TT and potentially to other TD antigens. These encouraging results suggest that additional studies with other clinically relevant antigens should now be conducted.

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