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A Possible Physiological Role of Caspase-11 During Germinal Center Reaction

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Abstract: Caspase-11 has been known as a dual regulator of cytokine maturation and apoptosis. Although the role of caspase-11 under pathological conditions has been well documented, its physiological role has not been studied much. In the present study, we investigated a possible physiological function of caspase-11 during immune response. In the absence of caspase-11, immunized spleen displayed increased cellularity and abnormal germinal center structure with disrupted microarchitecture. The rate of cell proliferation and apoptosis in the immunized spleen was not changed in the caspase-11-deficient mice. Furthermore, the caspase-11-deficient peritoneal macrophages showed normal phagocytotic activity. However, caspase-11-/- splenocytes and macrophages showed defective migrating capacity. The dysregulation of cell migration did not seem to be mediated by caspase-3, interleukin-1α or interleukin-1β which acts downstream of caspase-11. These results suggest that a direct regulation of immune cell migration by caspase-11 is critical for the formation of germinal center microarchitecture during immune response. However, humoral immunity in the caspase-11-deficient mice was normal, suggesting the formation of germinal center structure is not essential for the affinity maturation of the antibodies.

Key words: caspase-11, germinal center reaction, immune response, immune cell migration

Caspase-11 is an inducible cysteine protease that regulates apoptosis and cytokine maturation (Wang et al., 1996; Kang et al., 2000). There have been many studies examining a role of caspase-11 mediating cell death and inflammation under pathological conditions such as systemic inflammation (Wang et al., 1996), brain ischemia (Kang et al., 2000), experimental autoimmune encephalomyelitis (Hisahara et al., 2001), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease (Furuya et al., 2004). The regulation of inflammatory response by caspase-11 is mediated by activation of caspase-1 (Wang et al., 1998). Caspase-11 activates caspase-1 by physical interaction and the activated caspase-1 processes pro-interleukin (IL)-1α/β or pro-IL-18 into mature forms (Wang et al., 1996; 1998). In certain conditions, caspase-11 cleaves and activates caspase-3 and thereby induces apoptosis (Kang et al., 2000; Hisahara et al., 2001). However, its physiological role has been paid little attention. Recently, a study reported that caspase-11 regulates immune cell migration by promoting Aip/Cofilin-mediated actin depolymerization (Li et al., 2007). This implies that caspase-11 may regulate immune cell function other than innate immunity.

The germinal center (GC) is a microanatomical structure formed in secondary lymphoid tissues during T cell-dependent immune responses. Antigen-stimulated B cells first proliferate in the T cell rich area called periarteriolar lymphoid sheath and migrate into B cell follicles to form GC, where the oligoclonal, activated B cells rapidly proliferate and then further migrate out to be selected based on the specificity of the surface immunoglobulins in a process known as affinity maturation by apoptosis or survival signals (reviewed by Tarlington and Smith, 2000). The control of this process is mediated by interaction of B cells with helper T cells and dendritic cells harboring antigens or survival signals. All these components of the GC reaction depend on the tight regulation of cellular migration.

The ability to undergo proper migration is a basic requirement for immune cells to survey many secondary lymphoid tissues throughout the body for foreign antigens. Tight regulation of the migration of specific cell types provides homeostatic control of the cellular migration and functional compartmentalization as well as the inflammatory response control. In response to a foreign antigen, a complex pattern of migration of the lymphocytes is orchestrated to facilitate specific cell-cell interaction for an

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effective immune response (Reviewed by Moser et al., 2001; Mackay, 2001). In the present study, we investigated whether caspase-11 plays a role in the GC reaction to understand a physiological function of caspase-11. Caspase-11-deficient mice displayed disrupted GC structure and increased spleen cellularity following immunization induced by 4-hydroxy-3-nitrophenylacetyl hapten conjugated to chicken gamma globulin (NP-CGG). The rate of cell death or proliferation in the spleen after NP-CGG injection and phagocytic activity of peritoneal macrophages were normal. In accordance with the previous report by Li et al. (2007), caspase-11 lymphocytes showed defective migratory capacity following immunization. However, humoral immunity in the caspase-11-deficient mice was normal. Our results confirm that caspase-11 regulates immune cell migration in vivo.

MATERIALS AND METHODS

Materials

Anti-caspase-11 antibodies were described previously (Wang et al., 1996). Anti-CD3, B220, and anti-MacI antibodies were purchased from Caltag laboratories. All of the secondary antibodies were purchased from Jackson ImmunoResearch. All other antibodies and reagents were purchased from Sigma-Aldrich unless otherwise stated.

Immunization

Mice at 6-8 wks of age were intraperitoneally injected with 100 μg of aluminium potassium sulfate (alum)-precipitated 4-hydroxy-3-nitrophenylacetyl-Chicken Gamma Globulin (NP_{21}-CGG). Briefly, 1 mL of NP_{21}-CGG (1 mg/mL in PBS) was precipitated by adding 4 mL of 10% alum and then 3.4 mL of potassium hydroxide drop wise. After washing three times with PBS, alum/NP_{21}-CGG was resuspended in sterile PBS. Concentration of unprecipitated immunogen was measured by BioRad protein assay and 80 μg of precipitated immunogen suspension was used for one animal. At indicated time, serum was prepared from the blood and spleens were taken out for analysis.

Cell sorting

To examine the cell type of caspase-11 expressing cells wild type mice were injected with LPS (20 mg/kg body weight) and spleens were taken out after 12 hrs. Red blood cell-free single cell suspensions were prepared and pooled cells of 10^8 were stained with anti-B220-PE for B cells, and anti-CD3-FITC for T cells. Another pool of cells was stained with anti-MacI-PE. Then the cells were sorted according to their cell surface markers using MoFloI cell sorter. The resulting cells were lysed in the SDS sample buffer and immunoblot-assayed for the detection of caspase-11.

Immunohistochemistry and TUNEL staining

Frozen sections of spleens were prepared using using Leica CM3000 cyostat. For the detection of Bcell follicles, macrophages and germinal centers, sections were stained with anti-B220, anti-MacI or F4/80, and peanut agglutinin (PNA) and visualized using ABC kit and Vector VIP substrate kit (Vector Laboratories) according to the manufacturer's protocol. TUNEL was performed using ApopTag (Millipore) according to the manufacturer's protocol.

In vivo cell proliferation assay

To check the proliferation in the spleen after immunization, mice were first immunized with 80 μg of NP_{21}-CGG precipitated with alum and BrdU (1 mg/mL) was added into drinking water for 2 days before sacrifice. RBC-free splenocytes taken at day 2, 4, 6, and 8 were seeded onto 96 wells at a density of 5×10^5 cells/well and the level of BrdU incorporation was measured using BrdU Cell Proliferation Assay kit (Oncogene Research Products).

Antibody titration

To measure the affinity of the antibody, the serum taken from mice at day 0, 3 or 12 after the immunization was serially diluted and incubated on 96-well plates coated with antigen, NP_{21}-BSA or NP_4-BSA (50 ng/well). The amount of bound antibody was measured from the readings at 490 nm using plate reader (Molecular Devices) following horseradish peroxidase-conjugated secondary antibodies specific for each immunoglobulin isotype and phenylenediamine substrate incubation.

Peritoneal macrophage isolation

To isolate peritoneal macrophages, mice were peritoneally injected with thioglycollate medium (4%, 1 mL/mouse). After 6 days, mice were sacrificed and peritoneal exudate cells were collected from the peritoneum using an 18-guage needle and 1% FBS/RPMI medium. Cells were plated and incubated for 3 hrs and unattached cells were washed away. Attached peritoneal macrophages were used for further experiment.

Migration assay

Isolated splenocytes (10^6 cells/well) or peritoneal macrophages (10^5 cells/well) were seeded on the top well of Transwell plates (Costar) in RPMI medium. Bottom chamber was filled with medium containing chemokines, SDF-1α or BLC (R&D systems). For lymphocytes, Transwell with 5 μm-pore membrane was used and for peritoneal macrophages, 8 μm. After 4 hrs of incubation, migrated lymphocytes were collected from the bottom chamber and counted using a hemacytometer. In case of peritoneal macrophages, attached cells at the top of the upper chamber

membrane were scraped off and the cells migrated to the bottom side of the membrane was fixed with 4% paraformaldehyde and stained with anti-MacI antibody. After the staining, the membrane was carefully removed from the well using a scalpel, mounted on the slide glass and counted under a fluorescence microscope.

Phagocytosis assay

Peritoneal macrophages were plated (10^5/mL) in 10% FBS/RPMI and red fluorescent latex beads (2 μm diameter, 10^6/35 mm dish) were added (cells:beads=1:10). After 6 hrs of incubation, cells were washed extensively with RPMI medium and fixed with 4% paraformaldehyde dissolved in PBS. Cells were then stained with anti-MacI antibody and the number of cells that ingested fluorescent beads was counted under fluorescent microscope. In another set of experiments, dead lymphocytes were used instead of beads. RBC-free splenocytes were isolated from caspase-11 mice, UV-irradiated (200 mJ, 10 sec), and cultured for 6 hrs in 10% FBS/RPMI medium. Cell death of more than 95% was confirmed by trypan blue staining. Dead lymphocytes were stained with Hoechst dye, extensively washed and added to the cultures of peritoneal macrophages (5:1 ratio). After 6 hrs of incubation, cells were washed, fixed and stained with anti-MacI. Then the number of MacI-positive cells that ingested Hoechst-stained lymphocytes was counted.

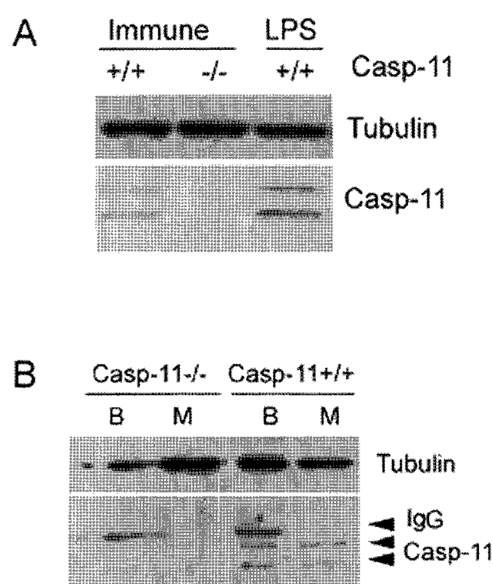

Fig. 1. Caspase-11 is expressed in B cells and macrophages in the spleen after immunization. A. To examine if caspase-11 was induced during the GC reaction, the wild type (Casp-11 +/+) and caspase-11-deficient (Casp-11-/-) mice were immunized with NP_{21}-CGG (80 μg/mouse) precipitated with Alum. At day 12 postimmunization, spleens (Immune) were analyzed for the caspase-11 expression using anti-caspase-11 antibody (Casp-11). As a positive control, spleen lysates from LPS challenged mice were loaded together (LPS). The blot was reprobed for anti-tubulin as a loading control. B. The cell type of caspase-11 expressing cells was examined by analyzing the sorted cells by immunoblot assay. The sorted cells were analyzed by immunoblot assay using anti-caspase-11 antibody. The blot was reprobed with anti-tubulin antibody for a loading control. Note the expression of caspase-11 in the B cells and macrophages.

RESULTS

Caspase-11 is expressed in B cells and macrophages after immunization

To investigate a possible physiological role of caspase-11 in the immune cells, caspase-11 mice were examined for the germinal center reaction where orchestrated immune cell proliferation, apoptosis, phagocytosis, and migration occur. Mice were intraperitoneally injected with nitrophenol (NP)-haptenated chicken gamma globulin NP_{21}-CGG precipitated with alum. At day 12 postimmunization, spleens were taken and the caspase-11 expression was examined by immunoblot assay. As shown in Fig. 1A, caspase-11 was induced in the immunized spleen. As in the LPS-challenged spleen, caspase-11 was expressed in B cells and macrophages when examined by immunoblot assay after cell sorting (Fig. 1B), suggesting caspase-11 may be involved in the germinal center reaction where B cells undergo activation and differentiation.

Caspase-11-deficient mice show increased cellularity and impaired germinal center formation in the spleen following immunization

Twelve days after immunization with NP_{21}-CGG, caspase-11-/- spleen showed significantly enlarged size compared to wild type (Fig. 2A). Before immunization, caspase-11-/- spleens were indistinguishable from those of wild type as stained by B cell marker (B220) or macrophage markers (MacI, shown; F4/80, not shown) (Fig. 2B). At day 12 after the immunization, wild type spleen exhibited well-developed germinal center structure as stained by peanut agglutinin PNA which is a specific marker for the germinal center (Fig. 2C). A compact B cell follicle typically seen during GC reaction is surrounded by marginal zone (Fig. 2C) and macrophages are confined to the extrafollicular space (Fig. 2C). In contrast, postimmune caspase-11-/- spleen displayed disrupted follicles and B cells were scattered or present as small patches (Fig. 2C). Macrophages were also scattered (Fig. 2C). When the spleen section was stained with PNA, caspase-11-/- showed few, poorly developed germinal centers (Fig. 2C), suggesting caspase-11 is required for the formation of proper germinal center architecture.

The role of caspase-11 during GC reaction is not mediated by caspase-3 or IL-1

As a first step to investigate the downstream mechanism by

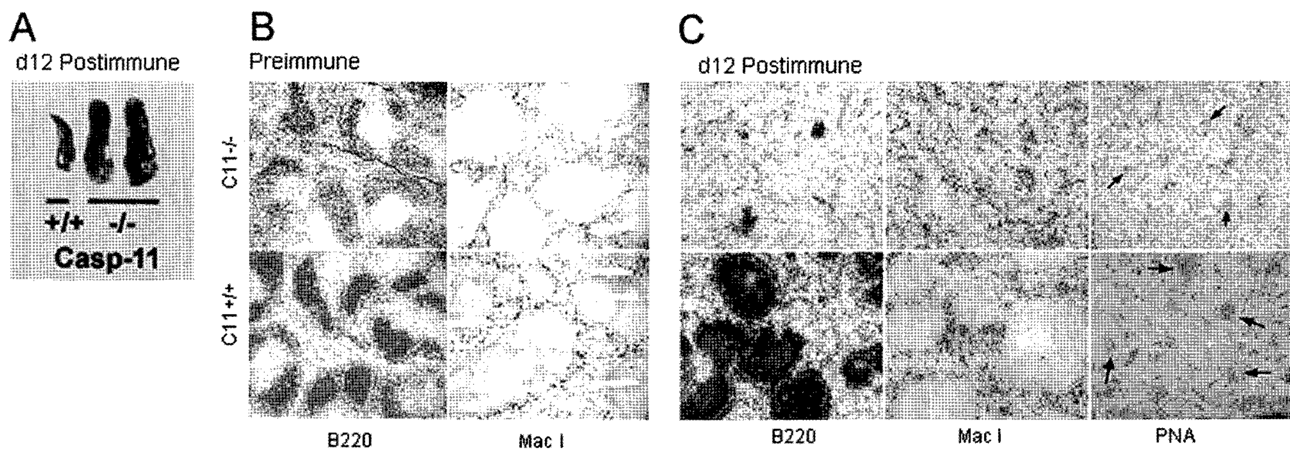

Fig. 2. The caspase-11-deficient spleen shows increased cellularity and disrupted follicular structure after immunization. A. Caspase-11-deficient (Casp-11-/-) and wild type littermate (Casp-11+/+) mice were injected with NP_{21}-CGG (80 μg/mouse)/Alum and the spleens were taken at d12 postimmunization. Note the enlarged spleens from the caspase-11-deficient mice. B-C. The spleen sections from the control mice (B) and mmunized mice (C) were stained with antibodies against marker for B cells (B220) and macrophages (MacI), and with PNA to visualize germinal centers (arrows). Note the absence of germinal centers as well as B cell follicles in the caspase-11-deficient spleen.

which caspase-11 regulates GC formation, we examined the GC reaction in mice deficient in the genes that are known to be downstream of caspase-11. Since caspase-11 can regulate caspase-3 (Kang et al., 2000) and cause the maturation of cytokines like IL-1α and IL-1β by activating caspase-1 (Wang et al., 1998), mice deficient in IL-1α, IL-1β, IL-1α/β, caspase-1 or caspase-3 were examined for the GC response. If caspase-11 is acting through one of these possible downstream effectors in regulating GC reaction, the mice deficient in the downstream effectors will exhibit the defective GC structure as in the caspase-11-deficient mice. Mice were immunized with NP_{21}-CGG and at 12 days after immunization, spleen sections from these mice were stained with markers for B cells (anti-B220), macrophages (MacI), and germinal centers (PNA). As shown in Fig. 3A, caspase-3-/- spleen after immunization showed normal phenotype with well-developed germinal centers. This implies that the role played by caspase-11 during GC reaction is not mediated by caspase-3. However, we cannot rule out the possibility that the functional redundancy of caspases resulted in the normal phenotype of caspase-3-/- spleens. If caspase-7 compensates for the absence of caspase-3 in the caspase-3-/- mice, it may be difficult to dissect out a possible involvement of caspase-3 by studying caspase-3-/- mice in vivo. Mice deficient in other possible downstream mediators, IL-1α, IL-1β and IL-1α/β, all showed normal GC structure (Fig. 3B), showing well-organized B cell follicles and PNA-positive GCs. Only caspase-1 exhibited the caspase-11-phenotype after the immunization (Fig. 3B), lacking distinguishable B cell follicles and PNA-positive GCs. Since caspase-1 deficient mice do not express caspase-11 (Kang et al., 2000), it cannot be resolved whether this defect is mediated by the absence of caspase-1 or caspase-11. However, these results rule out the involvement of the cytokine pathway, at least IL-1α or β in the role played by caspase-11 specifically during germinal center reaction. Thus, caspase-11 may regulate GC reaction by a mechanism different from that by which caspase-11 regulates apoptosis or inflammatory response under pathological conditions.

The rate of proliferation per cell is not changed in the caspase-11-/- spleen during immunization

As shown in Fig. 2, caspase-11-/- spleen showed increased cellularity and disrupted architecture after immunization. This defect seems to be immunization-specific, since LPS challenged spleen retainsed normal primary follicular structures even though the spleen was enlarged (data not shown). Possible causes for this post-immunization phenotype of caspase-11 can be defects in cell proliferation, cell death, clearance or migration, or a combination of these defects together. In an attempt to elucidate the mechanism that led to these altered phenotypes, we first examined cell proliferation during immunization in vivo. Mice were immunized with NP_{21}-CGG and fed with BrdU-containing water (1 mg/mL) for two days before being sacrificed. Spleens were taken at day 2, 4, 6 and 8 after the immunization and the splenocytes were examined for the incorporation of BrdU by ELISA. As shown in Fig. 4, there is no significant difference in the BrdU incorporation between wild type and the caspase-11-deficient mice, raising a possibility that cell proliferation is not responsible for the postimmune phenotype of the spleen in the caspase-11-/- mice. However, since the caspase-11-/- spleen displays increased cellularity, total number of BrdU-incorporated cells per spleen will be bigger in the caspase-

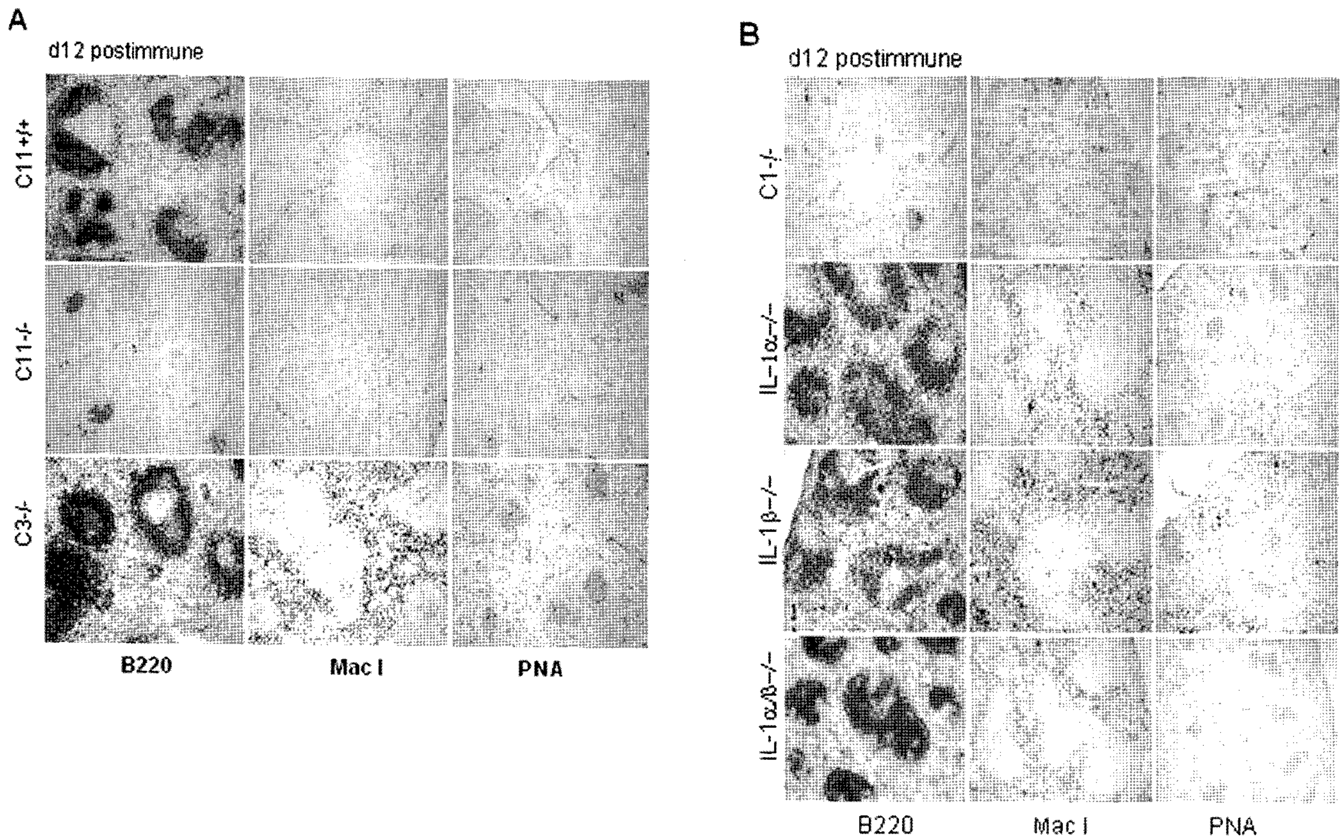

Fig. 3. The caspase-1-deficient mice displayed the same defective splenic architecture as that of caspase-11-deficient animals while caspse-3-/-, IL-1α-/-, IL-1β-/-, or IL-1α/β double knockout mice exhibited normal GC reaction after the immunization. A. Mice deficient in caspase-11 (C11-/-) and caspase-3 (C3-/-) and wild type mice (WT) were injected with NP_{21}-CGG (80 μg/mouse)/Alum and spleen sections from these mice at day 12 postimmunization were stained with antibodies against markers for B cells (B220) and macrophages (MacI) and with a marker for germinal centers (PNA). B. Mice deficient in caspase-1 (Casp-1), IL-1α (IL-1α-/-), IL-1β (IL-1β-/-) and IL-1α/β (IL-1α/β-/-) were immunized and splenic microarchitecture was examined as in panel A. Note the mice deficient in IL-1α, IL-1β, or IL-1α/β display normal splenic architecture and germinal centers while caspase-1-deficient mice have disrupted B cell follicles and no germinal centers. Note the disruption of B cell follicles and few structurally defined germinal centers in caspase-11-deficient and caspase-1-deficient spleens. Preimmune spleens have well-defined primary follicular structures in both wild type and caspase-11-deficient spleens.

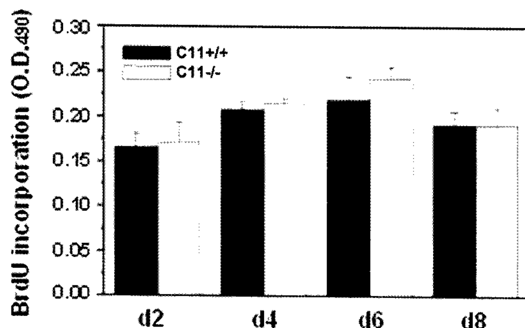

Fig. 4. The rate of proliferation per cell is not changed in the Caspase-11-/- spleen during immunization. Caspase-11-deficient (C11-/-) and wild type littermate (C11+/+) mice were injected with NP21-CGG (80 μg/mouse) and sacrificed at day 2, 4, 6, and 8 after the injection. For the last 48 hrs, BrdU (1 mg/mL) was added into the drinking water. RBC-free splenocytes taken at indicated time were analyzed for the incorporation of BrdU by ELISA. Means from the triplicate readings from 2 mice (total n=6) for each time point are shown. Readings from the same number of splenocytes from the non-immunized and 48-hr-BrdU labeled mice were served as zero point for all the readings from immunized samples.

11 spleen compared to that of the wild type although the proliferation rate per cell may not be different regardless of the presence of caspase-11. A dynamic nature of the spleen with a constant influx and efflux of immune cells makes the interpretation of the present result difficult. If there is no difference in the net influx of cells in the wild type or caspase-11-/- spleen, we can conclude that caspase-11-/- cells proliferated more. However, if there is more influx of cells in the caspase-11-/- spleen, the increase in the total number of BrdU-incorporated cells does not indicate the enhanced proliferative capacity of the caspase-11-/- cells. Another problem from the analysis of the whole spleen may arise from the inability to distinguish different response of different subpopulation of the splenocytes. Therefore, further analysis of the proliferation of the total isolated splenocytes and different subpopulations of the splenocytes in response to known mitogens such as IL-4 and/or anti-CD40 in vitro is necessary to draw more definitive conclusions.

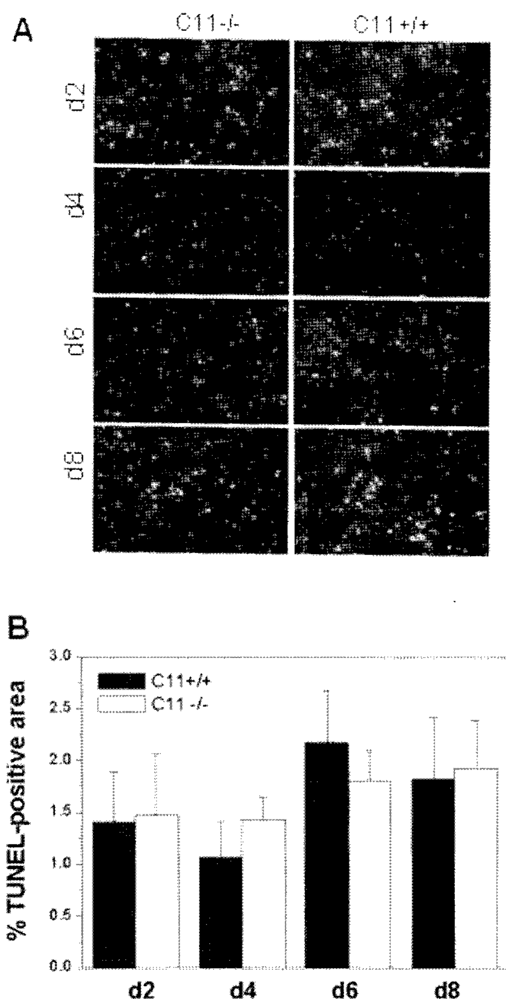

Fig. 5. Cell death is not defective in caspase-11-/- spleen after immunization. A. The caspase-11-deficient (C11-/-) and wild type littermate (C11+/+) mice were examined for cell death by TUNEL at day 2, 4, 6, 8, and 12 after the immunization with NP_{21}-CGG (80 μg/mL) precipitated with Alum. B. Cell death rate was calculated from the TUNEL-positive area scanned from 5 fields from 5 different sections (total 25 fields) of each sample. Mean TUNEL-positive area is shown. There is no significant difference in the apoptosis rate between the wild type and caspase-11-/- splenocytes.

Cell death is not defective in both caspase-11 and 3 spleen after immunization

Another possible cause of caspase-11-/- spleen abnormality is a defect in cell death. To determine the rate of cell death during immunization, we examined the spleen sections from day 2, 4, 6, and 8 postimmune mice by TUNEL assay (Fig. 5A) and quantified the TUNEL-positive area in the stained sections (Fig. 5B). Intriguingly, caspase-11-/- and wild type spleen did not show any significant difference in the cell death rate, thereby ruling out altered cell death as a possible cause for increased postimmune spleen cellularity of caspase-11-/- mice. During the examined time period, cell death rate increased slightly in later time points (d6 and d8 postimmunization) in both wild type and caspase-11-/- spleen but the change was not significant. It was evident that apoptotic cells are scattered in the early phase but become clustered in later time points (Fig. 5A, arrows), probably indicating apoptotic cells engulfed by tingible body macrophages (Han et al., 1997).

Caspase-11-/- macrophages do not have defects in phagocytotic activity

Since neither cell proliferation nor cell death can be accounted for the abnormal phenotype of the caspase-11-deficient mice, phagocytotic capacity was compared between wild type and caspase-11-/- macrophages. Peritoneal macrophages were isolated from thioglycollate-stimulated mice and incubated with red fluorescent latex beads. After staining the cells with anti-MacI, the number of macrophages that had phagocytosed the fluorescent beads was determined (Fig. 6A). As shown in Fig. 6B, caspase-11-/- macrophages did not show any defect in their ability to phagocytose the beads.

To further compare the clearance process in the wild type and caspase-11 -/- mice in the context of intercellular recognition, we used dead splenocytes as prey instead of beads in another experiment. The splenocytes from wild type and caspase-11-/- mice were killed by UV-irradiation, stained with Hoechst dye, and then fed to peritoneal macrophages in culture. After staining the peritoneal macrophages with anti-MacI, the number of MacI-positive cells that had Hoechst-stained nuclei of dead splenocytes was determined. As shown in Fig. 6C, wild type and caspase-11-/- macrophages did not show any difference in engulfing the dead splenocytes from both genotypes. These results rule out phagocytosis as a likely mechanism that caused the caspase-11-/- phenotype in the postimmune spleen.

Caspase-11-/- splenocytes and peritoneal macrophages show defective migration in response to chemokines

Another possible cause of the splenic architecture disruption and increased cellularity is a defect in cell migration as caspase-11 has been recently shown to regulate cell migration (Li et al., 2007).

To compare the migratory capacity of wild type and caspase-11-/- immune cells, we examined migration of total splenocytes taken from the immunized mice and peritoneal macrophages by Transwell migration assay. The isolated cells were seeded in the pored upper chamber containing plain medium and the chamber was placed on top of the bottom chamber containing chemokines such as SDF-1α or BLC. When the number of cells migrated to the bottom chamber was counted after 4 hrs, interestingly caspase-11-/- splenocytes and macrophages both showed defective migration towards chemokines compared to wild type cells (Fig. 7A and B). The difference was more significant in the

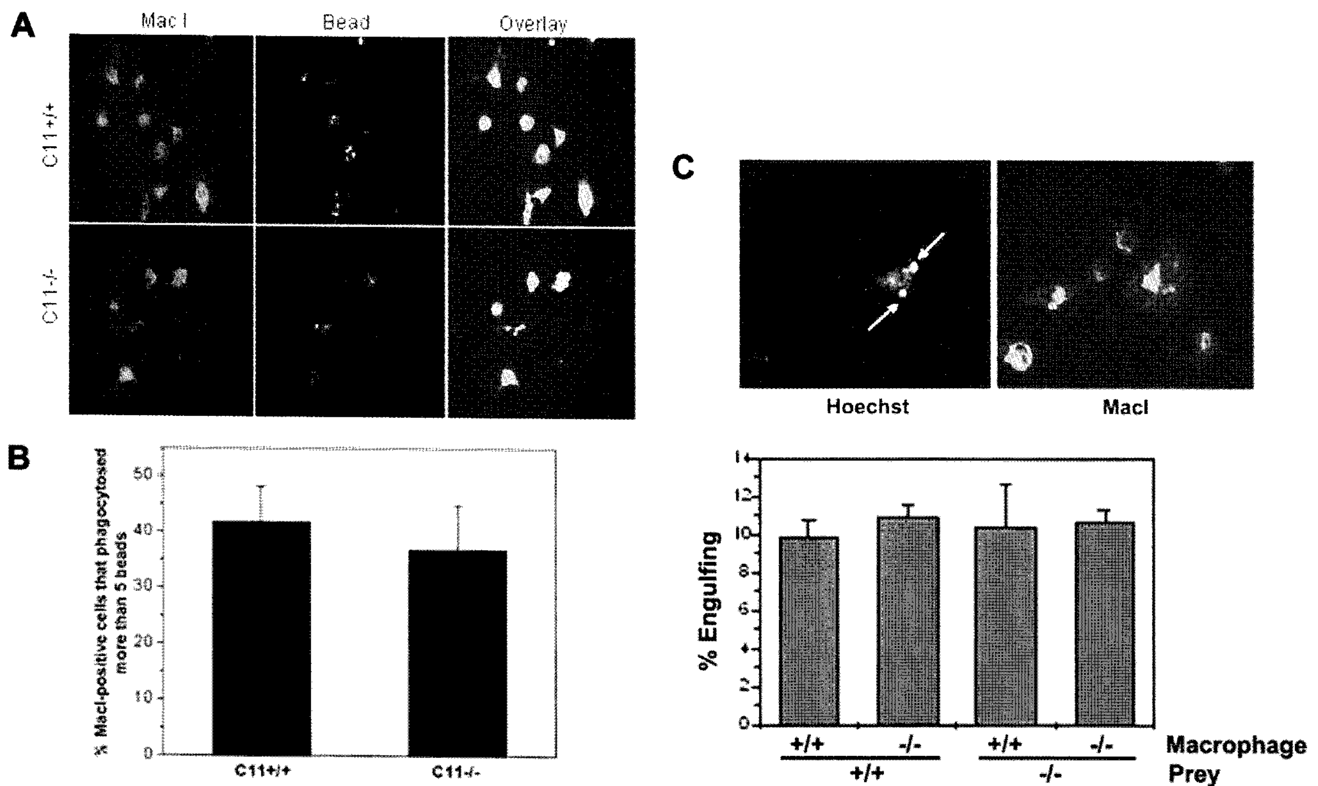

Fig. 6. The caspase-11-/- macrophages do not have defects in phagocytotic activity. A. The caspase-11-/- (C11-/-) and wild type (C11+/+) peritoneal macrophages were isolated from thioglycollate-stimulated mice and red fluorescent beads (2 μm diameter) were added into the culture medium (bead:cell = 10:1) of the peritoneal macrophages. After 4 hrs of the incubation, cells were washed and stained with macrophage marker (MacI). Shown are the beads phagocytosed by peritoneal macrophages of caspase-11-/- (C11-/-) and wild type littermate (C11+/+) mice. B. The number of macrophages that phagocytosed more than five beads in one field (200×) was determined. The average from counts of 7 fields is shown. C. To examine the engulfment of apoptotic cells by macrophages, dead splenocytes were fed to peritoneal macrophages in culture. RBC-free splenocytes were killed by UV-irradiation, stained with Hoechst dye and then incubated with peritoneal macrophages in culture (dead splenocytes:macrophages =5:1). MacI-positive peritoneal macrophages that phagocytosed Hoechststained splenoycytes (arrows) are shown. The percentage of MacI-positive cells that phagocytosed dead splenocytes (prey) was calculated from the counts of 10 fields. Note there is no difference in phagocytosis regardless of the genotype of the macrophages or prey cells.

splenocytes. Since the assay was carried out in 4 hrs, it is unlikely that cell death or proliferation affected the results. This result suggests that caspase-11 may be responsible for proper control of immune cell migration required for normal splenic architecture during immune response.

Antibody production is not impaired in caspase-11-/- mice

Since germinal center structure is known to be required for the proper differentiation of B cells and maturation of antibody affinity (Kelsoe and Zheng, 1993; Tarlington and Smith, 2000), the functional output of the disrupted germinal center structure in caspase-11-/- mice was examined by measuring affinities of the antibodies in the serum. Fig. 8A shows the affinities of total IgG against the antigen NP_{21} in the serum of caspase-11-/-, caspase-3 -/-, and wild type mice at day 13 postimmunization. Surprisingly, serum IgG of caspase-11-/- as well as caspase-3-/- mice showed normal levels of affinity as compared to that of wild type. When the binding to NP_{21} and NP_4 was compared in these serum samples, there was no detectable difference in the abilities of the IgG's to differentiate the low molecular number hapten (NP_4) and high molecular number hapten (NP_{21}) in all the samples (data not shown). These results suggest that antibody isotype switching and affinity maturation occurred normally. To further examine the isotype class switching, we measured antibody affinity of different isotypes of immunoglobulins. As shown in Fig. 8B-H, caspase-11-/- and wild type mice displayed the same affinity in all the isotypes examined: IgG1, IgG2a, IgG2b, IgG3, λ, IgM, and IgA. This clearly shows that antibody isotype class switching and affinity maturation occurred in the absence of germinal centers in the caspase-11-/- mice.

DISCUSSION

In the present study, a possible physiological function of caspase-11 was investigated using the GC reaction as a model system. The induction of caspase-11 in the spleen after immunization suggested a potential role during GC

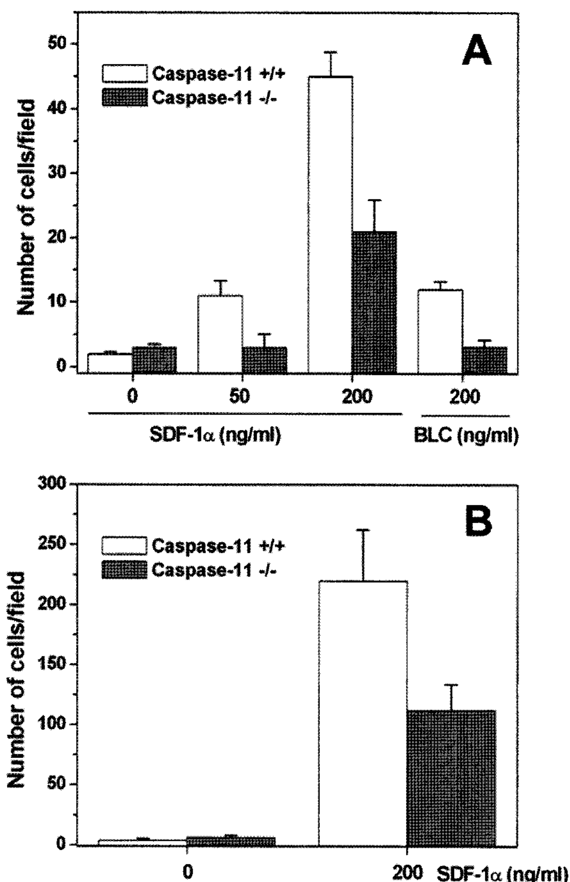

Fig. 7. The caspase-11-/- splenocytes and peritoneal macrophages display defective migration in response to chemokines. RBC-free splenocytes taken from immunized mice (A) and peritoneal macrophages (B) were assayed for the ability for migration using Transwell. Isolated splenocytes or macrophages were seeded on the upper chamber in a plain medium and placed on top of the lower chamber containing chemokines (SDF-1α or BLC) at indicated concentrations. After 4 hrs of incubation, the number of cells migrated to the bottom chamber was determined. Shown is the average of 7 counts in each group.

reaction. The deficiency of this enzyme led to the postimmunization disruption of microarchitecture, loss of GC structure, and increased cellularity in the spleen, suggesting caspase-11 has a critical role in immune response. Interestingly, however, antibody isotype switching and affinity maturation were not affected by the absence of caspase-11. The mechanism leading to this altered phenotype in caspase-11-deficient mice seemed to be defective cell migration: isolated splenocytes and peritoneal macrophages from caspase-11-/- mice showed defective migration towards chemokines, suggesting caspase-11 normally regulates cell migration as recently reported (Li et al., 2007). This study revealed a new role for caspase-11 in organizing splenic architecture during the immune response.

However, unlike other known mutant mice with defects in GC reaction, caspase-11-deficient mice did not show abnormality in lymphoid tissue organogenesis. Under normal conditions without immunization, caspase-11-/- mice developed all the lymph nodes and the spleen showed normal architecture. However, only after the immunization, the splenic architecture was strikingly disrupted. The abnormal structure of the spleen seems to be immunization-specific since LPS challenge did not perturb the splenic architecture (data not shown). Perhaps in septic shock conditions caused by LPS injection, massive and potent upregulation and activation of caspase-11 is all geared to initiate programmed cell death and other toxic proinflammatory cytokines are in synergistic action to complete the total demolition. During GC reaction, however, the activation of caspase-11 may be at a low level or a different set of regulatory molecules is involved to impart a different role to caspase-11. TNF-α, for example, has been best known for its cytotoxic function in many pathological conditions including septic shock. However, the knockout animal revealed a different role in secondary lymphoid organogenesis and organizing splenic architecture (Le Hir et al., 1996; Pasparakis et al., 1996; Pasparakis et al., 1997; Cook et al., 1998). Most of the mutant mice with defective GC reaction showed abnormal splenic architecture even at normal conditions. For example, in the spleen of TNFα-/- mice, marginal zones were expanded and follicles and follicular dendritic cells (FDCs) were absent (Pasparakis et al., 1996; 1997). Mice deficient in lymphotoxin (LT) showed even more defective lymphoid organ structure: LTβ-/- mice lacked lymph nodes and Peyers patches in addition to follicles and FDCs and LTα-/- mice showed the same defects as LT-/- mice except in mesenteric and cervical lymph nodes (De Togni et al., 1994; Banks et al., 1995; Alimzhanov et al., 1997). Since caspase-1-deficient monocytes have a partial defect in secretion of TNFα (Kuida et al., 1995), the phenotype of caspase-11-deficient mice upon immunization could be partly due to the decreased level of TNFα. This possibility can be examined by comparing the level of TNF-α in the postimmune spleens of wild type and caspase-11-/- mice. However, the fact that TNFα-/- mice show abnormality at the organogenesis level whereas caspase-11-/- mice show only stimulus-specific defects indicates that the mechanism by which caspase-11 regulates GC reaction is different from that of TNF-α action. Interestingly, CD40-deficient mice display normal structure in all secondary lymphoid tissues during development but the GC formation is defective (Kawabe et al., 1994), raising a possibility that the defect caused by the absence of CD40 may share the similar pathway as the caspase-11-deficient animals.

It is intriguing that the antibody production was normal in the caspase-11-/- mice even though GC structure was not formed properly. Most of the genetic mutants with abnormal GC structure showed defects in humoral immunity.

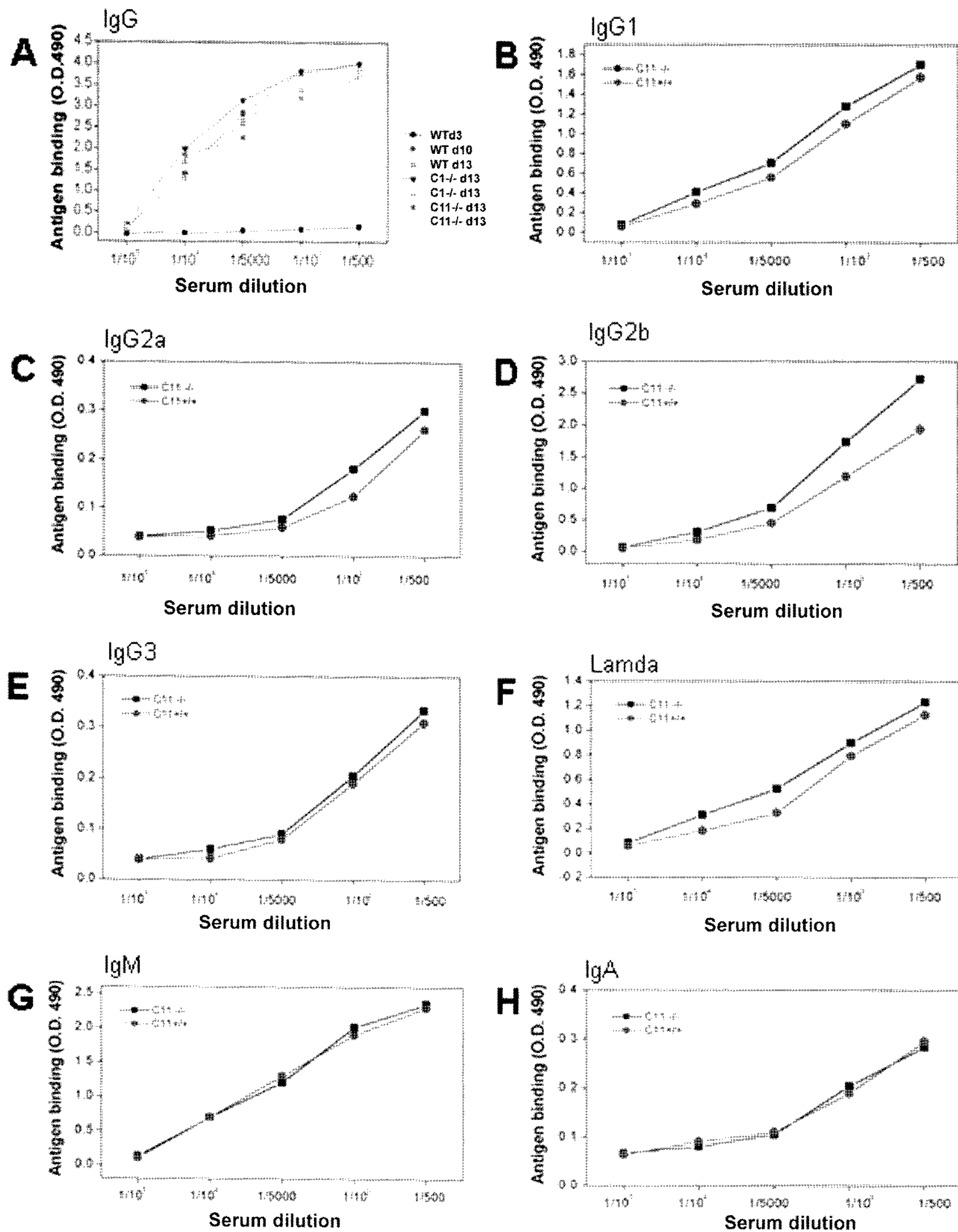

Fig. 8. Humoral immune response is not impaired in caspase-11-/- mice. Serum immunoglobulin affinities against NP were measured by ELISA to examine the isotype switching and affinity maturation in caspase-11-/- mice. The serum from the immunized mice was serially diluted and incubated with the antigen, NP_{21}-BSA or NP_4-BSA. The amount of bound antibody was measured from the readings at 490 nm following secondary antibodies specific for each immunoglobulin isotype (from B to H; IgG1, IgG2a, IgG2b, IgG3, lamda, IgM, and IgA). Note the serums from caspase-3-/- (A) or caspase-11-/- (A to H) mice have the same level of immunoglobulin affinity for the antigen in all isotypes examined. The ratio of NP_{21}:NP_4 binding was also the same in all isotypes tested (not shown).

However, LTα-/- showed somatic hypermutation and affinity maturation of the serum antibody (Matsumoto et al., 1996) although these mice lack peripheral lymph nodes and Peyer's patches, display an abnormal splenic architecture and inability to form GC after immunization (Matsumoto et al., 1996). This and our results suggest that the typical GC structure is not an absolute requirement for the antibody isotype switching and affinity maturation.

The recent work by Li et al. (2007) suggests that caspase-11 regulates cell migration by promoting Aip1/

Cofilin-mediated actin depolymerization. The regulation of actin dynamics by caspase-11 in this case is cell autonomous. However, we cannot rule out the possibility that the defective migration of the immune cells in the caspase-11-/- mice is due to the reduced downstream cytokines or chemokines other than IL-1. In a report by Cumberbatch et al. (2001) IL-18 induced Langerhans cell migration following skin sensitization in mice and this migration was dependent on the presence of TNFα and IL-1β. The same group also reported caspase-1-deficient mice are defective in the Langerhans cell migration from epidermis to draining lymph nodes (Antonopulos et al., 2001). Thus, IL-18 or other unknown cytokines regulated by caspase-11 could mediate the regulation of cell migration by caspase-11 in a non-cell autonomous way.

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