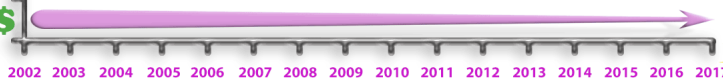




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Distinct Roles of Adenylyl Cyclase VII in Regulating the Immune Responses in Mice

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The second messenger cAMP plays a critical role in regulating immune responses. Although well known for its immunosuppressive effect, cAMP is also required for the development of optimal immune responses. Thus, the regulation of this second messenger needs to be finely tuned and well balanced in a context dependent manner. To further understand the role of cAMP synthesis in the functions of the immune system, we focus on a specific adenylyl cyclase (AC) isoform, AC VII (AC7), which is highly expressed in the immune system. We show that mice deficient of AC7 are hypersensitive to LPS-induced endotoxic shock. Macrophages from AC7-deficient mice produce more of the proinflammatory cytokine, TNF- α , in response to LPS. The inability to generate intracellular cAMP response to serum factors, such as lysophosphatidic acid, is a potential cause for this phenotype. Thus, AC7 functions to control the extent of immune responses toward bacterial infection. However, it is also required for the optimal functions of B and T cells during adaptive immune responses. AC7 is the major isoform that regulates cAMP synthesis in both B and T cells. AC7-deficient mice display compromised Ab responses toward both T cell-independent and T cell-dependent Ags. The generation of memory T cells is also reduced. These results are the first to ascribe specific functions to an AC isoform in the immune system and emphasize the importance of cAMP synthesis by this isoform in shaping the immune responses. *The Journal of Immunology*, 2010, 185: 335–344.

The critical role of the ubiquitous second messenger, cAMP, in the immune system has been known for over several decades (1–3). During the development of the immune system, cAMP regulates cell proliferation, differentiation, and apoptosis (4, 5). Elevation of cAMP concentration induces arrest of T cell proliferation or cell death (5, 6); whereas, several types of B cell lymphoma are associated with reduced concentration of intracellular cAMP (7). With respect to the development of immune responses, cAMP is generally known as a potent immunosuppressant (8). Its immunosuppressive effects range from suppression of proinflammatory cytokine production by T cells and macrophage cells (9, 10) to inhibition of T cell proliferation, T cell and B cell activation (6, 11), and neutrophil chemotaxis (9). However, cAMP has also been shown to be required for the generation of optimal immune responses. Elevation of cAMP concentration in B cells has been shown to result in upregulation of B7-2 (CD86) expression (12, 13), a costimulatory factor that is important for T cell activation. cAMP can also promote class switching in B lymphocytes during Ab responses (14, 15). In dendritic cells, cAMP enhances the production of IL-23, which in turn promotes

secretion of IFN- γ and IL-17 by memory T cells (16). Recently, Conche et al. (17) elegantly demonstrated that a transient rise of cAMP during T cell adhesion to APCs is necessary for optimal T cell activation.

The seemingly contradictory roles of cAMP in the regulation of immune responses may be due to the dynamic regulation of this second messenger. For example, it may be that transient rise of cAMP is important for signaling certain changes in immune responses; whereas, sustained increase of cAMP dampens immune responses in general. Clearly, further elucidation of cAMP regulation in the immune cells is needed to better understand the various crucial roles of cAMP in the immune system.

The intracellular concentration of cAMP is dynamically regulated between the activities of adenylyl cyclases (ACs), which catalyze the conversion of ATP to cAMP upon activation by the heterotrimeric G_s protein, and the actions of phosphodiesterases (PDEs), which degrade cAMP to inactive 5'-AMP. Both ACs and PDEs exist in multiple isoforms, and each isoform has distinct regulatory properties and tissue distributions (18–22). The roles of some PDE isoforms in the immune system have been revealed in mouse models by studies using gene knockout, RNAi knock-down, or perturbation with small molecule inhibitors. Mice lacking PDE4B show impaired response to LPS-induced shock (23). Both PDE4B and PDE4D appear to be required for proper neutrophil chemotaxis and recruitment to the sites of inflammation (24). Another PDE isoform, PDE7A, may play a role in T cell proliferation (25).

In contrast, the participation of specific isoforms of AC in immune responses has not been studied. There are nine transmembrane AC isoforms in mammalian cells. All the isoforms are activated by G_s. Yet, the activity of each can be further modulated by other G protein coupled pathways (20, 21, 26). One of the isoforms, AC VII (AC7), is highly expressed in lymphocytes and macrophages (27–29). Interestingly, we recently showed that AC7 serves as a key integrator of cAMP regulation in bone marrow-derived macrophages (BMDMs) (30). BMDMs respond to G_s-stimulating

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Abbreviations used in this paper: AC, adenylyl cyclase; AC7, adenylyl cyclase VII; AM, assay medium; BMDM, bone marrow-derived macrophage; FoB, follicular B cell; GM, growth medium; ISO, isoproterenol; LBP, LPS-binding protein; LPA, lysophosphatidic acid; MzB, marginal zone B cell; PDE, phosphodiesterase; poly-I: C, polyinosinic-polycytidylic acid; Ro20, Ro20-1724; TD, T cell-dependent; TER, terbutaline; TI, T cell-independent.

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ligands, such as isoproterenol and PGE₂, with a rise of intracellular cAMP concentration. Ligands that are coupled to other G protein pathways, including G_q, G_i, and G_{12/13}, can synergize with the G_s pathway and transiently enhance intracellular cAMP concentration in wild-type BMDMs. However, BMDMs lacking AC7 are particularly insensitive to the synergistic regulations by these non-G_s pathways. The expression and unique regulation of AC7 in the immune system implies potential function of this AC isoform in immune responses. Therefore, we examined the functions of AC7 in both inflammatory and Ab responses *in vivo*. Because AC7-deficient mice display severe embryonic lethality (>90%), we used bone marrow transplants to generate chimeric mice in which AC7 is only deleted in the hematopoietic system. AC7-deficient bone marrow cells were able to regenerate the immune system and the ratio of B cells and T cells appeared to be normal. However, the chimeric mice showed reduced total numbers of lymphocytes, suggesting AC7 might play a role in hematopoiesis. These chimeric mice displayed hypersensitivity to LPS-induced endotoxic shock. *In vitro* analyses using BMDMs suggest that AC7 senses factor(s) present in the serum to control the extent of TLR signaling via intracellular cAMP changes. In contrast, chimeric mice carrying AC7-deficient immune system displayed compromised Ab responses to challenges with either T cell-independent (TI) or T cell-dependent (TD) Ags. We show that AC7 is the major AC isoform that regulates cAMP synthesis in both B cells and T cells. Our results demonstrate that the specific AC isoform, AC7, has multifaceted roles in regulating both innate and adaptive immune responses. It prevents hyperactive immune responses to bacterial infection while enabling optimal Ab responses. This is the first study that ascribes the functions of a specific AC isoform in the immune system and it highlights the importance of the dynamic regulation of cAMP in the development of immune responses.

Materials and Methods

Reagents

LPS (R595Re) was purchased from List biological. LPS binding protein (LBP) was purchased from R&D Systems (Minneapolis, MN). Polyinosinic-polycytidylic acid (poly-I:C) was obtained from Amersham. LPS (055:B5), Ro 20-1724, and IFA were obtained from Sigma-Aldrich (St. Louis, MO). TNP-LPS, NP-Ficoll, KLH, TNP-KLH, FITC-KLH, NP-BSA, and TNP-BSA were purchased from Biosearch Technologies (Novato, CA). HRP conjugated anti-IgM, IgG1, IgG2c, or IgG3 Abs were obtained from Southern Biotechnology Associates (Birmingham, AL). ELISA kit for TNF- α (88-7324) was purchased from eBioscience (San Diego, CA).

Animal handling

AC7 knockout mice were purchased from The Jackson Laboratory (005774) (Bar Harbor, ME) and bred by intercrossing heterozygous males and females. C57BL/6J mice were purchased from the mouse breeding core at University of Texas Southwestern Medical Center. All experimental procedures involving animals in this study were reviewed and approved by the Institutional Animal Care and Research Advisory Committee at University of Texas Southwestern Medical Center.

Genotyping

Tissue samples were digested in lysis buffer (10 mM Tris•HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.45% NP-40, 0.45% Tween-20) containing 60 μ g/ml proteinase K (Clontech Laboratories, Mountain View, CA) at 55°C overnight. Genotyping by PCR was performed as described previously (30).

Generation of chimeric mice by bone marrow transplantation

Bone marrow cells from AC7 knockout mice and their wild-type littermates were isolated from femurs, humerus, and backbones. After lysing the RBCs, the remaining cells were counted and resuspended in sterile PBS at 5×10^7 cells per ml. Recipient wild-type C57BL/6J mice were irradiated with gamma-ray at 600 rads twice with a 4 h interval between exposures. Then

the irradiated mice were injected with 1×10^7 wild-type or AC7-deficient bone marrow cells through the tail vein. For each experiment, 5–10 recipient mice were used for each genotype of donor cells. The mice were allowed to recover and regenerate their immune systems from the injected bone marrow cells for 6–8 wk before being used for experiments. There was no difference between wild-type and AC7^{+/-} heterozygous mice or chimeras on initial analyses therefore they were used for controls interchangeably.

Purification of B and T cells

Spleens were dissected out from euthanized mice and dispersed through a 70- μ m nylon mesh to generate a suspension of single cells. RBCs were lysed and removed through osmotic shock. To purify B cells, the remaining splenocytes were incubated with anti-CD43 and anti-CD11b Ab-conjugated microbeads (Miltenyi Biotec, Auburn, CA) and sorted using an AutoMacs magnetic cell sorter. The negative fraction contains the resting B cells. The purity of the B cells is >95% as determined by cell surface expression of B220 using FACS. To purify T cells, the splenocytes were incubated with anti-B220 and anti-CD11b Ab-conjugated microbeads and sorted through AutoMacs. The negative fraction contains the T cells and the purity is ~80–90% as determined by cell surface expression of CD3.

cAMP assay

Purified B cells or T cells (1 million cells per condition) were cultured in serum-free medium for 1 h at 37°C. Ligands (10 \times) were then added to stimulate the cells for 1 min and reactions were stopped by removal of medium and cell lysis with 65% ethanol. Cell lysates were then dried and assayed using the cAMP Biotrak EIA kit (GE Healthcare Life Sciences, Piscataway, NJ). cAMP response in BMDMs was measured using a BRET assay as previously described (29).

BMDM isolation

Bone marrow-derived primary macrophages were isolated from mouse femurs and cultured as described (30, 31).

Cytokine measurement

BMDMs were plated at $2-3 \times 10^5$ cells per well on a 24-well plate the day before the assay. The medium was replaced with assay medium (AM) (DMEM, 20 mM HEPES, 0.01% BSA) with no FBS or fresh growth medium (GM) 1 h before ligand addition. LPS and LBP were combined and incubated at 37°C for 30 min to form complexes. The LPS/LBP mixture was added to the cells to reach a final concentration of 10 ng/ml LPS and 25 pM LBP. After incubation for 4 h at 37°C, medium was collected and secreted cytokines were quantified using ELISA kit according to the manufacturer's protocols. Cells remaining in the wells were disrupted with lysis buffer containing 1% Triton X-100 and total protein was measured using Precision Red (Cytoskeleton, Denver, CO).

LPS injection and endotoxic shock

Mice were weighed and injected i.p. with LPS at 15 μ g/g or 20 μ g/g body weight. Blood was collected 6 h after LPS injection via tail vein bleeding. Mouse serum was diluted 2- to 10-fold and the concentration of TNF- α was determined using the mouse TNF- α ELISA kit from eBiosciences. The mice were subsequently monitored every 6–24 h for a 6-d period. Time of death was recorded.

Ag challenges and measurement of Ab responses

Six to 8 wk after bone marrow transplant, chimeric mice were subjected to a variety of Ag challenges. The Ags were delivered via i.p. injections at indicated doses. Blood was collected through tail vein bleeding at the indicated times and Ab levels in the serum were measured using ELISA. Briefly Ag-specific Abs were captured using BSA-Ag conjugates. HRP conjugated anti-IgM, IgG1, IgG2c, or IgG3 Abs were used to distinguish the species of the Igs. Oxidation of *o*-phenylenediamine by HRP was detected by the change in OD at 490 nm.

Assessment of Ab responses in T cell-primed animals

Wild to type C57BL/6J mice were immunized with the TD Ag KLH emulsified in IFA at 100 μ g per mouse. Three days later, these mice were subjected to 750 rad of gamma-ray irradiation, which is sufficient to eliminate the B cells without affecting the KLH-primed T cells. These mice subsequently received 1×10^7 of either wild-type or AC7-deficient splenocytes through i.v. injection. Four hours later they were challenged by i.p.

injection of TNP-KLH in the absence of adjuvant. Blood was collected 14 d later and Ab production was measured by ELISA.

Flow cytometry

Cells from spleen, lymph nodes (inguinal and mesenteric), bone marrows, and peripheral blood were isolated and dissociated into single-cell suspension. After lysis and removal of RBCs using osmotic shock buffer, the total number of leukocytes from the spleen were counted using a hemocytometer. Half a million cells were used for each subsequent Ab staining. A fixed amount of splenocytes from BALB/C mice labeled with PE anti-IgM^a was added to each sample as normalization control. The remaining spleen leukocytes were used for B cell or T cell purification. For cells from the lymph nodes and bone marrows, Ab staining was performed with the same volumes of cells of each genotype. The cells were stained with various Abs at predetermined optimal concentrations and were analyzed with a FACSCalibur flow cytometer and FlowJo software (Tree Star, Ashland, OR). Subsets of B and T lymphocytes and NK cells were distinguished using fluorochrome-labeled Abs against mouse Ags, including FITC-labeled anti-B220/CD45R, CD19, CD21; PE-labeled anti-NK1.1 (BioLegend, San Diego, CA), CD23, CD86, TLR4/MD-2 complex; biotinylated anti-B220/CD45R, NK1.1 (BioLegend), CD3ε, CD8, CD18, CD21, CD43, MHC-II (eBiosciences), and streptavidin-PerCP; APC-labeled anti-B220/CD45R, CD3ε, CD4, CD11b. Matching isotype controls were used for all staining. All Abs were from BD Biosciences (San Jose, CA) unless otherwise noted.

Immunohistochemistry

Spleens were isolated from chimeric mice and frozen in OCT compound. A total of 8 μm cryosections were stained directly with anti-IgM-FITC and anti-CD4-PE Abs without fixation.

Statistical analysis

The Mann-Whitney rank sum test was used to calculate the *p* values and determine the significance of differences between data sets.

Results

AC7-deficient bone marrow cells regenerate the immune system with reduced numbers of leukocytes

More than 93% of AC7^{-/-} homozygous mice die during embryogenesis, making it difficult to directly examine the functions of AC7 in the immune system. To obtain adult animals with AC7-deficient immune systems, we transplanted the hematopoietic stem cells obtained from the rare adult AC7^{-/-} homozygous mice into lethally irradiated wild-type animals. All chimeric mice survived the transplant procedure and appeared healthy. Six weeks after transplantation, we examined the genotype and the composition of the PBLs. The genotype of the PBLs matched the donor genotype, indicating that the immune system is fully regenerated from the donor bone marrow cells (Supplemental Fig. 1A). Both B cells and T cells were regenerated and the percentages of B and T lymphocytes and NK cells as determined by cell surface marker staining were comparable among all three genotypes of donor cells (Supplemental Fig. 1B).

However, the absolute number of total splenocytes was reduced by >60% in the chimeras generated from AC7-deficient bone marrows (Table I). The amount of both B cells and T cells appeared to be proportionally reduced and the ratio of CD4⁺ T and CD8⁺ T cells remained unchanged (Fig. 1A, 1B, Table I). When the lymphocyte composition was analyzed in the lymph nodes, similar pattern was observed (Table I). Immunohistochemical staining of spleen sections revealed normal organization of follicular B cells and CD4⁺ T cells (Fig. 1C). Further examination of the subtypes of B cells in the spleen showed that both the follicular B (FoB) and the marginal zone B (MzB) cells were present but the ratio of FoB/MzB was decreased in AC7-deficient animals (Fig. 1B, Table I).

The reduction of total leukocytes numbers without alteration of the proportion of B and T lymphocytes suggests a potential

Table I. Comparison of immune regeneration from wild-type and AC7-deficient bone marrows

	AC7 ^{+/+} Chimeras	AC7 ^{-/-} Chimeras
Spleen (<i>n</i> = 5)		
Total leukocyte (×10 ⁶)	147 ± 38	48 ± 8*
B cell (% control)	100	30 ± 6*
T cell (% control)	100	41 ± 10*
CD4 ⁺ T/CD8 ⁺ T (ratio)	2.1 ± 1.0	2.3 ± 0.8
FoB/MzB (ratio)	12.3 ± 3.2	5.9 ± 1.7**
Lymph nodes (<i>n</i> = 5)		
B cell (% control)	100	38 ± 27**
T cell (% control)	100	26 ± 16**
CD4 ⁺ T/CD8 ⁺ T (ratio)	2.2 ± 1.0	3.2 ± 1.1

p* < 0.001; *p* < 0.01 as calculated by the Mann-Whitney rank sum test.

proliferative defect in AC7-deficient hematopoietic stem cells. Regardless, both mature B cells and T cells were generated in AC7-deficient chimeras. These chimeric mice can be used for further studying the functions of the AC7-deficient leukocytes during immune responses.

Mice harboring AC7-deficient hematopoietic cells are more sensitive to endotoxic shock

To determine the physiological functions of AC7 in vivo, we first examined the response to bacterial infection by challenging the chimeric mice with a semilethal dose of LPS (15 μg/g body weight). Response was measured by their survival rate. As shown in Fig. 2A, mice harboring an AC7-deficient immune system died at a faster rate than mice with a wild-type immune system. After 6 d, only 22% of AC7-deficient mice survived the LPS challenge, compared with 67% of wild-type mice. When the LPS dose was increased to 20 μg/g body weight, 60% of mice with an AC7-deficient immune system died within the first 24 h; whereas, only 10% of wild-type mice died during the same period (Fig. 2B). At the end of day 6, all the AC7-deficient mice were dead, but 20% wild-type mice survived this challenge. Thus, AC7-deficient mice appear to be hypersensitive to endotoxic shock.

A hallmark of septic shock response is the profound production of proinflammatory cytokines, which in severe cases causes organ failure and death. When the serum concentration of TNF-α was measured at 6 h after challenge with LPS, mice harboring an AC7-deficient immune system were found to produce much greater amounts of this proinflammatory cytokine than wild-type mice (Fig. 2C). These results suggest that AC7 functions to suppress proinflammatory cytokine responses on endotoxic shock.

AC7-deficient BMDMs displayed defective cytokine responses

Macrophage cells are among the first cells in line to defend the host from microbial infection. To test whether AC7-deficient macrophage cells could be the cause for the hypersensitive response to endotoxic shock, we isolated BMDMs from wild-type and AC7 knockout mice and examined their cytokine responses to LPS in vitro. Without LPS treatment, there was no detectable amount of TNF-α present in medium (<8 pg/ml). Upon activation of TLR4 receptors by LPS, wild-type BMDMs secreted the proinflammatory cytokine, TNF-α, into the medium (Fig. 3A). In contrast to wild-type cells, AC7-deficient BMDMs produced significantly higher amounts of this proinflammatory cytokine (Fig. 3A), consistent with the in vivo observations. The increase in TNF-α production is not caused by increased detection of LPS by AC7-deficient BMDMs for two reasons. First, both wild-type and mutant cells displayed similar amount of cell surface expression of TLR4 before and after LPS treatment (Supplemental Fig.

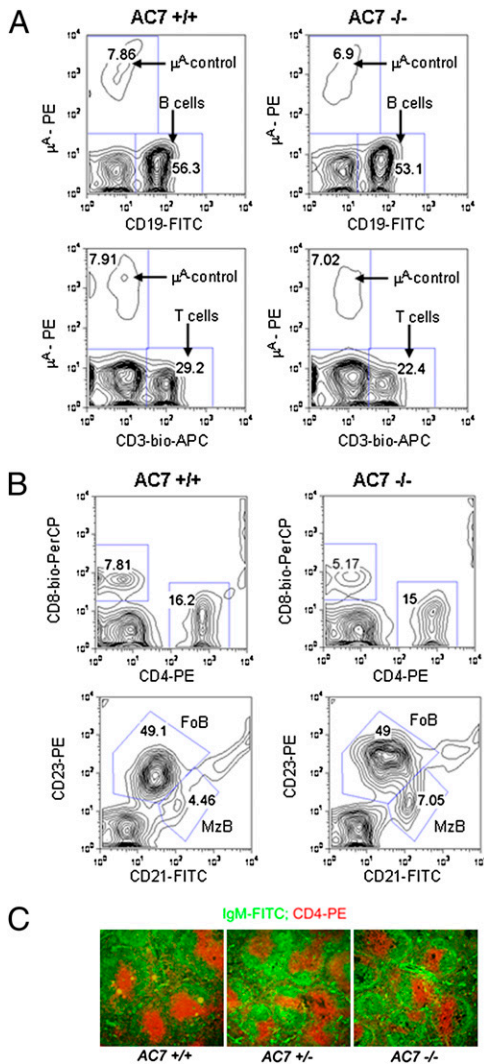


FIGURE 1. Regeneration of the immune system from AC7-deficient bone marrow cells. **A**, Half a million spleen leukocytes taken from wild-type or AC7-deficient chimeras were stained with anti-CD19-FITC and anti-CD3-Biotin. A fixed amount of splenocytes from BALB/C mice labeled with PE anti-IgM^h (μ^A) were added to each sample as control lymphocytes and normalization standard for FACS analysis. Both wild-type and mutant mice showed similar proportions of B cells and T cells. **B**, Half a million spleen leukocytes were stained with anti-CD4-PE and anti-CD8-FITC or with anti-CD23-PE and anti-CD21-FITC. The ratio between CD4⁺ T and CD8⁺ T cells is comparable between wild-type and mutant mice but the ratio of FoB/MzB is reduced in mutant mice. Graphs shown here are representative of five experiments; detailed analysis is given in Table I. **C**, Spleen sections from the chimeric mice were stained with anti-IgM-FITC (green) and anti-CD4-PE (red) to reveal the follicular structures. A representative photograph of three experiments is shown (original magnification $\times 40$).

2). Second, similar phenotypes were observed when the cells were stimulated with poly-I:C, which activates the TLR3 receptors (Supplemental Fig. 3).

Interestingly, when stimulation with LPS was carried out in the absence of FBS, TNF- α production was greatly increased in both cells. Importantly, the difference in TNF- α production between the wild-type and mutant cells was eliminated (Fig. 3B). Responses to poly-I:C were similarly regulated (Supplemental Fig. 3). This result argues that the difference in responses to TLR3 or TLR4 ligands between wild-type and AC7-deficient BMDMs does not lie in TLR signaling per se; rather it is caused by regulation from unknown factors present in the serum. It has

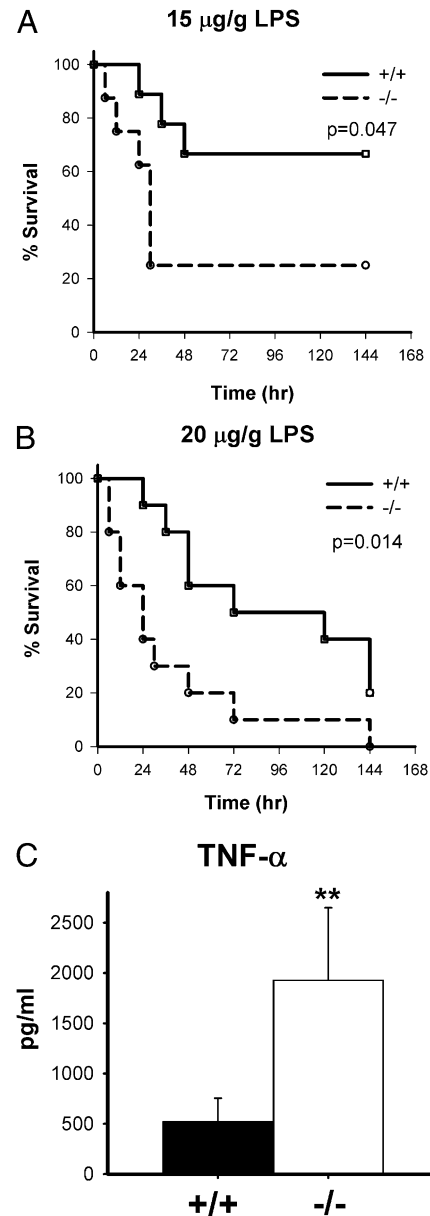


FIGURE 2. Mice carrying an AC7-deficient immune system are more sensitive to endotoxic shock. Chimeric mice were generated by bone marrow transplant using AC7- deficient or wild-type bone marrow cells as donor and gamma-ray irradiated wild-type mice as recipient. Two months after bone marrow transplant mice were challenged with i.p. injection of LPS at 15 $\mu\text{g/g}$ body weight (**A**) or 20 $\mu\text{g/g}$ body weight (**B**). Each group consists of 8–10 mice. Survival was monitored every 6 or 12 h. The differences between wild-type and AC7-deficient chimeras are significant in both experiments; the p values are calculated by the Kaplan-Meier survival log-rank test. **C**, Six hours after injection of 20 $\mu\text{g/g}$ LPS, blood was collected through tail vein bleeding. The serum level of TNF- α was measured by ELISA. There was no detectable level of serum TNF- α prior to LPS treatment in all animals (< 8 pg/ml). ** $p < 0.0001$, based on Mann-Whitney rank sum test.

been reported that factors present in serum can regulate TLR signaling pathways and inhibit the production of TNF- α (32). Our result indicates that part of this regulation is dependent on AC7. The fact that the factor(s) is present in the FBS suggests that it is unlikely to be a protein.

It is well documented that cAMP can suppress LPS-induced TNF- α production in macrophages (10, 33, 34). Presumably AC7-deficient BMDMs have a lower concentration of intracellular

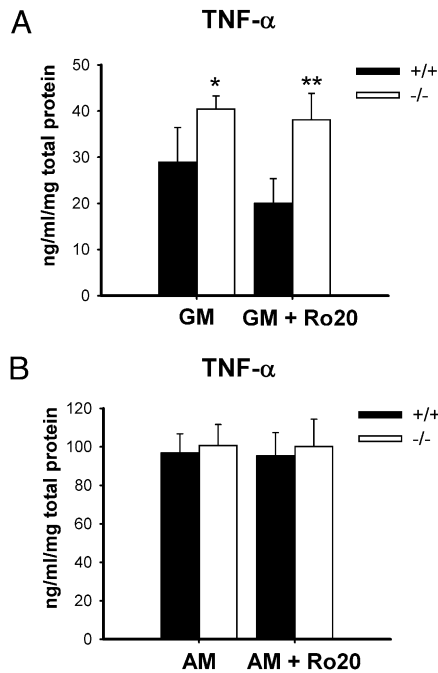


FIGURE 3. Regulation of LPS-induced TNF- α production in BMDMs. BMDMs were isolated from 8- to 10-wk-old mice deficient of AC7 or their wild-type littermates. After 7–10 d of culturing, the cells were treated with 10 ng/ml LPS and 25 pM LBP (LPS-binding protein) under various conditions: (A) in the presence of serum (growth medium, GM) with or without the presence of a PDE inhibitor, 10 μ M Ro20-1724 (Ro20); (B) in the absence of serum (assay medium, AM) with or without 10 μ M Ro20. Cytokine accumulation in the medium was measured after 4 h of exposure to LPS/LBP using ELISA. There was no detectable TNF- α in medium prior to LPS/LBP treatment (<8 pg/ml). The cytokine level was normalized against the amount of total proteins per well (indicator of total numbers of cells). Error bars indicate the SD of two to three independent experiments (each from a different isolation of BMDMs). * p < 0.01; ** p < 0.001 when responses are compared between wild-type and mutant cells

cAMP and without this suppression they are able to produce higher amounts of TNF- α in response to LPS stimulation. To confirm that the difference in the TNF- α responses to LPS in the presence and absence of serum factors could be caused by cAMP, a PDE inhibitor was used to cause increased accumulation of cAMP in cells and further suppression of TNF- α production. Treatment of cells with a PDE inhibitor, Ro20-1724, had no effect on LPS-induced TNF- α production in the absence of serum (Fig. 3B). However, in the presence of serum, inhibition of PDE activity further reduced the production of TNF- α in wild-type cells but not in AC7-deficient BMDMs (Fig. 3A). This result suggests that a lack of cAMP response to factors present in the FBS may be a cause for the overproduction of TNF- α to LPS stimulation in AC7-deficient BMDMs.

LPA as a potential factor to regulate TNF- α production in an AC7/cAMP-dependent manner

To determine whether factors present in the serum can indeed elicit cAMP responses in BMDMs in an AC7-dependent manner, we measured the responses using a BRET sensor for intracellular cAMP (29). When wild-type BMDMs were stimulated with 10% FBS, the cells responded with a small rise of intracellular cAMP concentrations (Fig. 4A). AC7-deficient BMDMs failed to produce such a response to FBS stimulation (Fig. 4A). No change of intracellular cAMP concentration was detected when wild-type BMDMs were treated with LPS.

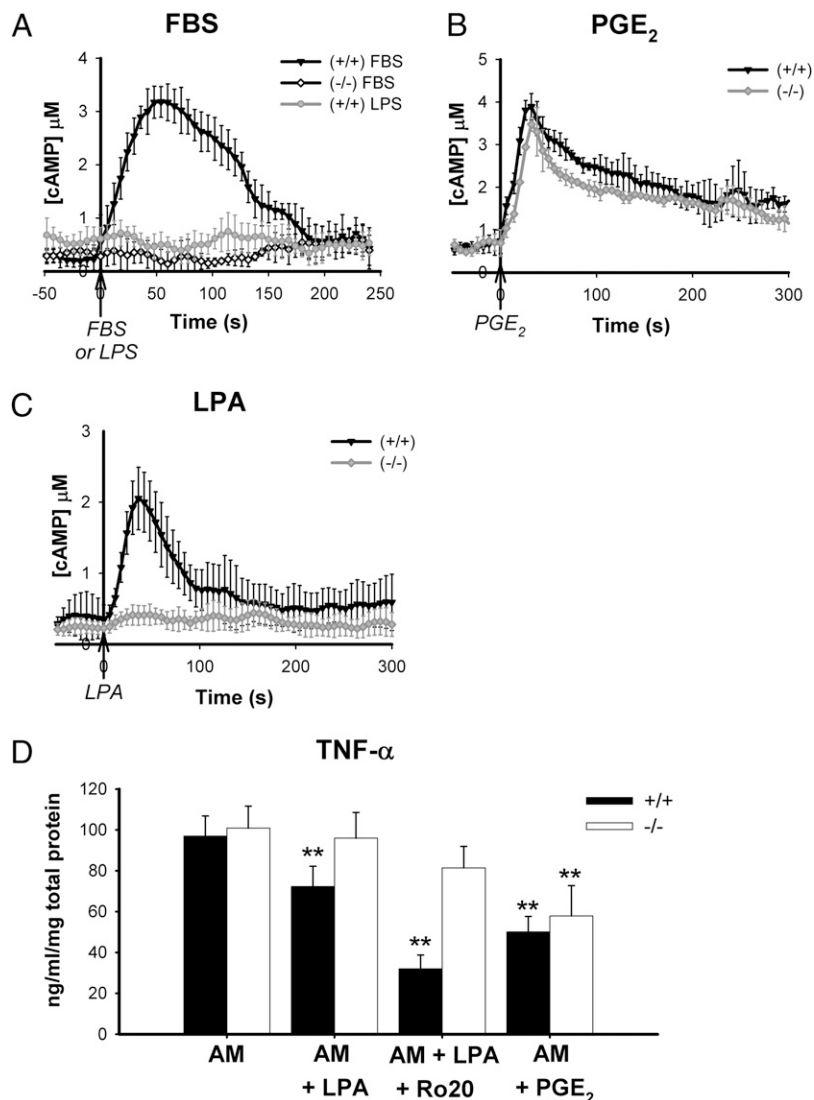
We have previously shown that the cAMP responses to G_s -stimulating ligands, such as isoproterenol (ISO), are comparable in AC7-deficient and wild-type BMDMs (30). Similarly, when challenged with another G_s -stimulating ligand, PGE₂, AC7-deficient BMDMs and wild-type cells produced similar cAMP responses (Fig. 4B). Presumably other AC isoforms expressed in BMDMs provide redundant capacity for G_s -stimulated cAMP synthesis. However, the cAMP response to ligands that are coupled to other G-protein pathways appeared to be AC7-dependent (30). One such ligand, lysophosphatidic acid (LPA), triggered a small but significant rise of cAMP concentration in wild-type BMDMs; this response was absent in AC7-deficient BMDMs (Fig. 4C).

We hypothesized that the bioactive lysophospholipids, LPA, would mimic the effect of serum in LPS-induced TNF- α production and explain the difference in TNF- α productions between wild-type and mutant BMDMs. We focused on this bioactive phospholipid as a potential factor based on two reasons. First, serum usually contains a variety of phospholipids, such as LPA (35). Interestingly, these lysophospholipids have been shown to mediate a variety of immune responses both in vitro and in vivo (36). Second, like FBS, LPA stimulates intracellular cAMP responses in BMDMs in an AC7-dependent manner. To test this hypothesis, wild-type cells were stimulated with LPS in conjunction with LPA. The production of TNF- α was indeed reduced in the presence of LPA compared with that treated with LPS alone (Fig. 4D). The reduction, although not as strong as that caused by the presence of serum, was significant. If the cells were also treated with a PDE inhibitor, the production of TNF- α was further decreased (Fig. 4D). Thus the regulation of LPS-induced TNF- α production by LPA is qualitatively similar to that induced by serum factors. Importantly, such regulation by LPA was absent in AC7-deficient BMDMs (Fig. 4D), consistent with the lack of a serum effect and lack of a cAMP response in those mutant cells (Figs. 3A, 4C). As a control, the production of TNF- α was also assessed in cells treated with LPS in conjunction with PGE₂. PGE₂ directly activates the G_s pathway to stimulate cAMP synthesis and AC7 is not essential for this regulation in BMDMs (Fig. 4B). As a result, it is not surprising that the addition of PGE₂ inhibited LPS-induced TNF- α production in both wild-type and mutant BMDMs (Fig. 4D).

AC7-deficient B and T lymphocytes show little intracellular cAMP responses

To assess AC7 functions in the lymphocytes, we first examined the cAMP responses in wild-type and mutant cells. Although AC7 is highly expressed in several cell lineages in the immune system, including B cells, T cells and macrophage cells, several other AC isoforms are also present in those cells (27–29) (Supplemental Fig. 4). To determine the contribution of AC7 to intracellular cAMP responses in those cells, we compared cAMP responses in wild-type and AC7-deficient lymphocytes. Purified wild-type B cells responded to terbutaline (TER) or ISO, agonists for the G_s -coupled β -adrenergic receptor, with a robust elevation of cAMP concentration (Fig. 5A). LPA, which can activate multiple G protein pathways, induced a slight increase of cAMP concentration in wild-type B cells (Fig. 5A). When the wild-type B cells were treated with TER and LPA simultaneously, a synergistic cAMP response was produced that is much greater than the sum of the responses to each ligand alone (Fig. 5A). In contrast, there was no cAMP change when AC7-deficient B cells were challenged with LPA. AC7-deficient B cells responded to TER with only a small rise of cAMP (~10–20% that of wild-type cells) and the synergy between TER and LPA was abolished (Fig. 5A). Similar results were obtained in enriched T cells (Fig. 5B). Therefore, in both

FIGURE 4. Serum factors regulate cAMP responses and LPS-induced TNF- α production in an AC7-dependent manner. *A–C*, Freshly isolated BMDMs were infected with retrovirus carrying the cAMP BRET sensor, CAMYEL. Intracellular cAMP responses were measured using a BRET assay. Wild-type or AC7-deficient cells were treated with 10% FBS or 10 ng/ml LPS and 25 pM LBP complex (*A*), 1 μ M PGE₂ (*B*), or 500 nM LPA (*C*) at time 0. Each trace is the average of three independent experiments and error bars represent the SD. *D*, Cultured BMDM cells were treated with 10 ng/ml LPS and 25 pM LBP in conjunction with 2 μ M LPA, 10 μ M Ro20, 250 nM PGE₂, or combinations of ligands and inhibitors as indicated. Cytokine accumulation in the medium was measured after 4 h of exposure to LPS/LBP using ELISA. There was no detectable TNF- α in medium prior to LPS/LBP treatment (<8 pg/ml). The cytokine level was normalized against the amount of total proteins per well (indicator of total numbers of cells). Error bars indicate the SD of two to three independent experiments (each from a different isolation of BMDMs). ***p* < 0.001 when responses are compared with responses in AM of cells carrying the same genotype.



B cells and T cells AC7 appears to be the major AC isoform for regulating cAMP synthesis. Without this enzyme, the B and T cells are largely incapable of generating cAMP synthesis.

T cell-independent responses are compromised in the AC7-deficient immune system

To determine the potential functions of AC7 in the adaptive immune responses, we characterized Ag-elicited Ab responses in the chimeric mice. TI Ags were used to examine B cell functions in vivo. There are two types of TI Ags that engage different signaling mechanisms. Type I TI Ags, such as LPS conjugated to a hapten, can activate TLRs in concert with the BCRs and provide the second signal for B cell activation, clonal expansion, and Ab production. Type II TI Ags, such as TNP-Ficoll, rely on help from cytokines (presumably from T cells) to fully activate B cells on extensive cross-linking of the BCR and activation of its downstream signaling pathway.

When immunized with the TI-I Ag, TNP-LPS, the chimeric mice with a wild-type immune system mounted a robust IgM response. This response was significantly reduced in chimeric mice bearing an AC7-deficient immune system (Fig. 6A). However, the IgG2c and IgG3 responses appeared to be comparable to those of wild-type animals (Fig. 6A). The chimeric mice carrying the AC7-deficient immune system showed reduced total number of B cells

in the spleen, which conceivably could account for the reduction in the IgM response. However, the normal IgG responses argue against the defect being simply quantitative. Rather, AC7 is qualitatively needed for optimal Ab responses.

When the TI-II Ag, NP-Ficoll, was used to challenge the chimeric mice, we found that not only the production of NP-specific IgM was reduced in AC7-deficient animals but also the levels of NP-specific IgG1, IgG2c, and IgG3 were all significantly reduced (Fig. 6B).

Together, these results indicate that AC7-deficient B cells were capable of mounting Ab responses against TI Ags. However, the strength of the responses was significantly reduced compared with AC7 heterozygous or wild-type animals. Because IgM production was compromised in response to both Ags, it is likely that the BCR signaling pathway is affected by the lack of AC7. The IgG responses to the two types of Ags differ. The intact switching to IgG2 and IgG3 in response to TI-I but not TI-II Ags suggests that the BCR signaling defect may be partially overcome by TLR signaling.

T cell-dependent responses are reduced in the AC7-deficient immune system

To determine whether AC7-deficient T cells can function normally, we examined Ab responses to a T cell-dependent Ag, TNP-KLH.

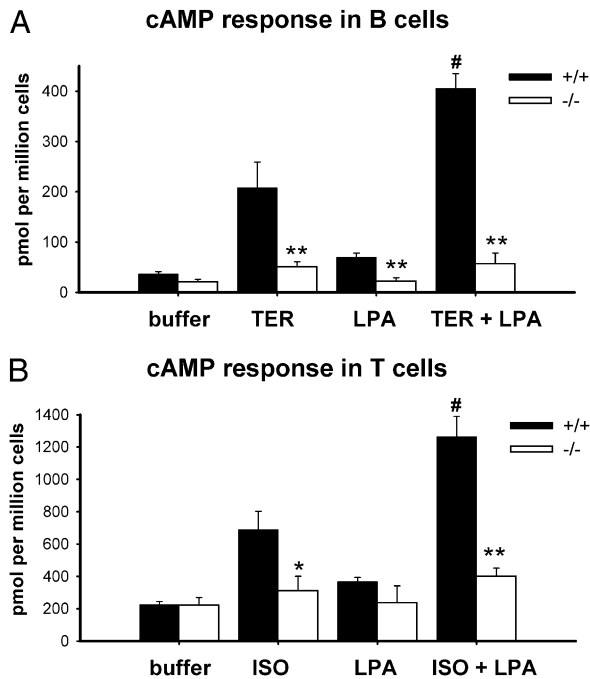


FIGURE 5. Measurement of cAMP responses in B cells and T cells. B cells and T cells were purified from the spleen through negative selection using auto-MACS. *A*, B cells were serum starved for 1 h then challenged with 10 μ M TER, 100 nM LPA, or combination of the two ligands. *B*, T cells were challenged with 100 nM ISO, 100 nM LPA, or combination of the two ligands. After 1 min the reaction was stopped by cell lysis and total cellular cAMP was measured using the Biotrak cAMP EIA kit. Results shown are the average of four experiments from two independent cell preparations. For significant differences when mutant cells are compared with wild-type cells; * $p < 0.01$; ** $p < 0.001$. # $p < 0.001$ when responses to double ligands are compared with the sum of single ligand responses in wild-type cells.

For effective T cell help, Ag captured via the BCR has to be presented to T cells. This results in the upregulation of costimulatory ligands as well as cytokines, which are required for the activation of B cells. When the chimeric mice were challenged with TNP-KLH Ag, all TNP-specific IgG responses were largely reduced in the chimeric mice generated from AC7-deficient bone marrow cells (Fig. 7A).

The reduced Ab responses could be caused by defective B cell responses or by a diminution in T cell help. To distinguish between these two possibilities, we examined the ability of wild-type T cells to facilitate AC7-deficient B cells for Ab production. A cohort of wild-type mice was first challenged with TD Ag, KLH, to prime both B cells and T cells. Three days later, these mice were sublethally irradiated resulting in survival of only radio-resistant T cells. These mice were then injected with splenocytes from AC7-deficient mice or their wild-type littermates as a source of functional B cells. The reconstituted mice, containing naive B cells from the donor and primed T cells from the wild-type host, were subsequently challenged with TNP-KLH. As a control, two mice were irradiated but without subsequent injection of splenocytes. Those mice failed to mount any Ab response (OD_{490} remained at basal level, ~ 0.05 – 0.1 , for all Ig subtypes measured), indicating the complete elimination of B cells. Mice injected with the splenocytes were able to generate Ab responses against TNP-KLH. Importantly, mice with either wild-type or AC7-deficient B cells produced similar levels of IgG1 and IgG2c in their sera as shown in Fig. 7B. This result indicates that primed wild-type T cells can rescue the reduced IgG1 and IgG2c responses to TD Ags,

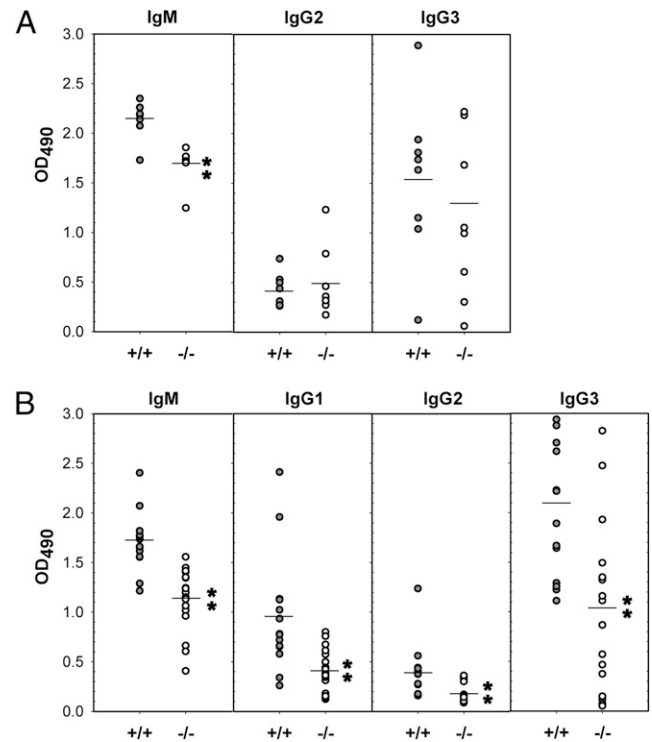


FIGURE 6. Compromised Ab responses toward T cell independent Ags in AC7-deficient mice. Six to 8 wk after the bone marrow transplant, the chimeric mice were challenged with TI-I Ag, TNP-LPS, at 10 μ g per mouse (*A*) or with TI-II Ag, NP-Ficoll, at 40 μ g per mouse (*B*) via i.p. injection. At day 7 (*A*) or day 12 (*B*), blood was collected from each chimeric mouse through tail vein bleeding. Serum IgM and IgG levels specific to the Ags (TNP in *A*, NP in *B*) were examined by ELISA. Prior to Ag exposure, the serum level of Ag-specific Abs were < 0.2 for IgM and < 0.1 for IgGs. Small horizontal bars indicate the average of each group. Each circle represents one mouse; one representative of two independent experiments (from two independent bone marrow transplants) is shown. ** $p < 0.005$ calculated by the Mann-Whitney rank sum.

suggesting a specific defect in T cell help due to AC7 deficiency. However, the production of TNP-specific IgG3 remained significantly reduced in mice harboring mutant B cells (Fig. 7B). The persistent reduction of IgG3 level, despite wild-type T cell help, may be due to B cell defects that cannot be rescued by T cell help.

Memory T cell function is dampened due to AC7 deficiency

The ability of AC7-deficient animals to generate T cell memory was examined by using the B cell response to register the extent of T cell help. Animals that had been immunized with TNP-KLH were challenged with FITC-KLH in the absence of adjuvant 6 wk later. Because the same carrier was used, the response to FITC-KLH should be a measure of whether memory for KLH was generated for a primary B cell response to FITC. As shown in Fig. 8, the production of FITC-specific IgM and IgG levels were all significantly reduced in mice with an AC7-deficient immune system. Together with the defects in TD responses, both primary and secondary T cell responses are compromised in AC7 defective animals.

Discussion

The role of cAMP in regulating immune functions is multifaceted and depends on the signaling context. Thus, cAMP can promote cell proliferation or induce apoptosis (4, 7). It can facilitate or inhibit cell–cell interactions (6, 17, 37). For cAMP to exert its proper functions in a context dependent manner, the temporal

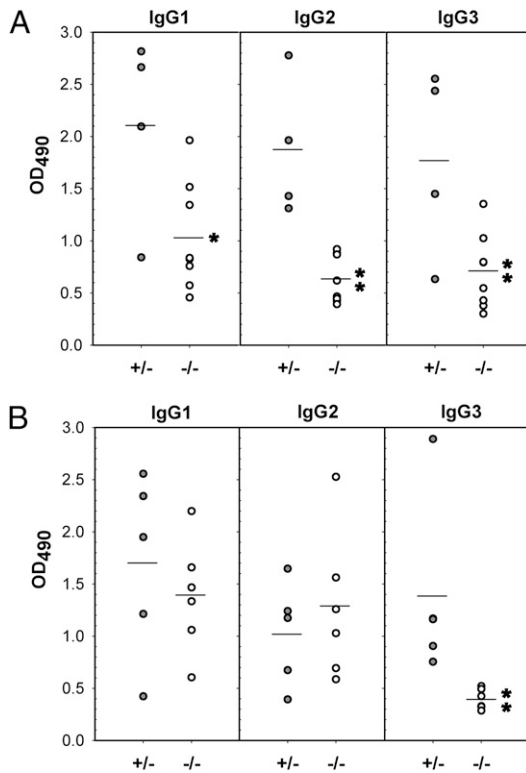


FIGURE 7. Defective T cell-dependent Ab responses in AC7-deficient chimeric mice. *A*, Three months after the bone marrow transplant, the chimeric mice were challenged with TD Ag, TNP-KLH, emulsified in incomplete Freund's adjuvant at 100 μ g per mouse via i.p. injection. At day 14, serum was collected from each chimeric mouse through tail vein bleeding. *B*, A cohort of age-matched and sex-matched wild-type mice were first primed with TD Ag, KLH, emulsified in ICF adjuvant at 100 μ g per mouse via i.p. injection. Three days later these mice were subjected to 750R of gamma-ray irradiation, under which condition the B cells were destroyed and primed T cells remained intact. Three hours later, those mice were injected through their tail veins with 10^7 total spleen cells isolated from an AC7-deficient mouse or its wild-type littermate. The mice were immediately challenged with TNP-KLH without any immune adjuvant at 100 μ g per mouse. Fourteen days later, blood was collected from each chimeric mouse through tail vein bleeding. Serum IgG levels specific to TNP were examined by ELISA. Prior to Ag exposure, the serum level of Ag-specific Abs were <0.2 for IgM and <0.1 for IgGs. Small horizontal bars indicate the average of each group. Each circle represents one mouse. One representative of two independent experiments (from two independent bone marrow transplants) is shown in *A*; the rescue experiment was done once. * $p < 0.05$; ** $p < 0.005$, as calculated by Mann-Whitney rank sum test.

regulation of its intracellular concentration may be crucial. It has been established that prolonged cAMP signaling suppresses immune responses in general. This has been demonstrated by the use of cAMP analogs or attenuation of PDE activities through application of small molecule inhibitors or gene knockout (6, 8, 38). The effect of these manipulations is sustained elevation of intracellular concentrations of cAMP. However, evidence exists that optimal immune responses also require transient rises of this second messenger (13, 15, 17). Such dynamic responses depend on cAMP synthesis controlled through activation of ACs by hormones. This study is the first to explore the function of a specific isoform of AC in the immune system.

The generation of bone marrow chimeras circumvented the embryonic lethal phenotype of AC7 knockout mice and provided a venue to study the functions of this specific AC isoform in the

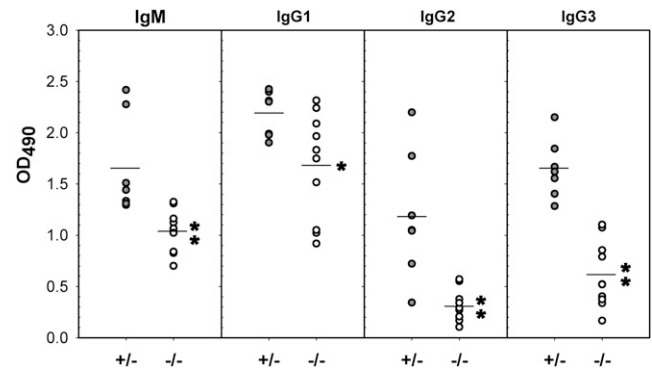


FIGURE 8. Memory T response is also compromised in mice with an AC7-deficient immune system. Six weeks after the initial TD challenge with TNP-KLH in the presence of ICF, the chimeric mice were further challenged with FITC-KLH without immune adjuvant to examine the functions of memory T cells. Fourteen days later, blood was collected from each chimeric mouse through tail vein bleeding. Serum IgM and IgG levels specific to FITC were examined by ELISA. Prior to Ag exposure, the serum level of Ag-specific Abs were <0.2 for IgM and <0.1 for IgGs. Each circle represents one mouse; one representative of two independent experiments (from two independent bone marrow transplants) is shown. * $p < 0.05$; ** $p < 0.005$, as calculated by the Mann-Whitney rank sum test.

development of the immune system and immune responses. AC7-deficient bone marrow cells were able to regenerate the full spectrum of the immune cells although the total leukocytes number was reduced. The reduction of total leukocytes number without altering the proportion of B and T lymphocytes could potentially be caused by a proliferative defect of AC7-deficient hematopoietic stem cells. It has been shown that PGE₂ regulates the developmental specification and regeneration of hematopoietic stem cells through the cAMP/PKA pathway (39, 40). Conceivably, AC7 could be the major isoform that mediates cAMP response in hematopoietic stem cells as it does in B cells and T cells (Fig. 5). Consistent with this notion, we found that in AC7-deficient bone marrow cells there was a reduction in immature B cells; whereas, the mature B cells appeared normal (E. Sadat, unpublished observation).

The chimeric mice were used to explore the functions of AC7 in bacterial infection and adaptive immune responses. We show here that mice harboring an AC7-deficient immune system are more susceptible to endotoxic shock and display a higher mortality rate. AC7 deficiency led to overproduction of the proinflammatory cytokine, TNF- α , both in vivo and in vitro (Figs. 2, 3). Interestingly, mice lacking PDE4b showed the exact opposite phenotype; the mice were resistant to endotoxic shock and produced little amount of TNF- α in response to LPS (41). The corroborative phenotypes emphasize the importance of keeping intracellular cAMP concentration in balance. Both cAMP synthesis and degradation appear to be regulated in an isoform specific manner. Dysregulation of either process leads to serious physiological consequences.

Using the BMDMs in vitro, we showed that this phenotype is not due to defect in TLR signaling per se. Rather, it requires the presence of certain serum factors. These factors regulate LPS-induced TNF- α production in a cAMP and AC7-dependent manner (Fig. 3). We show that one potential factor could be the bioactive lysophospholipid, LPA, which regulates cAMP responses in macrophages in an AC7-dependent manner (Fig. 4). Therefore, AC7 functions to keep inflammation under control during bacterial infection by sensing the presence of serum factors, such as LPA. Without this regulation by AC7, the inflammatory response

becomes hyperactive and detrimental. We have previously shown that AC7 has a unique role in regulating cAMP concentration in macrophages that is nonredundant from other AC isoforms expressed in these cells (30). The current study provides insight into the physiological function of this regulation.

Contrary to its immunosuppressive role in bacterial infection, AC7 is required to generate optimal immune responses against a variety of Ags (Figs. 6–8). Without this enzyme most of the Ab responses were dampened although the specific pathways differ depending on whether the response is T cell dependent. In response to T cell independent type II Ags, AC7-deficient animals showed significantly reduced productions of Ag-specific Abs (Fig. 6B), suggesting a defect when responding to extensive cross-linking of the BCR. This phenotype resembles that of mutant mice defective in BCR signaling pathway, such as mice lacking subunits of PI3K or Btk (42–46), suggesting that the AC7/cAMP pathway might modulate BCR signaling. Further genetic and cellular analyses are needed to determine whether these two pathways intersect and how the AC7/cAMP pathway regulates B cell functions.

In contrast, in the context of T cell help, in which the B cell activation is not solely dependent on ligation of the BCR, the functions of B cells are no longer dependent on AC7 (Fig. 7). Thus, although AC7-deficient mice displayed greatly compromised Ab responses to TD Ags; however, when primed wild-type T cells were used to provide costimulatory factors to mutant B cells, AC7-defective B cells were able to produce Ag-specific Abs and switch to downstream isotypes to the same extent as wild-type B cells do. This result, together with the finding that generation of memory cells is compromised, strongly suggests that the defects in TD responses are largely due to defective T cell help in AC7-deficient animals. The reciprocal experiment has been difficult to perform due to the fact that the majority of AC7 knockout mice die before birth. A conditional knockout strain that is being developed in the laboratory will help address this question directly.

In both B cells and T cells, AC7 is the major isoform that regulates cAMP synthesis in response to activation of the G_s as well as other G protein pathways (Fig. 5). Loss of AC7 in those cells resulted in impaired signaling in both cell types, suggesting cAMP is needed to ensure adequate adaptive immune responses. However, it is not clear under which conditions and in what cellular context that AC7 exerts its function to regulate immune responses. We have begun to address this question by examining B cell and T cell functions in vitro under defined signaling conditions. Our preliminary result suggests that there is no obvious defect in BCR and TCR signaling when activated by its respective ligand(s). This is not surprising based on how AC7/cAMP regulates TLR signaling in macrophages. It is likely that AC7/cAMP functions during specific cell–cell interactions where multiple signaling pathways are involved. One such example was demonstrated elegantly by Conche et al. (17). They showed that a transient rise of cAMP in T cells on T cell interaction with dendritic cells is important for optimal T cell activation. We are in the process of determining whether this cAMP response is dependent on AC7 and what ligand stimulates this activity.

Taken together, our results solidify the notion that cAMP functions in a context dependent manner to either suppress or support immune responses. We have shown that AC7 is the key AC isoform in mediating cAMP response and its downstream physiological functions in the immune system. Further understanding of how AC7 activity is regulated and how the AC7/cAMP pathway integrates into the signaling events will help provide a better understanding of the intricate tuning and balancing of multiple pathways that lead to proper immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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