

Induction of long-lived allergen-specific plasma cells by mucosal allergen challenge

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Background: Allergen-specific IgE antibodies are responsible for the pathogenesis of type I hypersensitivity. In patients with allergy, IgE titers can persist in the apparent absence of allergen for years. Seasonal allergen exposure triggers clinical symptoms and enhances allergen-specific IgE. Whether allergen-specific plasma cells originating from seasonal allergen exposures can survive and become long-lived is so far unclear.

Objective: We analyzed the localization and lifetimes of allergen-specific IgE-secreting, IgA-secreting, and IgG₁-secreting plasma cells after allergen inhalation in an ovalbumin-induced murine model of allergic asthma.

Methods: Ovalbumin-specific IgG₁-secreting, IgA-secreting, and IgE-secreting cells in lungs, spleen, and bone marrow were isolated and tested for antibody secretion by the ELISpot technique. Longevity of ovalbumin-specific plasma cells was determined by cyclophosphamide treatment, which depletes proliferating plasmablasts but leaves plasma cells untouched. Ovalbumin aerosol-induced infiltrates in lungs were localized by confocal microscopy.

Results: Long-lived ovalbumin-specific plasma cells were generated by systemic sensitization and survived in bone marrow and spleen, maintaining systemic ovalbumin-specific titers of IgG, IgA, and IgE. On inhalation of ovalbumin-containing aerosol, sensitized mice developed airway inflammation and more ovalbumin-specific IgG₁-secreting, IgA-secreting, and IgE-secreting cells in the lungs and in secondary lymphoid organs. These plasma cells joined the pool of ovalbumin-specific plasma cells in the bone marrow and became long-lived—that is, they are resistant to cyclophosphamide. Termination of ovalbumin inhalation depleted ovalbumin-specific plasma cells from the lungs, but they persisted in spleen and bone marrow.

Conclusion: Our results show that inhalation of aerosolized allergen generates long-lived, allergen-specific IgG₁-secreting,

IgA-secreting, and IgE-secreting plasma cells that survive cytostatic treatment. (*J Allergy Clin Immunol* 2009;124:819-26.)

Key words: Plasma cells, allergy, inflammation, memory, lung, mucosa

IgE can survive even without allergen contact for long periods bound to Fc receptors of basophilic granulocytes or mast cells.¹ The half-life of IgE antibodies in serum is only 3 to 12 hours,^{2,3} implying that IgE plasma cells (PCs) have to be maintained over time to provide persistent serum IgE titers. The persistence of IgE-secreting PCs has been a matter of controversy. If PCs were short-lived end-products of B-cell differentiation, IgE-secreting PCs would have to be replenished continuously from activated B lymphocytes in a chronic immune reaction driven by residual allergen or by cytokine signals.^{4,5} On the other hand, evidence has been available for quite some time that at least some IgE-secreting cells in allergic animals are long-lived.⁶ Recently, more evidence was raised that long-lived PCs can survive in the bone marrow or in inflamed tissue over years, providing the antibodies of protective humoral memory.⁷⁻⁹ These long-lived PCs do not proliferate, and they are refractory to antigen and immunosuppression.^{8,10,11}

In systemic immune responses, antigen-specific antibody-secreting cells (ASCs) have been generated in secondary lymphoid organs. Most of these cells then migrated into the bone marrow as plasmablasts.^{10,12,13} These plasmablasts are proliferating precursors of PCs and can therefore be depleted from the system by cytostatic drugs such as cyclophosphamide. In bone marrow, migratory plasmablasts differentiate finally into resident PCs and survive over extended periods.^{7,8,10,14} Survival of these long-lived PCs is likely to be a result of signals from their environment,^{9,15,16} forming a PC survival niche. PCs relocated from their survival niches will probably die, because they are no longer migratory and are deprived of their survival signals.^{9,13}

Most type I immune responses are complex immune reactions including both systemic and local mucosal components. The persistent production of specific IgE in the apparent absence of its antigen/allergen is a hallmark of type I hypersensitivity in human beings.¹⁷ The resistance of type I hypersensitivity to immunosuppression, and the persistence of allergen-specific IgE in the face of immunosuppressive therapy,^{18,19} are in line with the notion that long-lived PCs maintain allergen-specific IgE titers and are thus the molecular basis for disease.⁹ It can even be speculated that these long-lived IgE PCs reside in the bone marrow, because allergen-specific IgE production can be transferred by the transplantation of bone marrow from individuals with allergy to individuals without allergy.²⁰

Here we wanted to answer whether inhalation of allergen can lead to the formation of long-lived, allergen-specific PCs.

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Abbreviations used

ASC: Antibody-secreting cell
PC: Plasma cell

We analyzed the generation and longevity of allergen-specific PCs after ovalbumin aerosol inhalation in a murine model of type I hypersensitivity, the ovalbumin asthma model,²¹ by using techniques such as fluorescence microscopy, flow cytometry, and ELISpot. The systemic allergen-specific immune response was analyzed by ELISA. We showed that ovalbumin-specific ASCs, generated during systemic sensitization, homed to and survived preferentially in the bone marrow, but also in the spleen. Inhalation of ovalbumin-containing aerosol in already sensitized animals induced a profound boost of IgE, but also IgG₁ and IgA secretion. The mucosal allergen challenge induced ovalbumin-specific PCs in the lung, cells that vanished rapidly after termination of aerosol treatment. However, numbers of ovalbumin-specific IgE-secreting, IgG₁-secreting, and IgA-secreting, cyclophosphamide-resistant cells in the bone marrow increased on aerosol treatment, indicating that aerosol delivery of allergen induces long-lived specific PCs. This article thus shows that inhalation of aerosolized allergen generates long-lived, allergen-specific IgG₁-secreting, IgA-secreting, and IgE-secreting PCs that survive cytostatic treatment in the spleen and bone marrow.

METHODS**Mice and sensitization protocol**

Female BALB/c mice 6 to 8 weeks old (Harlan Winkelmann, Borcheln, Germany) were sensitized intraperitoneally with ovalbumin 10 µg (Sigma, St Louis, Mo)/1.5 mg Al(OH)₃ (Pierce) on days 1, 14, and 21. Mice were challenged with ovalbumin aerosol (1% wt/vol in PBS) at the indicated time points. Mice received 35 mg/kg body weight cyclophosphamide intraperitoneally. Animal experiments were approved by the institutional review committee of the University of Marburg.

Quantification of ASCs by ELISpot technology

Single-cell suspensions of spleen, bone marrow, and lung were resuspended in RPMI 1640 medium/10% FCS (Invitrogen, Carlsbad, Calif). Lungs were perfused with PBS/BSA to remove draining blood cells. Ninety-six-well plates (Multiscreen HTS; Millipore, Billerica, Mass) were coated with rat antimouse IgE (84-1C) or ovalbumin in sodium carbonate buffer and blocked with RPMI 1640, 10% FCS, and PBS/3% BSA. Cell dilutions were titrated in medium and incubated for 4 hours at 37°C/5% CO₂. Cells were removed with PBS/1% BSA/0.05% Tween 20. ASC were detected by using rat antimouse IgE-Bio (EM95.3), IgG1-Bio, and IgA-Bio in PBS/1% BSA and streptavidin-horseradish peroxidase (SA-HRP) (Sigma). Spots were visualized with 100 µL/well AEC solution (Sigma) and counted (C.T.L. ImmunoSpot Analyzer and ImmunoSpot software 4; Cellular Technology, Bonn, Germany).

ELISA

For total IgE, 96-well plates (Nunc, Langensfeld, Germany) were coated with 5 µg/mL antimouse IgE (84-1C) and blocked with PBS/3% milk powder. Ovalbumin-specific IgE was assayed on 5 µg ovalbumin (Sigma). For ovalbumin-specific IgE measurements, sera were pretreated with GammaBind-G-Sepharose (Amersham Biosciences, Freiburg, Germany). Specific and total IgE was assayed with biotinylated rat antimouse IgE (EM95.3)/SA-HRP and developed with 3, 3', 5, 5'-tetramethylbenzidine

(TMB) (Sigma). Standard curves were calculated by using known amounts of purified IgE (BD, Heidelberg, Germany). All assays were performed in duplicate.

Localization of ovalbumin-specific PCs by fluorescence microscopy

Lungs were flushed with PBS to remove circulating cells and inflated with 50% optimal cutting temperature medium (Sakura Finetek, Heppenheim, Germany) in PBS. Sections of 6 µm were fixed in acetone and blocked with PBS/3% BSA and antimouse Fcγ receptor. For κ/λ stainings, fluorescein isothiocyanate-conjugated antimouse κ (Southern Biotech, Birmingham, Ala) and antimouse Ig λ₁, λ₂, and λ₃ light chain (BD) were used. CD3-positive cells were stained with rat antimouse CD3-Alexa Fluor 488 (Serotech, Duesseldorf, Germany). For IgE stainings, 5 µg monoclonal biotinylated rat antimouse IgE antibody (84-1C)/streptavidin-Alexa₅₄₆ was used. Ovalbumin-specific PCs were stained with Cy5-labeled ovalbumin and examined by confocal laser scanning microscopy (DMIRE2; Leica, Wetzlar, Germany). In addition, we stained IgG and IgE on single cryosections. The absence of any double-positive PCs proved the specificity of the IgE staining (data not shown).

Fluorescence-activated cell sorting analyses

Spleen suspensions were filtered (70 µm), incubated with anti-CD138-phycoerythrin (BD), fixed, and stained with ovalbumin-Cy5 (Sigma-Aldrich, Munich, Germany) intracellularly. Cytometric analyses were performed by using an LSR II cytometer with FACSDiva software (BD).

Statistical analyses

Data are expressed as means ± SEMs. Results were calculated with GraphPad Prism software (San Diego, Calif) and interpreted by using the 2-tailed Mann-Whitney *U* test. Differences were considered statistically significant at *P* < .05 (****P* < .001; ***P* < .01; **P* < .05).

RESULTS**Systemically induced long-lived ovalbumin-specific IgG₁, IgA, and IgE PCs survive in bone marrow and spleen**

Systemic sensitization with ovalbumin-alum induced an IgE-triggered allergic reaction in the course of a profound ovalbumin-specific IgG₁ and IgA immune response. To analyze the lifetime and location of ovalbumin-specific IgG₁, IgA, and IgE PCs, mice were sensitized systemically with ovalbumin-alum on days 1, 14, and 21. One group was treated with cyclophosphamide from days 31 to 34, leading to the elimination of proliferating cells—that is, short-lived plasmablasts and still-proliferating early PCs¹¹—and analyzed for surviving, nonproliferating PCs at day 100 (Fig 1, A). As shown in Fig 1, B, ovalbumin-specific IgE, IgG₁, and IgA PCs generated during systemic immunization survived in bone marrow and to a lesser extent in the spleen. These PCs were long-lived because they were cyclophosphamide-resistant.¹¹ The efficacy of cyclophosphamide treatment was shown at day 70 (Fig 1, C). For total ASC numbers, see this article's Fig E1, A, in the Online Repository at www.jacionline.org. IgA₁ PCs were also detectable among long-lived, cyclophosphamide-resistant PCs of bone marrow and spleen. For ovalbumin-specific PCs of any isotype, higher numbers of ASCs were detected in the bone marrow than the spleen. In nonimmunized animals, no ovalbumin-specific ASCs were detectable (data not shown). This article's Fig E1, B, shows an exemplary ELISpot plate.

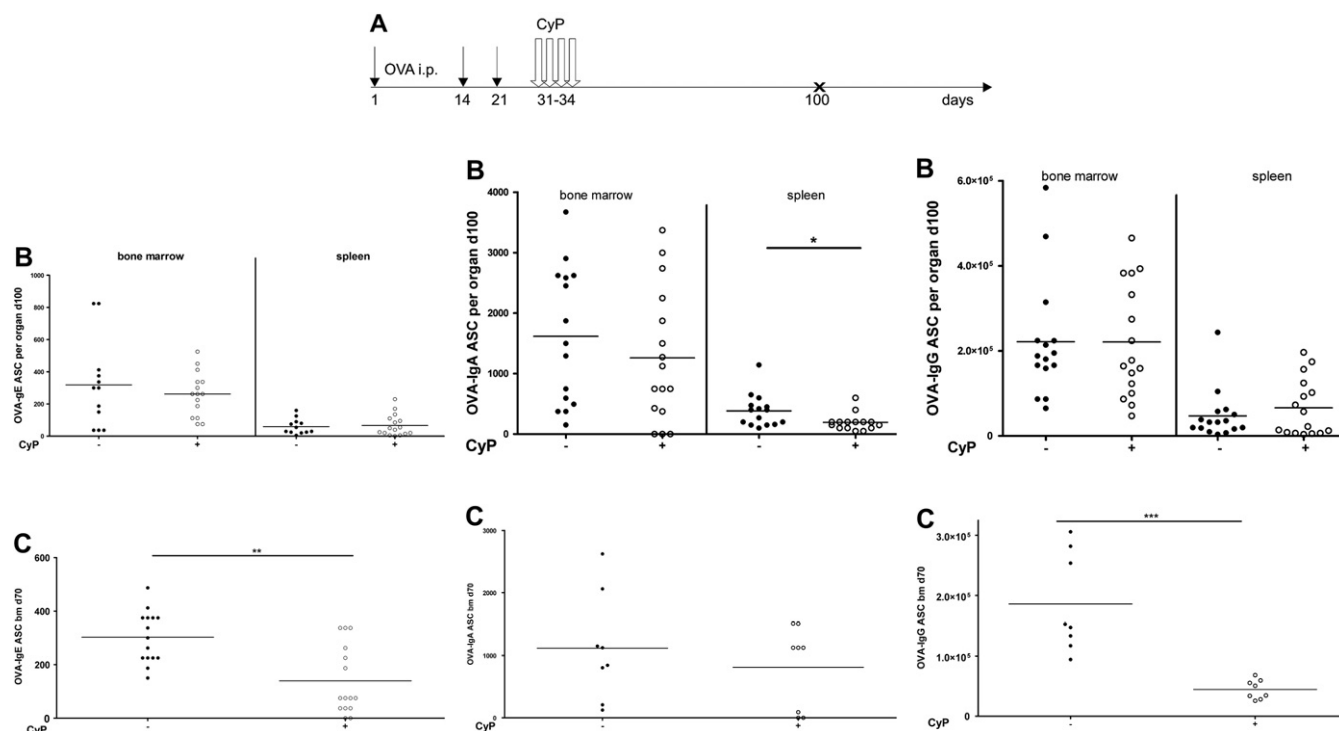


FIG 1. Cyclophosphamide (CyP)-resistant ovalbumin (OVA)-specific PCs in bone marrow and spleen. **A**, Experimental setup. **B**, Survival of OVA-specific immunoglobulin ASCs detected by ELISpot at day 100. **C**, Specific effect of CyP shown by reduction of ASCs at day 70. Each symbol represents the number of ASCs per organ per single mouse. Data from 2 independent experiments, 16 mice each. *i.p.*, Intraperitoneal.

Lymphocytes migrate into the lung after aerosol inhalation but do not persist there

It has been reported that after allergen uptake, airway dendritic cells migrate to the draining lymph nodes of the lung, upregulate the expression of costimulatory ligands, and interact with naive T lymphocytes, inducing an immune response.²² Airway inflammation induced by repeated allergen-in-aerosol exposure leads to the immigration of leukocytes into the lung.²¹ Plasmablasts have been reported to be able to migrate from secondary lymphoid tissue into inflamed tissue and survive there,⁹ but they also could be generated within inflamed tissue from precursors in ectopic immune reactions.²³ In the model system analyzed here (see this article's Fig E2, A, in the Online Repository at www.jacionline.org), already after the second allergen-aerosol inhalation, not only B cells (B220), T cells (anti-CD3), κ/λ -positive B cells, and ASCs, but also IgE-positive and IgG-positive B cells and PCs are detectable in the lungs, suggesting that these cells had immigrated rather than developed there (Fig E2, B). Here we show, however, that persistence of these PCs is dependent on continued allergen-aerosol stimulation of the lung (Fig 2, A and B); after termination of aerosol treatment, these PCs disappear (Fig 2, B), although they are still detectable elsewhere (Fig 2, C), where they continue to secrete specific antibodies.

Mucosal allergen challenge triggers a rapid IgE response in bone marrow and spleen

In the model system analyzed here, systemic sensitization was followed by a continued airway challenge with allergen aerosol (Fig 3, A). This treatment induces chronic allergic inflammation of the lung.²¹ Here we show that the local mucosal allergen

challenge also affected the systemic humoral memory for the allergen. Starting at day 28—that is, 7 days after the onset of allergen-aerosol inhalation—IgE PC numbers increased 5-fold in the spleen (Fig 3, B), and frequencies of ovalbumin-specific PCs increased from 0.4% to 13% (Fig 3, C). In the bone marrow, continued allergen inhalation led to an increase of specific IgE PCs over time, from about 500 ± 35 to more than 1235 ± 499 within less than 4 weeks (Fig 3, D). The difference in the number of ovalbumin-specific IgE PCs in spleen and bone marrow translated into drastic differences in the titers of ovalbumin-specific and total IgE in the serum (see this article's Fig E3 in the Online Repository at www.jacionline.org).

Mucosal allergen-challenge induces long-lived allergen-specific IgE PCs

To determine the impact of airway challenge with allergen on the humoral memory of systemically sensitized mice, sensitized mice inhaled allergen aerosol from days 56 to 70 every third day. Mice were treated with cyclophosphamide from days 74 to 77 to kill proliferating, short-lived PCs, and numbers of allergen-specific PCs of the various isotypes were determined on day 100 (Fig 4, A). Spleen, bone marrow, and lung were examined for the presence of surviving ovalbumin-specific PCs. After aerosol treatment, most of the IgG₁, IgA, and IgE PCs were detectable in the bone marrow, less in the spleen, and nearly none in the lungs (Fig 4, B). Challenge with allergen aerosol led to a 3-fold induction of ovalbumin-specific IgE, a 5-fold induction of ovalbumin-specific IgG₁, and a 40-fold induction of ovalbumin-specific IgA PCs in the bone marrow compared with mice that were sensitized only. As shown in this article's Fig E4 in

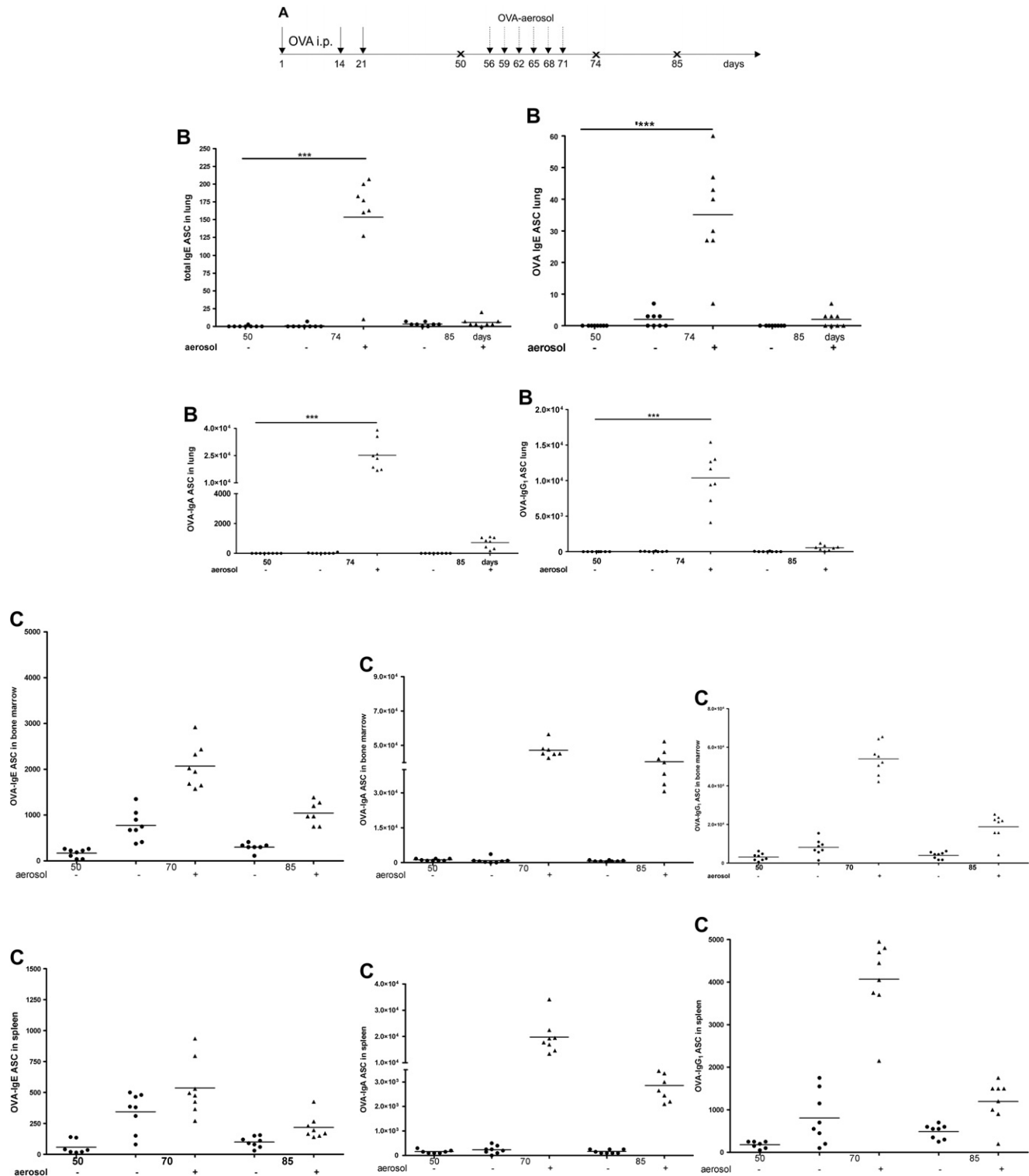


FIG 2. Immigration and kinetic of ovalbumin (OVA)-specific IgG₁, IgA, and IgE ASCs. **A**, Experimental setup. **B**, Lung ELISpot of OVA-sensitized (●) and OVA-sensitized plus OVA aerosol-challenged mice (▲). **C**, Bone marrow/spleen ELISpot of OVA-sensitized (●) and sensitized plus OVA-challenged mice (▲). *i.p.*, Intraperitoneal.

the Online Repository at www.jacionline.org. PC numbers nicely correlate with serum titers of ovalbumin-specific IgE and IgG₁. Except for IgA-secreting ovalbumin-specific PCs in the lung, spleen, and bone marrow, none of the PC populations was

affected by cyclophosphamide, indicating that nearly all IgG₁-secreting and IgE-secreting cells had been generated more than 3 weeks before day 100 and were long-lived. They survived in the spleen and bone marrow but not the lung. This was also

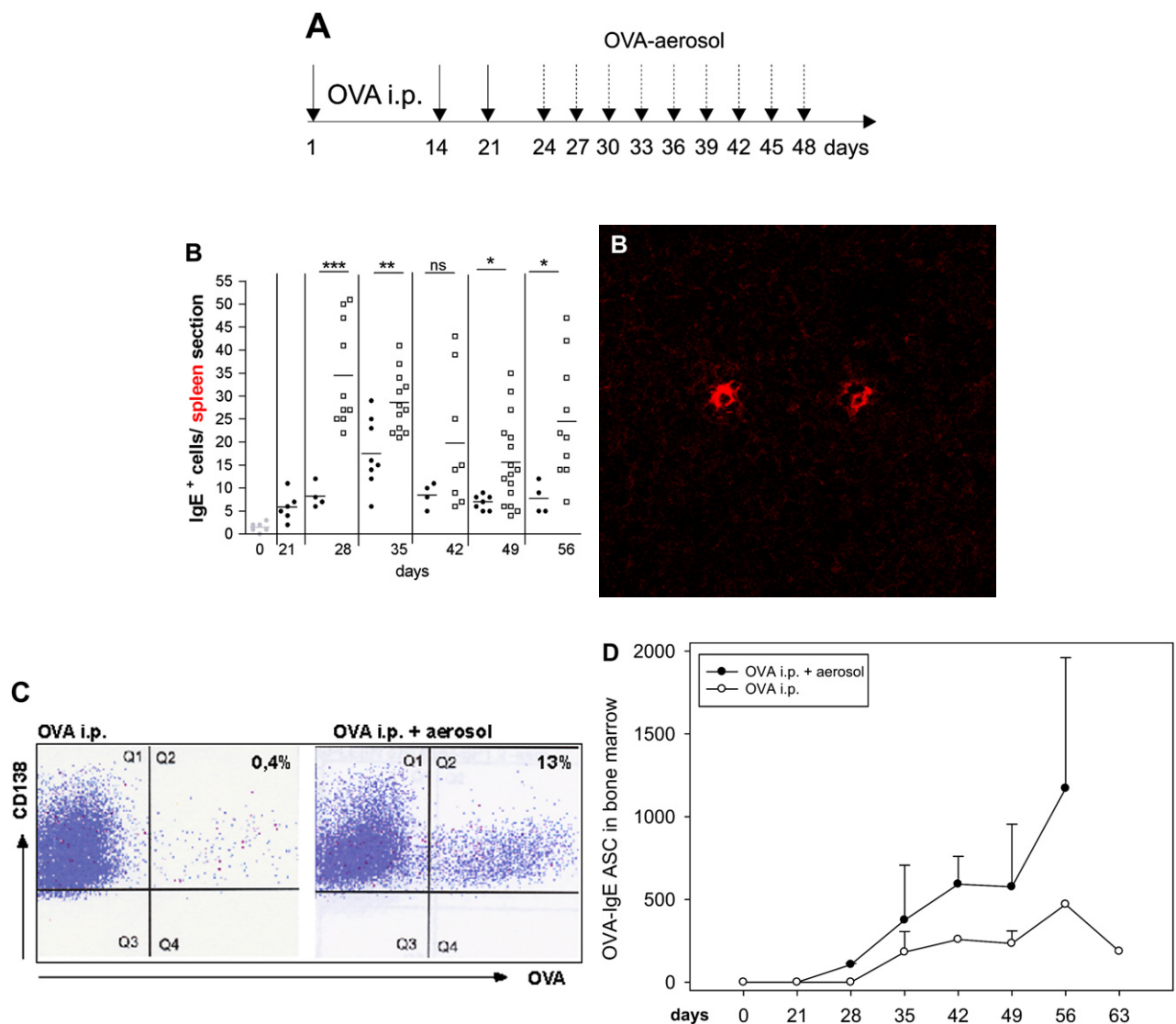


FIG 3. Ovalbumin (OVA) aerosol boosts systemic immune response. **A**, Experimental setup. **B**, Cryostaining of splenic IgE PCs with (□) and without (●) OVA inhalation. $n = 6-16$. Representative IgE PC staining. **C**, OVA-specific PCs in spleen with and without 2 weeks of OVA aerosol treatment. Gated on CD138. **D**, Increase of OVA-IgE ASCs in bone marrow during OVA aerosol by ELISpot. $n = 6$. *i.p.*, Intraperitoneal.

true for about 50% of the IgA-secreting cells. These results demonstrate that not only systemic sensitization but also mucosal challenge with allergen induce allergen-specific IgG₁, IgA, and IgE long-lived PCs that are resistant to immunosuppression by cyclophosphamide.

DISCUSSION

As the key molecule of type I hypersensitivity, IgE provides specificity for the allergen and links it to the allergic effector functions. Antibodies are secreted by plasmablasts and PCs. The fate of PCs is a subject of controversy, in particular with respect to their lifetime and persistence in the absence of allergen (see review⁹). In general, PCs were considered for a long time to be short-lived end products of B-cell differentiation, and many of them are short-lived,²⁴ although for more

than 20 years evidence has been available that IgE-secreting PCs can persist for months.^{6,25} The question whether PCs represent a distinct cellular entity of immunologic memory is of considerable therapeutic relevance, because long-lived PCs are resistant to immunosuppression, for example by cyclophosphamide,¹¹ x-ray irradiation,^{6,8} or anti-CD20 antibodies,^{26,27} whereas the chronic generation of short-lived PCs is suppressed by conventional immunosuppression.

In a murine model of type I hypersensitivity and chronic asthma,²¹ we here demonstrate that ovalbumin-specific long-lived PCs secreting IgG₁, IgA, or IgE, as induced by systemic sensitization, are present in the bone marrow, and to lesser extent in the spleen. We provide new evidence that on challenge of sensitized mice with ovalbumin-containing aerosol, the numbers of ovalbumin-specific long-lived PCs increase in the spleen and bone marrow, indicating that the local allergen contact via the mucosa

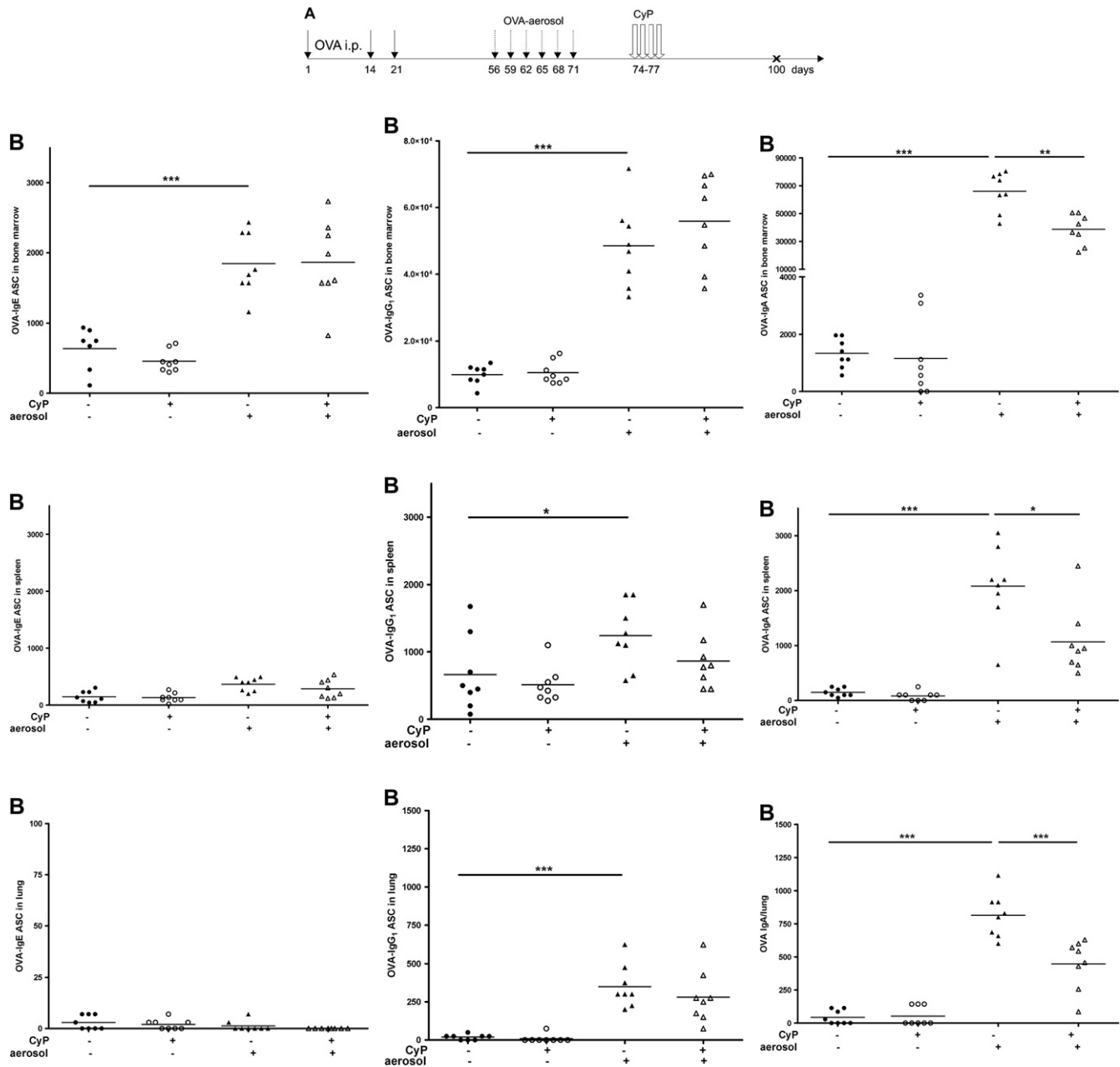


FIG 4. Survival of long-lived, ovalbumin (OVA)-specific PCs at day 100 after aerosol challenge. **A**, Experimental setup. **B**, Long-lived OVA-specific ASCs at day 100 in bone marrow, spleen, and lung, detected by ELISpot. (●) Systemically immunized, (○) systemically immunized plus cyclophosphamide (CyP)-treated (days 74-77), (▲) systemically immunized plus aerosol-challenged, (△) systemically immunized, aerosol and cyclophosphamide-treated. *n* = 8 per group. *i.p.*, Intraperitoneal.

generates long-lived, cyclophosphamide-resistant PCs at classic survival sites of systemic immune responses. Ovalbumin-specific immunoglobulin-secreting PCs also appear in the lung; however, their persistence is dependent on continued aerosol challenge.

For systemic immunization, it has been shown before that IgG-secreting cells generated in secondary lymphoid organs can survive in the bone marrow and a few also in the secondary lymphoid organs themselves.^{7,8,28,29} Here we have extended these findings by showing that ovalbumin-specific IgA-secreting and IgE-secreting cells induced by systemic immunization do migrate into the bone marrow and survive there for extended periods. As

yet, the life span of mucosal PCs has not been determined, because neither has the possible survival in the bone marrow of plasmablasts and PCs generated in mucosal immune responses.

Type I hypersensitivity is probably based on a systemic sensitization, generating an immunologic memory for the allergen. Allergic reactions are then triggered by mucosal challenge, such as exposure of airways to pollen. So far it is unclear to what extent the mucosal challenge also contributes to the allergic memory—that is, the generation of long-lived PCs secreting allergen-specific IgE. We know that allergic memory is established during sensitization. Holt et al⁶ have shown that

IgE-secreting cells survive the elimination of B cells by X-irradiation, and individuals with allergy have high titers of pollen-specific IgE in the serum even out of season.³⁰ On mucosal challenge, IgE-secreting cells have been detected in the nasal and lung mucosa of patients with hay fever and asthma.³⁰⁻³³ Allergen-specific IgE-secreting PCs were also detected in the lungs of intratracheally allergen-instilled mice.³⁴ Here we show that these IgE-secreting cells do not persist in the lung in the absence of allergen. Aerosol inhalation on 2 consecutive days is enough to induce infiltrates of lymphocytes in the lung tissue. Continued allergen-aerosol inhalation results in the presence of ovalbumin-specific IgG₁-secreting, IgA-secreting, or IgE-secreting cells. These cells vanish rapidly from the lung if the aerosol treatment is terminated. However, mucosal challenge with allergen also induces the generation of IgG₁-secreting, IgA-secreting, or IgE-secreting cells in secondary lymphoid organs such as the spleen. Allergen-specific IgE-secreting PCs generated on mucosal allergen challenge survive in spleen and bone marrow as long-lived PCs. Therefore, we provide evidence that allergic memory is enhanced by mucosal challenge with aerosolized allergen.

The presence of allergen-specific ASCs in the lung during allergen exposure does not necessarily mean that these cells are generated there. Allergen-specific plasmablasts also could have immigrated into the lung from secondary lymphoid organs in which the allergen-specific immune reaction took place, with dendritic cells probably importing the antigen.^{22,35} The spreading of mucosal allergen through the body by mucosal dendritic cells^{36,37} has been shown to be critical for induction of chronic airway inflammation in response to inhaled allergen.²²

Type I hypersensitivity cannot be cured by state-of-the-art immunosuppression. Allergen-specific IgE persists despite immunosuppressive therapy.^{18,19} IgE-secreting cells in bone marrow and to a lesser extent in spleen survive X-irradiation⁶ and, as we show here, treatment with cyclophosphamide, which has been shown to deplete short-lived PCs and to block their generation (through induction of DNA breaks in proliferating cells) but to leave long-lived PCs unaffected.¹¹ Long-lived allergen-specific IgE-secreting PCs of bone marrow and spleen will have to be targeted by novel therapies to achieve a cure for type I hypersensitivity. Such novel therapies could aim for specific surface markers of PCs and interfere with their survival signals or block migration of their plasmablast precursors from secondary lymphoid organs to the bone marrow, their prime residency.

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Key messages

- Inhalation of allergen-containing aerosol induces allergen-specific PCs not only locally in the lungs but also in secondary lymphoid tissues such as the spleen and bone marrow.
- A fraction of these PCs enters the pool of allergen-specific PCs in the bone marrow and becomes long-lived.
- Long-lived, allergen-specific PCs are resistant to cyclophosphamide.
- Termination of allergen inhalation depletes specific PCs from the lungs, but they persist in the spleen and bone marrow.

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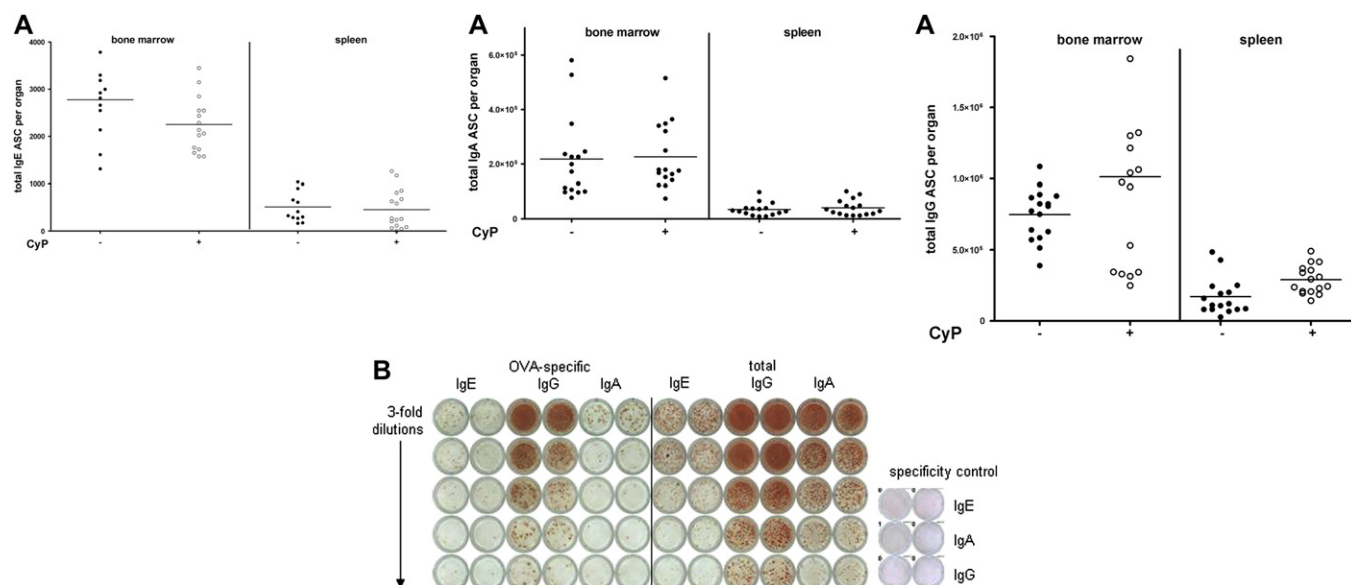


FIG E1. Cyclophosphamide-resistant ovalbumin-specific PCs in bone marrow and spleen. **A**, Survival of total immunoglobulin ASCs detected by ELISpot at day 100. **B**, Representative ELISpot.

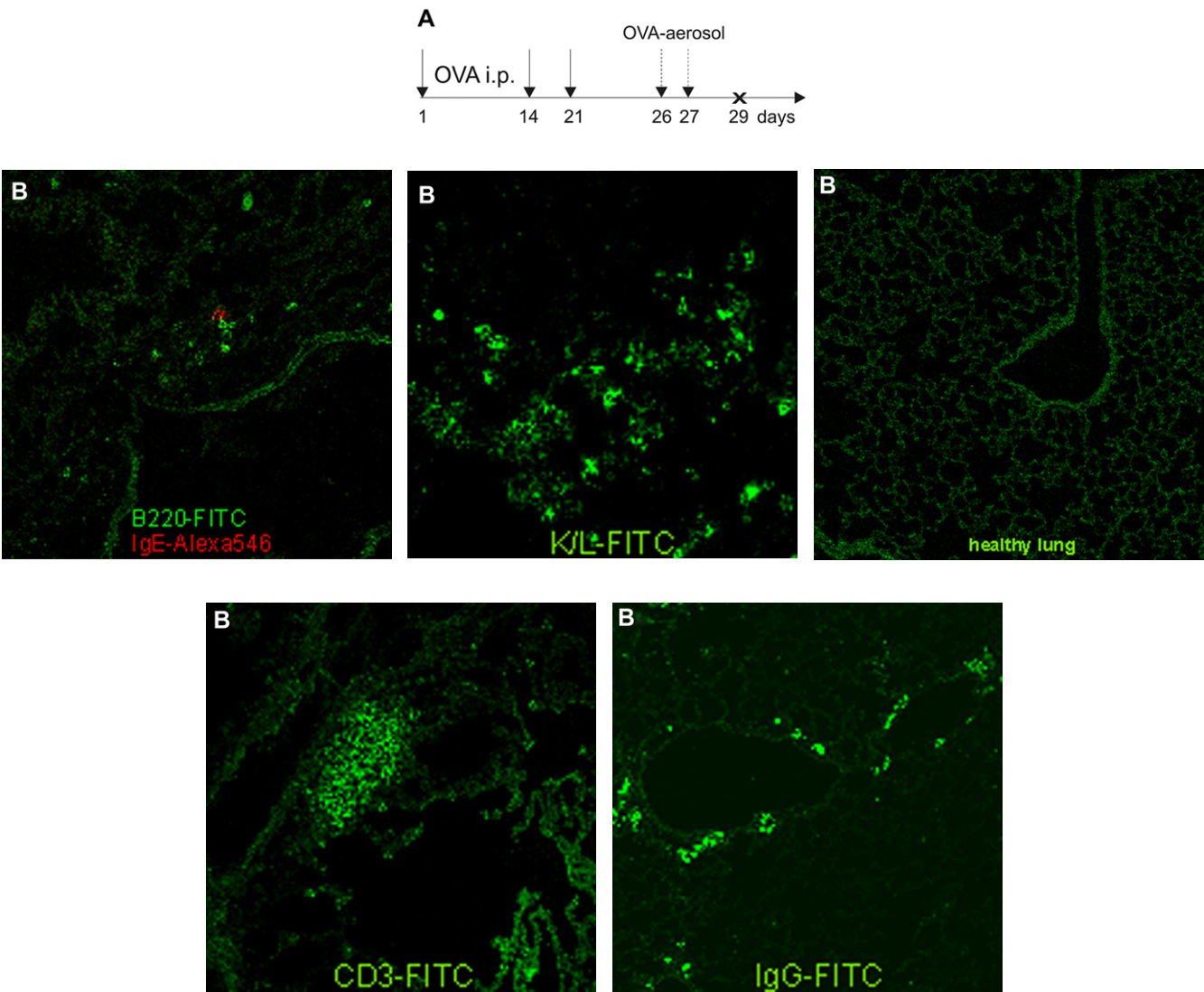


FIG E2. Immigration and kinetic of ovalbumin-specific IgG₁, IgA, and IgE ASCs. **A**, Experimental setup. **B**, Cryostaining from day 29 of T cells (anti-CD3), B cells (anti-B220), BCR on the surface or in the cytoplasm of B cells/plasmablasts/PCs (anti- κ/λ staining), IgE-positive and IgG-positive B cells and PCs (anti-IgE/anti-IgG).

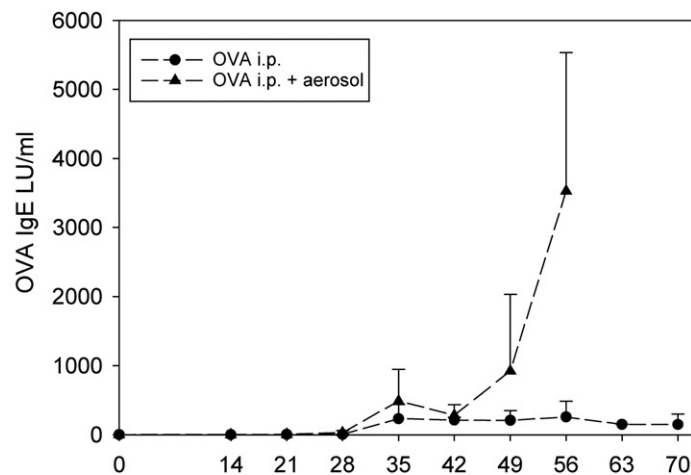
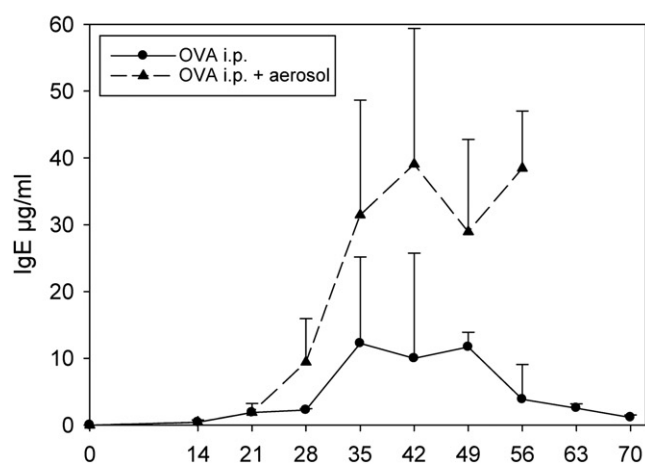


FIG E3. Ovalbumin aerosol boosts systemic immune response. Total and ovalbumin-specific ELISA. Data from 2 independent experiments. n = 12 each.

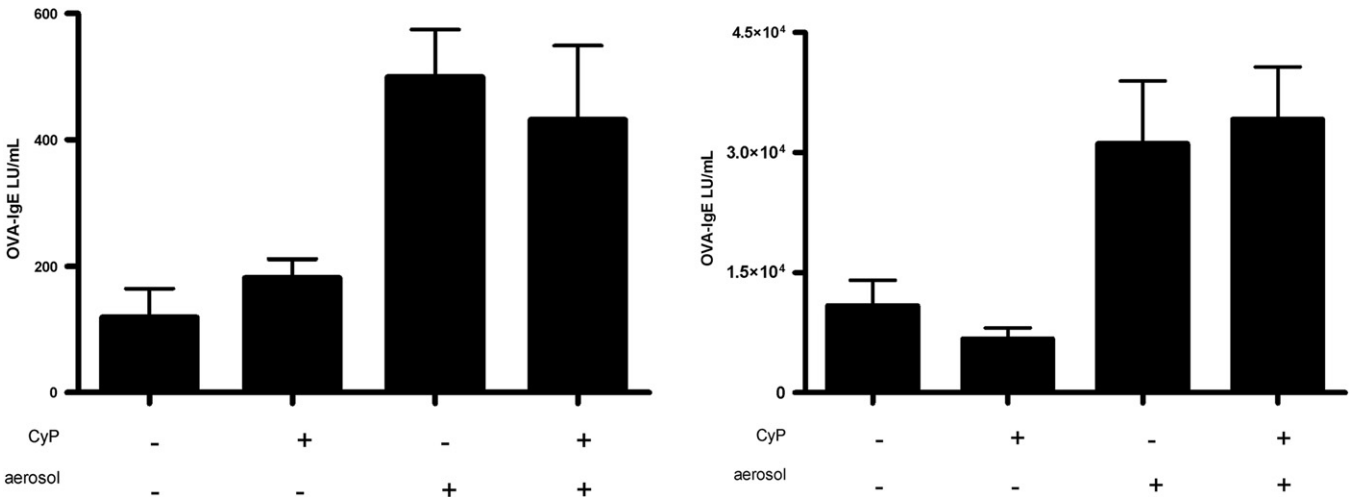


FIG E4. Survival of long-lived, ovalbumin-specific PCs at day 100 after aerosol challenge. Ovalbumin-specific IgE and IgG₁ ELISA Data from 2 independent experiments. n = 10.