Pivotal Advance: Eosinophils mediate early alum adjuvant-elicited B cell priming and IgM production

Hai-Bin Wang and Peter F. Weller

Department of Medicine, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA

Abstract: Alum, aluminum-hydroxide-containing compounds, long used as adjuvants in human vaccinations, functions by ill-defined, immunostimulatory mechanisms. Antigen-free alum has been shown to act via a previously unidentified, splenic Gr1⁺, IL-4-expressing myeloid cell population to stimulate early B cell priming. We demonstrate that the alum-elicited and -activated splenic myeloid cells are IL-4-expressing eosinophils that function to prime B cell responses. Eosinophils are the principal Gr1⁺, IL-4⁺ cells in the spleens 6 days following i.p. alum administration. Alum-elicited splenic B cell priming, as evidenced by MHC II cross-linking-mediated calcium mobilization developed in wild-type BALB/c mice, was absent in ΔdblGATA BALB/c eosinophil-deficient mice and could be reconstituted by adoptive eosinophil infusions into the eosinophil-deficient mice. Moreover, early antigen-specific IgM antibody responses in alum-antigen-immunized mice were impaired in eosinophil-deficient mice and were restored with adoptive transfers of eosinophils. Thus, eosinophils, leukocytes of the innate immune system that contain preformed cytokines, including IL-4, have novel, immunomodulatory roles in the initial priming of B cells elicited by the adjuvant alum and in the optimal early B cell generation of antigen-specific IgM. J. Leukoc. Biol. 83: 817–821; 2008.

Key Words: BALB/c · MHC II · innate immunity

INTRODUCTION

To enhance immune responses to vaccinations, aluminum compounds, including aluminum hydroxide-containing preparations (alum), have been used as vaccine adjuvants for over 70 years; to date, alum remains the principal adjuvant approved for use in human vaccines [1]. The Toll receptor-independent immunostimulatory mechanisms by which varied adjuvants, including alum, function remain uncertain [2]. For alum, one often invoked candidate mechanism, the adsorption of antigen (Ag) onto particulate alum, may be contributory but does not account for adjuvant effects of alum when administered at sites different from Ag [3] and does not account for Ag-independent effects of alum administration [4, 5]. Three decades ago, alum administration was noted to elicit T cell-dependent eosinophilia [6], but roles for eosinophils, cells of the innate immune system, in contributing to alum-adjuvant-mediated immune responses have never been defined. More recently, eosinophils have been recognized for their potential roles in innate immunity, in part, as a result of their preformed intracellular pools of multiple, readily secreted cytokine proteins, including IL-4 [7–11].

A novel candidate mechanism for alum to exert its adjuvant activity was indicated by the finding that immunization with alum, even without Ag, led to early in vivo priming of splenic B cell responses (e.g., increases in intracellular calcium following B cell surface MHC class II aggregation), as typically demonstrable in vitro with IL-4- or Ag-activated B cells [4]. This B cell “priming” activity of alum was mediated by a previously unrecognized population of Gr1⁺/CD11b⁺ IL-4-producing myeloid splenocytes that was required for in vivo priming, expansion of Ag-specific B cells, and optimal Ag-specific, early IgM antibody (Ab) production [4]. We now have evaluated whether eosinophils, as innate Gr1⁺, IL-4-producing cells, are necessary and sufficient to mediate early in vivo alum-elicited priming of B cells and initial Ag-specific IgM Ab responses.

MATERIALS AND METHODS

Mice

Wild-type (WT) BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA, USA). IL-5-transgenic BALB/c mice and eosinophil-deficient ΔdblGATA BALB/c mice were obtained from Drs. Alison Humbles and Craig Gerard (Children’s Hospital Medical Center, Harvard Medical School, Boston, MA, USA) and shipped to our animal facility from Charles River Laboratories. Mice used in this study were females at 8–10 weeks of age and were maintained in our animal facility under a protocol approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center (Boston, MA, USA).

Antibodies and reagents

The following mAb and isotype control Ab were purchased from BD PharMingen (San Diego, CA, USA): PE-rat anti-Gr1 (RB6-8C5) and PE-rat IgG2b, FITC-rat anti-IL-4 (11B11) and FITC-rat IgG1, PE-rat anti-B220 (RA3-6B2) and PE-rat IgG2a, biotin-anti-MHC class II (AMS-32.1) and 2.4G2 FcR.

1 Correspondence: Beth Israel Deaconess Medical Center, Harvard Medical School, Department of Medicine, Divisions of Allergy and Inflammation and Infectious Diseases, DA 617, 330 Brookline Avenue, Boston MA, 02215, USA. E-mail: pweller@bidmc.harvard.edu

Received June 12, 2007; revised December 10, 2007; accepted December 13, 2007. doi: 10.1189/jlb.0607392
blocking mAb. We purchased aluminum hydroxide-magnesium hydroxide (Inject Alum) from Pierce Biotechnology (Rockford, IL, USA). Indole 1 AM from Molecular Probes (Eugene, OR, USA), and IMDM from Cambrex Biosciences (Baltimore, MD, USA). Nitrophenyl (NP)15-OVA, NP18-BSA, HRP-conjugated goat anti-mouse IgM and IgG1 Abs, and IgM and IgG1 standards were from Southern Biotechnology Associates (Birmingham, AL, USA).

**Alum immunization and flow cytometric analyses**

Mice were injected i.p. with 100 μl (4.5 mg) alum, alone or with Ag, as specified. Six days after injection, mice were killed, and spleens and femurs were recovered. Spleens and marrow contents were made into single-cell suspensions. For eosinophil counts in splenocytes or bone marrow (BM) cells from mice with or without alum administration, cytocentrifuged cell smears were stained with Hema 3 differential stain (Fisher Scientific, Hampton, NH, USA). Eosinophils were counted in 600 cells per sample and expressed as percentages of total splenocytes or BM cells.

For analysis of expression of cell-surface Gr1 and intracellular IL-4 by subpopulations of splenocytes, cells in PBS containing 1% heat-inactivated FCS were incubated with PE-labeled anti-mouse Gr1 mAb for 30 min at 4°C in the dark. Upon addition of PBS/1% FCS, cells were fixed and permeabilized with a fixation/permeabilization kit (Becton Dickinson, San Jose, CA, USA) followed by a 30-min incubation with FITC-labeled anti-mouse IL-4 mAb. Appropriate fluorochrome-conjugated isotype mAbs, as noted above, were used as controls. Cells were analyzed on a Becton Dickinson LSR II flow cytometer. In some experiments, Gr1+ and IL-4+ double-positive cells were cell-sorted by flow cytometry (MoFlo, Dako, Carpinteria, CA, USA), stained with Hema 3 differential stain, and observed microscopically.

**Analyses of intracellular B cell calcium fluxes after cross-linking B cell MHC II proteins**

Six days after i.p. alum injection, spleens were harvested and made into single-cell suspensions. RBGs were lysed with hypotonic saline. Cells were resuspended at 5 × 10^6/ml in IMDM containing 2.5% FCS and incubated for 45 min at 37°C in 5 μM Indo 1 AM (1 μg/ml), PE-anti-B220 mAb (2 μg/ml), and biotinylated anti-MHC class II mAb (1 μg/ml). Cells, washed with IMDM, were resuspended at 2 × 10^6/ml for analyses on a Becton Dickinson LSR II flow cytometer. MHC class II-mediated calcium mobilization in splenic B220+ cells was triggered by addition of avidin. Ionomycin was used as a positive control stimulus. Data were analyzed by Becton Dickinson FACSDiva software and FlowJo (Tree Star, Ashland, OR, USA) software.

**Adaptive transfer of eosinophils into eosinophil-deficient BALB/c mice**

To confirm eosinophils were the critical cells mediating alum-induced in vivo B cell priming, 10 × 10^6 eosinophils, purified (>99% purity, as assessed by Hema 3 staining) from spleens of IL-5-transgenic BALB/c mice, as described previously [12, 13], were adaptively injected into the tail veins of eosinophil-deficient ΔdblGATA BALB/c mice immediately after i.p. alum challenge. Three days later, recipient mice were given second i.v. injections of 10 × 10^6 eosinophils to maintain eosinophil numbers in the spleens of reconstituted mice equivalent to those of WT mice 6 days after i.p. alum. Six days after alum administration and the first injection of eosinophils, mice were killed, and spleens were harvested and made into single-cell suspensions. MHC class II-mediated calcium mobilization in splenic B220+ cells was elicited by incubating at room temperature for 2 h with HRP-conjugated goat anti-mouse IgM and IgG1. HRP activity was detected using a peroxidase substrate kit (Bio-Rad, Hercules, CA, USA), and ODs were determined at 415 nm. Concentrations of NP-bound IgM and IgG1 were evaluated with mouse IgM and IgG1 Abs as standards that were coated directly onto the plates.

**Statistical analysis**

Results are expressed as means ± s.d. Statistical significance was calculated by Student’s t-test.

**RESULTS**

As B cell priming mediated by Gr1+/CD11b+ myeloid cells occurred 6 days after alum administration [4], we evaluated BM and splenic eosinophil numbers 6 days after i.p. Ag-free alum administration in WT BALB/c and ΔdblGATA BALB/c mice—the latter which lack eosinophils as a result of a mutation of a GATA-1 binding site in the gata-1 promoter [14, 15]. The absence of mature eosinophils in ΔdblGATA mice has been confirmed with cross-bred, transgenic IL-4-GFP 4get mice [16]. Six days after i.p. alum administration, eosinophil numbers in the BM and the spleen were increased significantly in BALB/c mice but not as expected in eosinophil-deficient ΔdblGATA BALB/c mice (Fig. 1). This enhanced BM eosinophilia after alum administration accords with that found previously in alum-administered mice, which was attributed to IL-5 released by CD34+ BM cells [5]. Moreover, we demonstrated that eosinophils were the predominant Gr1+ intracellular IL-4+ splenocytes 6 days after i.p. alum administration, as ascertained by flow cytometric analyses (Fig. 2, A–C). In addition, flow cytometric cell sorting of the Gr1+, IL-4+ splenocytes demonstrated that 80% were eosinophil-staining cells (Fig. 2D).

We next evaluated splenic B cell priming 6 days after alum administration, as assessed by fluxes in intracellular calcium.

**Measurement of serum NP-specific IgM and IgG1 antibodies by ELISA**

WT, eosinophil-deficient ΔdblGATA, or ΔdblGATA reconstituted with eosinophils per i.v. transfer (as described above) BALB/c mice were immunized i.p. with 100 μg alum-precipitated NP15-OVA and were bled from the tail vein on days 0, 3, 6, 9, and 12. NP-specific IgM and IgG1 Ab were measured by ELISA. In brief, flat-bottom, 96-well plates were coated with 10 μg/ml NP15-BSA at 4°C overnight. Coated plates were blocked with 3% BSA in PBS, and then diluted serum samples were added to individual wells and incubated at 37°C for 1 h. After washing with PBS, bound Ab were assayed by incubating at room temperature for 2 h with HRP-conjugated goat anti-mouse IgM and IgG1. HRP activity was detected using a peroxidase substrate kit (Bio-Rad, Hercules, CA, USA), and ODs were determined at 415 nm. Concentrations of NP-bound IgM and IgG1 were evaluated with mouse IgM and IgG1 Abs as standards that were coated directly onto the plates.

![Fig. 1](http://www.jleukbio.org)
elicited by MHC II cross-linking on B cells, a response not exhibited by naïve B cells but typical of IL-4- or Ag-activated B cells [4]. Splenic B220⁺ B cells, labeled with a biotinylated anti-MHC II Ab and activated by avidin-mediated cross-linking of the B cell-bound anti-MHC II Ab, exhibited B cell priming, as evidenced by increases in intracellular calcium, only following prior alum administration (Fig. 3, A and B). In marked contrast, splenic B220⁺ B cells, when derived from alum-administered, eosinophil-deficient ΔdblGATA BALB/c mice, failed to exhibit this primed rise in intracellular calcium (Fig. 3C). Thus, eosinophils, innate immune cells, apparently have a critical role in augmenting early B cell priming. To ascertain that eosinophils were indeed the critical cells mediating alum-induced B cell priming, we reconstituted eosinophil-deficient ΔdblGATA mice with eosinophils isolated and purified (99% purity) from IL-5-transgenic BALB/c mice. Adoptive reconstitutions with i.v.-delivered eosinophils fully reconstituted the alum-elicited in vivo priming (MHC II-mediated calcium mobilization) responses of splenic B cells (Fig. 3D).

Prior findings indicated that Gr1⁺ splenocytes were involved in early alum-Ag-elicited, Ag-specific IgM (but not IgG1) Ab responses within 1 week of alum-Ag administration [4]. We further investigated whether eosinophils mediated this early IgM Ab response of alum-primed B cells to thymus-dependent Ags. Alum-precipitated NP-OVA was used to i.p.-immunize WT, ΔdblGATA, or eosinophil-reconstituted ΔdblGATA mice, and serum levels of NP-specific IgM and IgG1 Ab were measured by ELISA at different time-points after immunization. Notably, in eosinophil-deficient ΔdblGATA mice, primary anti-NP IgM Ab levels on day 6 after NP-OVA/alum immunization were diminished in comparison with responses in WT mice (Fig. 4A). The reduced anti-NP IgM responses in ΔdblGATA eosinophil-deficient mice were completely recovered by i.v. reconstitution of eosinophils (Fig. 4A). In accord with the earlier report with splenic Gr1⁺ cells [4], primary anti-NP IgG1 responses were not significantly different between WT and Gr1⁺ eosinophil-deficient ΔdblGATA mice (Fig. 4B). Thus, these findings demonstrated that eosinophils play an important role in optimizing thymus-dependent, early Ag-specific IgM Ab responses.

DISCUSSION

Our results provide insights into the mechanisms of alum adjuvanticity and elucidate novel roles for eosinophils in early alum-activated priming of B cell responses, including their synthesis of Ag-specific IgM. Eosinophils, leukocytes of the innate immune system, have been recognized in mice and humans to constitute a source of cytokines, including, notably, IL-4 [8, 10, 11], as well as other cytokines that might modulate B cell responses [7]. Eosinophils are prominent leukocytes in Th2-mediated immune responses that underlie reactions to infections with helminthic parasites [17] and contribute to allergic inflammation [18]. Thus, the conventional focus on the functions of eosinophils has principally been on their roles as end-stage effector cells, potentially contributing to anthelmintic immune responses and to the immunopathogenesis of asthma and related allergic disorders. Eosinophils can also function as APCs to stimulate T cell responses pertinent to allergic and anthelmintic immune responses [12, 13, 19]. Our findings that eosinophils are the Gr1⁺, CD11b⁺, IL-4-expressing splenocytes that are required for mediating early alum-
Fig. 3. Eosinophils are required for in vivo alum-elicited, early priming of MHC II-mediated calcium mobilization responses in splenic B cells. Calcium mobilization was evaluated in WT mice (A), following i.p. alum administration (B), in eosinophil-deficient ΔdblGATA mice (C), and in ΔdblGATA mice that received adoptive reconstitutions with eosinophils by i.v. transfers on days 0 and 3 following i.p. alum administration (D). MHC II cross-linking elicited B cell calcium mobilization only in WT mice (B) and in eosinophil-reconstituted ΔdblGATA mice (D) following i.p. alum administration. Single-cell suspensions were prepared from spleens of the indicated mice 6 days after injection of i.p. alum, i.p. alum and i.v. eosinophils, or nothing. Cells were incubated in IMDM for 45 min at 37°C in the presence of Indo 1 AM, 2.4G2 FcR-blocking mAb, anti-B220-PE mAb, and biotinylated anti-MHC class II mAb. MHC class II-mediated calcium mobilization in splenic B220<sup>+</sup> cells was triggered by avidin cross-linking and monitored by B220<sup>+</sup>-gated flow cytometry (Becton Dickinson LSR II). Ionomycin was a positive control. Calcium mobilization responses in B220<sup>+</sup> B cells were analyzed by Becton Dickinson FACSDiva (left panel) or FlowJo software (right panel). Results are representative of one of three experiments.

Fig. 4. Impaired, early primary anti-NP-specific IgM, but not IgG1, Ab responses in eosinophil-deficient ΔdblGATA mice. WT, ΔdblGATA, or eosinophil-reconstituted ΔdblGATA (ΔdblGATA + Eos) BALB/c mice were immunized i.p. with 100 μg alum-precipitated NP<sub>15</sub>-OVA on Day 0 and were bled from the tail vein on days 0, 3, 6, 9, and 12. Serum IgM (A) and IgG1 (B) Ab specific for the NP-hapten were measured by ELISA. Data are means ± SD of three mice in each group. Results are from one of two independent experiments with identical findings. On day 6, serum NP-specific IgM levels were diminished significantly in eosinophil-deficient ΔdblGATA mice in comparison with WT mice and eosinophil-reconstituted ΔdblGATA mice (*, P<0.01).
elicited B cell priming provide evidence of the capacities of eosinophils to enhance B cell functions.

The significance of the early alum-elicited priming of B cell activation and enhanced Ag-specific IgM synthesis mediated by the previously unidentified IL-4+ myeloid splenocytes had been uncertain [20]. Our identification of eosinophils as the splenocytes mediating alum-elicited B cell activation may provide insights into the biologic significance of the early B cell activation and IgM synthesis. A multiplicity of immune cell responses ultimately contributes to alum adjuvant-elicited immunization responses. Eosinophil-deficient ΔdblGATA BALB/c mice did not exhibit deficits in serum anti-OVA IgE levels many months following alum-OVA i.p. immunizations [15], but comparable serum Ab levels may not detect early differences in eosinophil-mediated enhancement of B and T cell responses. Indeed, the prior finding that depletion of Gr1+ splenocytes leads to transiently impaired, Ag-specific IgM synthesis 5 days after i.p. alum-Ag immunization [4] was identical to what we found with a similar protocol in eosinophil-deficient ΔdblGATA BALB/c mice (Fig. 4A). Restoration of early IgM synthesis in eosinophil-reconstituted ΔdblGATA mice corroborated the role of Gr1+ eosinophils in mediating the early, Ag-specific IgM response.

Although roles for early Ag-specific IgM responses in the pathogenesis of allergic diseases remain to be delineated, in anhelminthic immunity, there are strong indications that early anhelminthic IgM responses are important [21, 22]. Notably, eosinophil deficits in IL-5 knockout (KO) mice abrogated development of IgM-mediated, protective immunity to larval Strongyloides stercoralis [23, 24]. Moreover, administration of eosinophils at the time of immunization in the IL-5 KO mice restored IgM-mediated, protective immunity [23, 24]. The actions of eosinophils were independent of direct eosinophil helminthotoxicity and instead, reflected a likely role for eosinophils in the induction of protective IgM Ab mediating the development of adaptive immunity to Strongyloides larvae [23, 24]. Our results directly confirm the capacity of eosinophils to enhance early IgM formation and support a role for eosinophils in facilitating initial IgM-based adaptive immune responses. Although the full consequences of the alum adjuvant-mediated, early priming of B cells by splenic IL-4+ eosinophils remain to be defined, our findings identify a broadened immunomodulatory function for eosinophils in contributing to B cell activation and early Ag-specific IgM synthesis.

ACKNOWLEDGMENTS

This work was supported by National Institute of Health grants AI20241, HL70270, and AI051645 to P. F. W. The authors have no competing financial interests.

REFERENCES


Wang and Weller Eosinophils mediate alum-elicited B cell priming 821