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# Gr1+IL-4-producing innate cells are induced in response to T<sub>H</sub>2 stimuli and suppress T<sub>H</sub>1-dependent antibody responses

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## Abstract

Alum is used as a vaccine adjuvant and induces T<sub>H</sub>2 responses and T<sub>H</sub>2-driven antibody isotype production against co-injected antigens. Alum also promotes the appearance in the spleen of Gr1+IL-4+ innate cells that, via IL-4 production, induce MHC II-mediated signaling in B cells. To investigate whether these Gr1+ cells accumulate in the spleen in response to other T<sub>H</sub>2-inducing stimuli and to understand some of their functions, the effects of injection of alum and eggs from the helminth, *Schistosoma mansoni*, were compared. Like alum, schistosome eggs induced the appearance of Gr1+IL-4+ cells in spleen and promoted MHC II-mediated signaling in B cells. Unlike alum, however, schistosome eggs did not promote CD4 T cell responses against co-injected antigens, suggesting that the effects of alum or schistosome eggs on splenic B cells cannot by themselves explain the T cell adjuvant properties of alum. Accordingly, depletion of IL-4 or Gr1+ cells in alum-injected mice had no effect on the ability of alum to improve expansion of primary CD4 T cells. However, Gr1+ cells and IL-4 played some role in the effects of alum, since depletion of either resulted in antibody responses to antigen that included not only the normal T<sub>H</sub>2-driven isotypes, like IgG1, but also a T<sub>H</sub>1-driven isotype, IgG2c. These data suggest that alum affects the immune response in at least two ways: one, independent of Gr1+ cells and IL-4, that promotes CD4 T cell proliferation and another, via Gr1+IL-4+ cells, that participates in the polarization of the response.

## Introduction

Recently, there has been a tremendous increase in the understanding of the innate immune system and its impact on specific immunity. Among the subjects that have received a great deal of attention are adjuvants, the means by which they activate innate immunity and the consequences of this activation for antigen-specific T and B cells. Adjuvants greatly improve specific immune responses by increasing the numbers of antigen-specific T and B cells, by increasing the titers of antibody made and by increasing the numbers of T and B cells that convert to memory cells (1, 2). Understanding how adjuvants work will enhance our understanding for the development of effective vaccines.

Some vaccines contain endogenous adjuvants in the form of microbial molecules. The innate immune system uses

pattern recognition receptors such as Toll-like receptors (TLRs) to broadly recognize molecules produced by invading organisms (3–5). Once engaged, directly or indirectly, by ligands, TLRs promote responses in innate cells that have the potential to affect developing adaptive immune responses. TLR ligands potently activate dendritic cells (DCs) to process antigen and up-regulate co-stimulatory molecules and cytokines to provide the required signals to activate naive T cells (6). Different adjuvants stimulate different responses from DCs and innate cells, and consequently the nature of the specific immune response to antigen is affected by the adjuvant present at the time (7).

Many vaccines contain alum, a TLR-independent adjuvant that induces T<sub>H</sub>2-polarized responses (8–10). Probably

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because of the bias in the type of T cell induced, alum markedly improves IgG1 and IgE response against co-injected antigens and suppresses production of antibody isotypes associated with T<sub>H</sub>1 responses such as IgG2a/c (10, 11). Like alum, some infectious agents make T cells differentiate into T<sub>H</sub>2 cells. Most notable among these are the helminths, including the eggs of the trematode worm, *Schistosoma mansoni*. *Schistosoma mansoni* eggs induce very powerful T<sub>H</sub>2 responses and high titers of antibody of the isotypes, which are stimulated by T<sub>H</sub>2, cytokines including IgG1 and IgE (12, 13).

Surprisingly, little is known about how alum and other T<sub>H</sub>2-driving substances are recognized by the body or how they act as adjuvants on the adaptive immune response *in vivo*. Unlike adjuvants that promote T<sub>H</sub>1 responses, T<sub>H</sub>2-promoting adjuvants do not induce DCs to produce T<sub>H</sub>1-polarizing cytokines such as IL-12 (7,14–16). Moreover, alum does not fully activate DCs *in vitro*, although it does induce caspase 1 activation and release of IL-1 $\beta$  and IL-18 cytokines by these cells, a property that may contribute to the effects of alum on CD4 cells *in vivo* (17–19). Alum may also directly enhance antigen presentation as DCs pulsed with alum-adsorbed antigen prime CD4 cells *in vitro* more effectively than DCs pulsed with soluble antigen do (20).

*In vivo*, T<sub>H</sub>2 responses can develop in the absence of IL-4 and IL-4<sup>-/-</sup> mice injected with alum-containing adjuvants are able to mount robust T<sub>H</sub>2 responses (21–24). However, in the absence of IL-4 or IL-4 receptor signaling, T<sub>H</sub>1 responses and T<sub>H</sub>1-dependent antibody isotypes also occur (23, 24). Thus, IL-4 plays an important role in suppressing T<sub>H</sub>1 development and for ultimately biasing the immune response against antigens with which alum is co-injected. In the primary response to alum-adsorbed antigen, IL-4 suppresses Tbet expression and T<sub>H</sub>1 responses as early as day 3 following injection, yet the means by which alum makes T cells differentiate into T<sub>H</sub>2 cells remain unknown (25). Although large numbers of CD4 cells themselves produce IL-4 during the primary response against alum-adsorbed antigen (26), it is clear that other innate cells in the spleen also express IL-4 in an antigen-independent manner (27). The relative importance of innate versus CD4 cell-derived IL-4 in the suppression of type 1 responses to alum-adsorbed antigen has not been dissected *in vivo*.

We have shown that increased numbers of innate, IL-4-expressing cells are detected in the spleen after exposure to alum (27). Two types of cells fall into this category, one expressing intermediate levels of Gr1 (Gr1<sup>+</sup>) and another population that is Gr1 negative (Gr1<sup>-</sup>). After alum injection, Gr1<sup>+</sup> cells are found in the red pulp of the spleen and near B cell follicles and IL-4-expressing cells in this population 'prime' B cells to respond to cross-linking of their MHC II proteins by fluxing calcium (Ca<sup>2+</sup>) rather than dying, the fate of a non-primed B cell on which MHC II has been cross-linked (27, 28). To find out whether the appearance of these cells is unique to alum exposure, we tested whether *S. mansoni* eggs, a natural T<sub>H</sub>2 stimulus, would also cause the appearance of innate IL-4-producing cells and consequent effects on MHC II-mediated signaling in splenic B cells. Here, we show that, like alum, *S. mansoni* eggs cause Gr1<sup>-</sup> and Gr1<sup>+</sup> IL-4-producing cells to appear in spleen. Like the cells induced by alum, the egg-induced Gr1-expressing

cells promote enhanced Ca<sup>2+</sup> fluxes in splenic B cells stimulated through their MHC II molecules. Alum greatly enhances expansion of antigen-specific CD4 cells to co-injected antigen while *S. mansoni* eggs do not. Thus, alum has several adjuvant effects, some of which occur by separate mechanisms. Enhanced expansion of antigen-specific CD4 cells to antigen co-injected with alum and the development of a T<sub>H</sub>2 response was independent of IL-4- and Gr1-expressing cells, but T<sub>H</sub>1 responses were enhanced when mice were treated with Gr1- or IL-4-depleting antibodies. This suggests that IL-4-expressing Gr1<sup>+</sup> cells play a role in suppressing type 1 immune responses potentiated by alum.

## Methods

### Mice

Female BALB/c and C57Bl/6 wild-type (WT) mice were obtained from The Jackson Laboratories. IL-4<sup>-/-</sup> mice on a B6 background were kindly provided by Erwin Gelfand at National Jewish Medical and Research Center. Green fluorescence protein (GFP) IL-4 reporter (4Get) mice on BALB/c and C57Bl/6 backgrounds were a gift from Richard Locksley at University of California at San Francisco Medical Center. IL-4 reporter mice and IL-4<sup>-/-</sup> were bred and maintained in a specific pathogen-free environment at the Animal Care Facility at National Jewish Biological Resource Center. All animals were housed and maintained in accordance with the research guidelines of the Institutional Animal Care and Use Committee.

### Antibodies and reagents

mAbs against Gr1 (RB6-8C5), IL-4 (11B11), IA<sup>b/d</sup> (MKD6 and D3.137), IA<sup>b</sup> (Y3P) and Fc $\gamma$ RIII/II (2.4G2) were purified from hybridoma supernatants in our laboratory. Alexa Fluor (AF) 408 was conjugated to Y3P antibodies using the AF408 protein conjugation kit (Molecular Probes). The following fluorochrome-labeled mAbs were purchased from BD Pharmingen: Allophycocyanin (APC)-Cy7 anti-CD4 (GK1.5), APC anti-CD44 (IM7), PE anti-B220 (RA3-6B2), PE-conjugated anti-IA<sup>b</sup> (AF6-120.1) and PE- and PerCP-labeled anti-Gr1 (RB6-8C5). Pacific blue (PB)-conjugated anti-Gr1 (RB6-8C5), F480 (BM9), B220 (RA3-6B2) and PE-Cy5-labeled anti-CD3 mAb (145-2C11) were purchased from eBiosciences. A647-labeled F480 (C1:A3-1) was from Serotec, PE-labeled anti-CCR3 (83101) was from R&D Systems and indo-1AM dye was purchased from Calbiochem. Alkaline phosphatase-conjugated antibodies against mouse IgG1 and IgG2c heavy chains were purchased from Southern Biotech. Chicken egg ovalbumin (OVA) protein for antibody experiments was purchased from Sigma or was from the Inject Maleimide. Activated OVA Kit (Pierce Biotechnology, Inc.) and was conjugated to a cysteine-linked 3K peptide (FEAQKAKANKAVDGGGC) (prepared at The Molecular Resources Center at the National Jewish Medical and Research Center) to form the 3K-OVA conjugate. PE-labeled IA<sup>b</sup>/3K tetramer was produced as described in our laboratory (29). *Escherichia coli* LPS was purchased from Difco. Alum preparations were performed as previously described (27). *Schistosoma mansoni* eggs were provided by Fred Lewis (Biomedical Research Institute, Rockville, MD, USA).

### Injections

To follow Gr1+IL-4+ cells and to assay B cell Ca<sup>2+</sup> responses to alum or helminth products in both BALB/c and B6 strains, WT or 4Get mice of both backgrounds were used. Age-matched females between 6 and 10 weeks of age were injected with 40 mg alum, *S. mansoni* eggs at indicated doses or 200 µl PBS intra-peritoneally (i.p.). In experiments designed to follow antigen-specific CD4 and antibody responses, 10 µg 3K-OVA or OVA protein either prepared in PBS, in combination with *S. mansoni* eggs, or precipitated with alum was injected i.p. into WT or IL-4<sup>-/-</sup> B6 mice. For depletion of Gr1+ cells or neutralization of IL-4 *in vivo*, mice were given 1 mg anti-Gr1 mAb, 500 µg anti-IL-4 mAb or purified rat IgG (Jackson ImmunoResearch Laboratories, Inc.) by intravenous injection 1, 3 and 5 days after immunization.

### Cell preparation

Spleens and peritoneal cells were harvested into cold balanced salt solution (BSS) at indicated times after injection. In some experiments, peritoneal cells were collected by peritoneal lavage in BSS. Blood was collected in some experiments and sera were stored at -20°C until analysis by ELISA. Splenocytes were processed into single-cell suspensions using nylon mesh and red cells lysed with ammonium chloride. Nucleated cells were enumerated using a Coulter counter.

### Flow cytometry

For analysis of Gr1+IL-4-expressing cells, peritoneal cells from challenged IL-4 reporter mice were stained with reagents against F480, cKit and Gr1 and splenocytes were stained with reagents against CD4, CD8, B220 and Gr1. For analysis of B cell Ca<sup>2+</sup> responses in response to MHC II ligation, splenocytes [10<sup>7</sup> ml<sup>-1</sup> in (Ca<sup>2+</sup> free) Supplemented Minimum Essential Media (SMEM) + 2.5% fetal bovine serum (FBS)] were incubated with 10% 2.4G2 hybridoma supernatant, biotinylated anti-MHC II antibody, 5 µM indo-1AM dye and fluoro-chrome-labeled mAb against B220 for 45 min (37°C in 5% CO<sub>2</sub>). Cells were washed in SMEM-2.5% FBS, re-suspended to 10<sup>7</sup> cells ml<sup>-1</sup> and allowed to rest 1 h at room temperature (RT) prior to data acquisition on an LSR flow cytometer using Cell Quest software (Beckton Dickinson). To cross-link MHC II molecules, 5 µg ml<sup>-1</sup> extravidin (Sigma) was added to each sample. Kinetic and population analysis of intracellular Ca<sup>2+</sup> responses was performed using Flow-Jo software (Tree Star, Inc.). To follow IA<sup>b</sup>/3K-specific CD4 cells, spleens were harvested 9 days after immunization. Splenocytes (1.25 × 10<sup>7</sup>) were added to the wells of 96 round well plates in 25 µl complete tumor medium and stained with 3K/IA<sup>b</sup> tetramers as described (29). APC-Cy7 anti-CD4, PB anti-B220, PB anti-F480, Alexa405 anti-IA<sup>b</sup> and APC anti-CD44 were added at 4°C for 20 min and cells were washed and analyzed immediately. Flow cytometry data were acquired on a Cyan cytometer using Summit software (Cytomation). Data were analyzed using Flow-Jo software.

### ELISAs

Ninety-six-well Immulon plates (Thermo) were coated with OVA protein at 100 µg ml<sup>-1</sup> in PBS. Plates were washed us-

ing ELX405 Auto Plate Washer (BioTek Instruments) and then blocked with 10% FCS/PBS for 2 h at RT. Plates were washed and antibody serum samples diluted in 10% FCS/PBS were added to the plates. To determine relative units, we used a positive control pooled serum sample from B6 and BALB/c mice that contained OVA-specific T<sub>H</sub>2 and T<sub>H</sub>1 isotypes. The samples were incubated overnight at 4°C. Plates were washed and alkaline phosphatase-conjugated anti-IgG1 (X56) or anti-IgG2a/c (R19-15) or biotinylated anti-IgE (R35-119) (PharMingen) detection antibodies were added for 2 h at RT. For IgE, alkaline phosphatase-conjugated streptavidin was added for 1 h at RT. Substrate (*p*-nitrophenyl phosphate) diluted in glycine buffer was added to each well and 405 nm absorbance values were collected on EI×808 microplate reader.

### Statistics

Data are presented as indicated in the figure legends and statistical significance determined using Student's two-tailed *t*-test with GraphPad Prism software version 4.

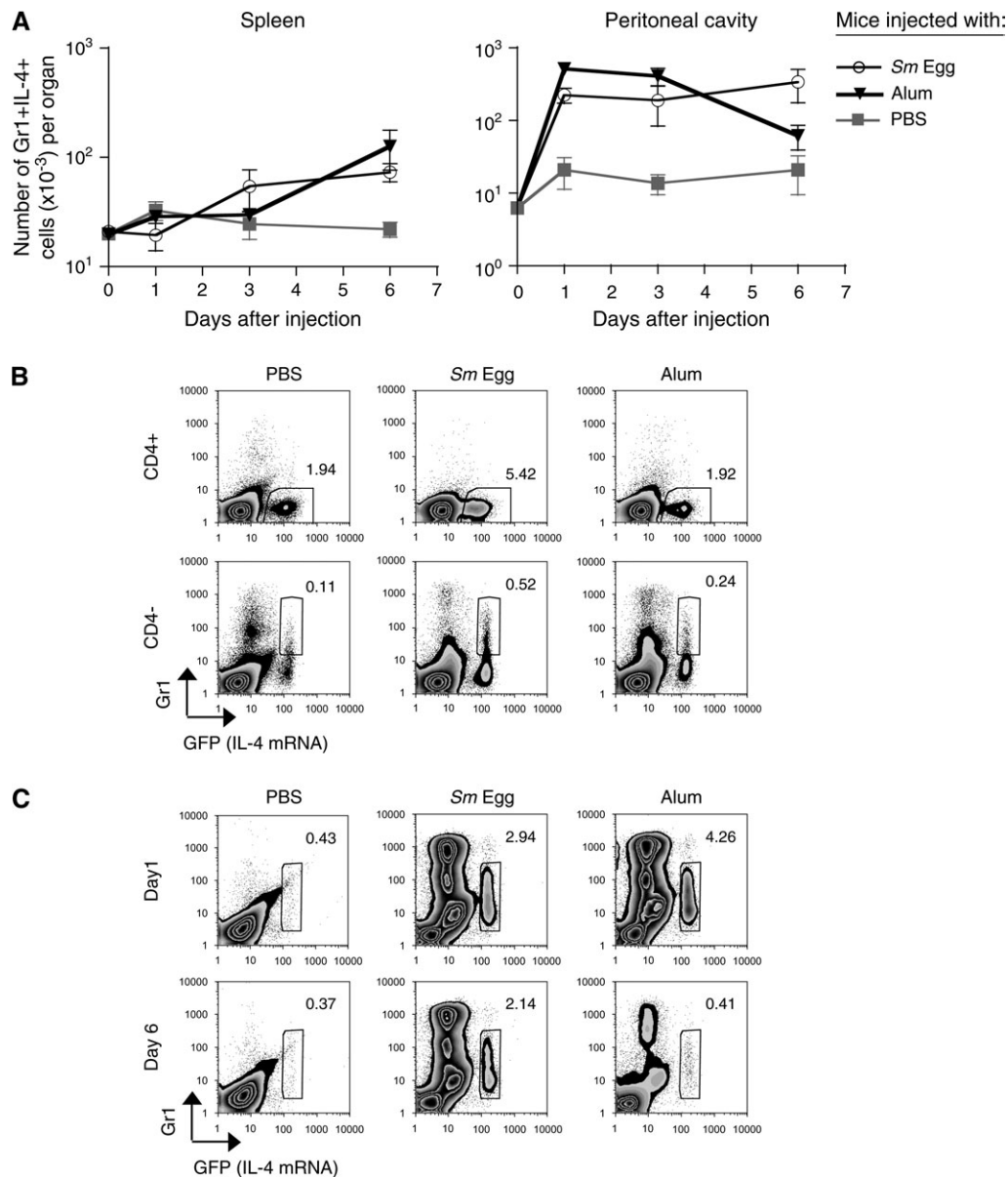
### Results

#### *A natural T<sub>H</sub>2-driving stimulus induces accumulation of similar IL-4-expressing cells as alum*

In mice injected with the adjuvant, alum, a population of innate cells expressing intermediate levels of Gr1 (Gr1+) and IL-4 accumulates in the spleen, reaching a peak 6 days after alum administration (27). To determine whether a similar population of cells accumulates in the spleen following exposure to a 'natural' material that is known to induce T<sub>H</sub>2 response, IL-4 transcriptional reporter (4Get) mice (30) were injected with *S. mansoni* eggs, alum or PBS. CD4-positive and CD4-negative cells in their spleens and the site of injection, the peritoneal cavities, were analyzed for GFP and Gr1 expression at various times thereafter. 4Get animals were used because they express GFP under the control of the IL-4 promoter and thus GFP expression by cells from these animals is an indication that their IL-4 locus is open (30).

Injection of either *S. mansoni* eggs or alum induced the appearance of increased numbers of GFP+Gr1+ cells in both locations over the time analyzed. The kinetics of accumulation of GFP+Gr1+ cells in the spleen was much later than the accumulation of GFP+Gr1+ cells in the peritoneal cavity. In the peritoneal cavity, the peak accumulation of GFP+Gr1+ cells occurred on day 1, while in the spleen accumulation of these cell peaked on day 6 [Fig. 1A and (27)]. In the spleens, 6 days after injection, both alum and *S. mansoni* eggs induced accumulation of CD4-negative cells that co-expressed GFP (Fig. 1B, lower three panels). These cells were found to be negative for B220, CD4 or CD8, and in the spleen consisted of two different populations, a Gr1-negative population and a Gr1+ population (Fig. 1B and data not shown).

In mice injected with *S. mansoni* eggs, an increased percentage of splenic CD4+ cells co-expressed GFP compared with mice injected with PBS or alum particles alone (Fig. 1B, top three panels). This is consistent with the known ability of *S. mansoni* egg components to drive T<sub>H</sub>2 responses against



**Fig. 1.** *Schistosoma mansoni* eggs, like alum, induce the appearance of Gr1+IL-4+ innate cells. (A) 4Get, IL-4 indicator mice were injected i.p. with alum, *S. mansoni* eggs or PBS. At the indicated times, cells were isolated from the spleens and peritoneal cavities of the animals and stained with antibodies against CD4, CD8, B220 and Gr1 (splenocytes) or F480, cKit and Gr1 (peritoneal cells). Shown are the means and standard errors of numbers of B220-, CD4- and CD8-negative splenocytes or F480- and cKit-negative peritoneal cells that were Gr1+IL-4+ (GFP+) isolated from three identically treated animals per group. (B) Expression of Gr1 and GFP (IL-4 mRNA) on CD4-negative or CD4-positive spleen cells 6 days after i.p. injection with alum, *S. mansoni* eggs or PBS. Numbers on graphs indicate the percentage of gated CD4- or CD4+ cells that fall within the gates on each individual FACS plot. (C) FACS plots show Gr1 and GFP (IL-4 mRNA) expression on F4/80- cKit- peritoneal cells from mice injected 1 or 6 days previously with PBS, *S. mansoni* eggs or alum. Numbers on graphs indicate the percentage of F4/80- cKit- cells that fell within the gate on each plot for the sample shown. For (B and C), data are representative FACS plots from three individual mice per group. Forty milligrams of alum or  $10^4$  *S. mansoni* eggs were used for injections in this experiment. Data are representative of three independent experiments.

intrinsic schistosome antigens. Although alum also is known to drive  $T_H2$  responses, no antigen was included in these experiments and so the alum was not expected to affect T cells. In addition, GFP+ CD4+ cells did not co-express Gr1 (Fig. 1B).

Examination of cells at the site of injection after i.p. injection of alum or parasite eggs revealed that both stimuli induced accumulation of IL-4-expressing cells in the

peritoneal cavity, as they did in the spleen. In the peritoneum, the cells were relatively homogeneous since >95% of these cells co-expressed Gr1 (Fig. 1C) and when sorted were found to be >95% eosinophils (data not shown). In contrast, only 50% of the Gr1+IL-4+ population in the spleen were eosinophils while the remaining 50% have a mononuclear morphology (27).

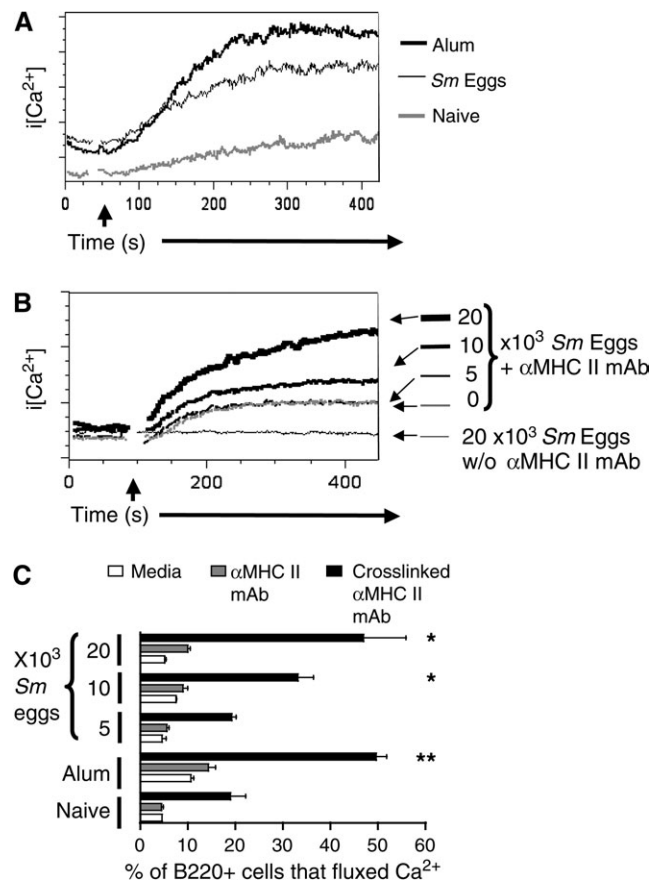
Exposure to  $T_H2$ -inducing stimuli increased MHC II-mediated signaling in a large percentage of splenic B cells

Aggregation of antigen receptors on B cells or exposure of B cells to IL-4 causes Ig $\alpha$ Ig $\beta$  signaling chains to associate with MHC II and thus enables MHC II, after cross-linking, to transduce signals leading to mobilization of intracellular  $Ca^{2+}$  and proliferation of the cells involved (28, 31). Recently, we discovered that injection of alum into mice causes up-regulation of MHC II expression and enhanced  $Ca^{2+}$  signals after cross-linking of these receptors on B cells in their spleens, a phenomenon which improves early IgM responses to co-injected antigens (27). Since anti-Gr1 depletion experiments implicated the Gr1+IL-4+ cells in this phenomenon, we wondered whether the Gr1+IL-4+ cells induced by *S. mansoni* eggs would have similar effects. Thus, we injected C57Bl/6 (B6) mice i.p. with *S. mansoni* eggs or alum and, 6 days later, tested the responses of the B cells in their spleens to cross-linking of the MHC II proteins on their surfaces. Like the B cells from alum-injected mice, B cells from *S. mansoni* egg-injected mice contained elevated concentrations of cytoplasmic  $Ca^{2+}$  in response to MHC II cross-linking when compared with B cells from untreated mice (Fig. 2A). In each mouse, we also compared the percentage of B220+ cells that fluxed  $Ca^{2+}$  at the peak of the response to MHC II cross-linking with the percentage that fluxed  $Ca^{2+}$  in the absence of stimulus. In mice that had been injected 6 days prior with alum or *S. mansoni* eggs, there was an up to 2.5-fold increase in the percentage of B cells fluxing  $Ca^{2+}$  than in untreated control mice (Fig. 2C). We did a dose response and found at least  $10^4$  *S. mansoni* eggs had to be injected to observe the effects on MHC II-mediated signaling (Fig. 2B). Thus, the polyclonal effects on B cells that we have previously found to accompany the presence of enhanced numbers of IL-4-expressing cells in mice exposed to alum also occurred in mice exposed to helminth products.

Depletion of Gr1+ or IL-4-expressing cells eliminated the in vivo effects of *S. mansoni* eggs or alum exposure on splenic B cell signaling

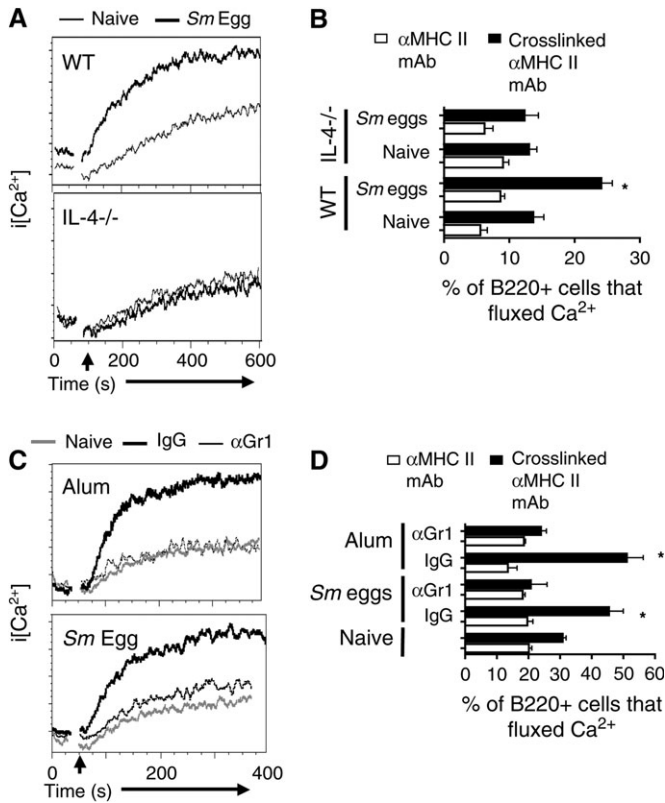
We have previously shown that IL-4 neutralization blocks the ability of alum to promote enhanced MHC II signaling in splenic B cells. To find out whether IL-4 is required for these polyclonal effects on B cells in response to *S. mansoni* eggs, IL-4 $-/-$  and WT B6 mice were injected with *S. mansoni* eggs i.p. and MHC II-mediated  $Ca^{2+}$  responses in splenic B cells were analyzed 6 days later. In contrast to the high MHC II-induced  $Ca^{2+}$  flux that occurred in B cells from *S. mansoni* egg-injected WT mice, intracellular  $Ca^{2+}$  levels increased only slightly following MHC II aggregation on B cells from egg-injected IL-4 $-/-$  mice (Fig. 3A) and *S. mansoni* egg injection did not increase the percentage of B cells that fluxed  $Ca^{2+}$  in IL-4 $-/-$  mice as it did in WT mice (Fig. 3B). These data suggest that helminth products, like alum, mediate polyclonal effects on B cells via an IL-4-dependent mechanism.

Alum cannot promote MHC II-mediated signaling in B cells if Gr1+ cells are removed with a Gr1-depleting antibody (27). To determine whether a Gr1-expressing cell type is involved in *S. mansoni* egg-induced effects on MHC



**Fig. 2.** MHC II-mediated  $Ca^{2+}$  responses in B cells are enhanced after exposure to  $T_H2$  stimuli. (A) Spleen cells were isolated from mice injected 6 days previously with nothing (gray histogram),  $10^4$  *Schistosoma mansoni* eggs (bold black histogram) or alum (fine black line histogram) and stained as described in Methods. MHC II molecules on the surface of the cells were cross-linked with biotinylated anti-MHC II followed by avidin. The time of addition of avidin is indicated on the figure by the arrow. Shown are the changes in intracellular  $Ca^{2+}$  levels in B220+ cells. (B) Mice were given the indicated numbers of *S. mansoni* eggs. Six days later, spleen cells from the mice were stained and their MHC II molecules cross-linked as described in Fig. 1(A). Shown are the changes in intracellular  $Ca^{2+}$  induced by the cross-linking. Data in (A and B) are representative of three mice per group in a single experiment. (C) Shown are the percentage of B220+ cells undergoing  $Ca^{2+}$  flux in the absence of any stimulation (white bars), after addition of anti-MHC II mAb but before avidin-mediated cross-linking (gray bars) and during the peak of the response after addition of avidin (solid bars) in mice injected 6 days previously with *S. mansoni* egg, alum or PBS. Means  $\pm$  standard errors for values from three mice are shown and asterisks indicate results that differed from those in naive mice with  $P < 0.05$  (\*) or  $P < 0.01$  (\*\*). Data in this figure are from a representative experiment of three.

II-mediated  $Ca^{2+}$  responses in B cells, we injected *S. mansoni* eggs into B6 mice that had been treated either with anti-Gr1 antibodies or with control rat IgG. We observed that after treatment with anti-Gr1 antibodies, MHC II cross-linking neither induced  $Ca^{2+}$  fluxes nor increased the percentage of B cells that increased their levels of intracellular  $Ca^{2+}$  (Fig. 3C and D). Thus, Gr1+IL-4+ cells probably play a role in polyclonal B cell effects induced by *S. mansoni* eggs, just as they do during responses to alum.



**Fig. 3.** The effects of *Schistosoma mansoni* egg or alum on B cells are dependent upon IL-4- and Gr1-expressing cells. (A) WT and IL-4<sup>-/-</sup> mice were injected i.p. with nothing (fine black line) or *S. mansoni* eggs (bold black lines). Six days later, spleen cells were isolated and stained and MHC II cross-linked as described in Fig. 2. Shown are the changes in intracellular Ca<sup>2+</sup> levels in B220<sup>+</sup> cells after MHC II cross-linking. (B) WT or IL-4<sup>-/-</sup> mice were injected with nothing or *S. mansoni* eggs and analyzed 6 days later as described in (A). Graphs show the percentages of splenic B220<sup>+</sup> cells from naive, *S. mansoni* egg-injected WT or IL-4<sup>-/-</sup> mice that fluxed Ca<sup>2+</sup> before (white bars) and during the peak (black bars) of the response to avidin-mediated cross-linking of their MHC II molecules. (C) Mice were injected i.p. with nothing (gray lines), alum or *S. mansoni* eggs (black lines). Alum- or *S. mansoni* egg-injected mice were treated with either control rat IgG (bold black lines) or anti-Gr1 mAb (fine black lines). Six days after alum or *S. mansoni* egg injection, spleen cells were isolated and their MHC II molecules cross-linked as described in Fig. 2. Shown are the changes in intracellular Ca<sup>2+</sup> levels in B220<sup>+</sup> cells after MHC II cross-linking. (D) Mice were injected with nothing, *S. mansoni* eggs or alum, later with control rat IgG or anti-Gr1 and their spleen cells analyzed 6 days after injection of *S. mansoni* eggs or alum as described in (C). Shown are the means  $\pm$  standard errors of the percentages of B220<sup>+</sup> cells that had increased levels of intracellular Ca<sup>2+</sup> before (white bars) or after (black bars) cross-linking of their MHC II proteins. Asterisks indicate a group with a percentage of cells fluxing Ca<sup>2+</sup> after MHC II cross-linking that was significantly different from that observed in cells from naive mice ( $P < 0.05$ ). Results in (A and C) are representative of two individual experiments and data in (B and D) are from a representative experiment of three.

#### *Alum promotes expansion of CD4 cells to co-injected antigen but S. mansoni eggs do not*

The enhanced ability of B cells to signal downstream of MHC II engagement could improve their ability to present antigen, thereby affecting the CD4 T cell response. To test

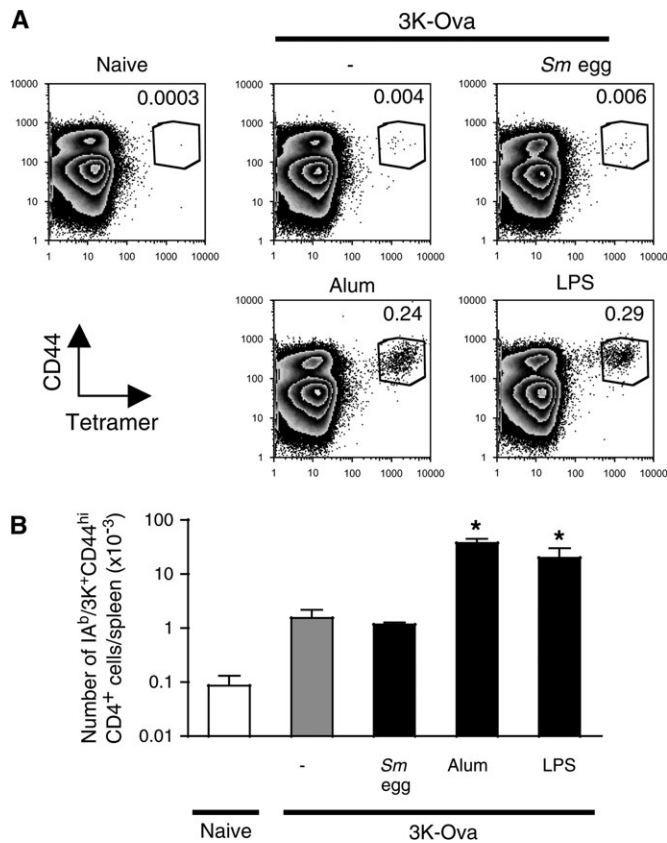
this, we decided to compare the effects of alum and *S. mansoni* eggs on T cell priming. In the past, such experiments have been done by following the consequences of T cell activation, such as helper function or cytokine release. However, here we wanted to measure T cell numbers. Such experiments can be performed by transfer of T cells expressing transgenic (Tg) TCRs, but artifacts have been associated with the use of fairly large populations of these cells, bearing a single TCR. In fact, we have noticed that such cells may not be good representatives of endogenous host T cells. For example, if too many TCR Tg T cells were transferred, they did not all increase their surface CD44 and differentiate into T<sub>H</sub>2, in mice given antigen + alum. To circumvent these problems, we decided to follow endogenous T cells specific for IA<sup>b</sup> bound to the 3K peptide, a combination for which excellent staining tetramers can be made. To allow us to follow both antibody and CD4 T cell responses, the 3K peptide was conjugated to OVA (3K-OVA). This combination still allowed presentation of 3K to T cells and OVA to B cells, since 3K-OVA induced the rapid expression of CD69 on IA<sup>b</sup>/3K-specific T cells *in vitro*, induced anti-OVA antibodies, detected by ELISA, in immunized mice and could be used to detect anti-OVA antibodies in capture ELISA reactions (data not shown).

We have estimated that naive B6 mice contain 50–100 T cells specific for IA<sup>b</sup>/3K (M. MacLeod, unpublished data). The numbers of IA<sup>b</sup>/3K-specific CD4 T cells peak 9 days after injection of 3K-OVA when either alum or LPS was used as adjuvant (M. MacLeod and A. McKee, unpublished data). Administration of 3K-OVA bound to alum, rather than free 3K-OVA alone, greatly increased the numbers of 3K-specific T cells in the spleen at this time (Fig. 4). We were surprised to find that alum promoted priming of CD4 cells as well as LPS, a TLR agonist often used as an adjuvant (32, 33). In contrast, there was no difference in the percentage or total number of IA<sup>b</sup>/3K-specific CD4 cells detected in the spleens of mice injected with 3K-OVA in the presence or absence of *S. mansoni* eggs (Fig. 4). Thus, the bystander enhancement of MHC II-mediated signaling in B cells, which occurs in the *S. mansoni* egg-injected animals, is not sufficient for enhanced CD4 priming to co-injected antigen. This implies that alum greatly promotes CD4 priming through another mechanism.

#### *Enhanced CD4 priming by alum adjuvant is independent of Gr1+ and IL-4-expressing cells*

Both alum and helminth eggs promoted recruitment of IL-4-expressing innate cells in the spleen, but only alum enhanced CD4 priming to co-injected antigen. To test directly whether or not the presence of Gr1-expressing cells affects CD4 priming, we injected B6 mice with 3K-OVA in the presence or absence of alum, treated them with either a Gr1-depleting antibody or control rat IgG and determined the number of IA<sup>b</sup>/3K-specific CD4 T cells in the spleen 9 days later. We found a similar number of activated IA<sup>b</sup>/3K-specific CD4 T cells in the spleens of 3K-OVA/alum-injected mice whether or not the mice were treated with the Gr1-depleting antibody (Fig. 5A and B).

This experiment did not test the need for IL-4 in T cell priming since Gr1<sup>+</sup> cells are not the only cells induced by



**Fig. 4.** Alum but not *Schistosoma mansoni* eggs promotes enhanced CD4 responses to co-injected antigen. (A) B6 mice were injected with 3K-OVA protein combined with nothing (-), *S. mansoni* eggs, alum or 7  $\mu$ g LPS and splenocytes were stained with antibodies against B220, F480, CD44, CD4 and IA<sup>b</sup>/3K tetramers 9 days later. Plots show staining with anti-CD44 and IA<sup>b</sup>/3K tetramers on CD4-positive, F4/80- B220- spleen cells. Numbers on graphs indicate the mean percentages of CD4 cells that were CD44<sup>hi</sup> IA<sup>b</sup>/3K tetramer positive for the sample shown. Data shown are representative of three identically treated mice in each group. Cells that stained as CD44<sup>hi</sup> and with IA<sup>b</sup>/3K tetramer in naive mice illustrate the level of background staining in these experiments. (B) Shown are the means  $\pm$  standard errors of the numbers of IA<sup>b</sup>/3K-specific CD44<sup>hi</sup> CD4<sup>+</sup> T cells detected per spleen for three mice per group. Asterisks indicate samples in which the numbers of IA<sup>b</sup>/3K-specific T cells that were detected were significantly different from the numbers detected in mice injected with 3K-OVA alone ( $P < 0.05$ ). Data are from a representative experiment of three.

alum that can produce the cytokine (Fig. 1A). Thus, to examine this issue, WT or IL-4<sup>-/-</sup> mice were injected with 3K-OVA in the presence or absence of alum and the numbers of IA<sup>b</sup>/3K-specific CD4 T cells counted 9 days later. The absence of IL-4 had no effect on the percentage or number of IA<sup>b</sup>/3K-specific cells detected in the spleens of mice injected 9 days previously with 3K-OVA/alum (Fig. 5C and D). Moreover, the fold increase of such cells in mice that had been injected with 3K-OVA/alum versus those in mice that received 3K-OVA alone was similar between the two types of mice. These results show that, as previously reported, the ability of alum to increase T cell primary responses does not involve IL-4.

#### Depletion of Gr1+ cells results in increased levels of T<sub>h</sub>1-associated antibody isotypes

Alum increases the primary T cell response to antigen. However, even on the peak day, animals given alum plus 3K-OVA still contained only rare cells specific for IA<sup>b</sup>/3K. Because of this, it has been difficult to assess by intracellular staining whether the cells induced in such experiments are T<sub>h</sub>1 or T<sub>h</sub>2 in nature. Therefore, to examine the effects of Gr1 depletion on the phenotype of the primed CD4 cells, we tracked antigen-specific CD4 cells in alum-injected 4Get mice. We found that the percentage of IA<sup>b</sup>/3K-specific cells that had the potential to make IL-4 was significantly larger in animals given antigen plus alum (Fig. 6A). This effect combined with the strong effect of alum on CD4 expansion (Fig. 4) resulted in greatly enhanced numbers of IA<sup>b</sup>/3K-specific, IL-4-expressing CD4 cells in 3K-OVA/alum-injected mice (Fig. 6B). Moreover, treatment of immunized mice with anti-Gr1 did not affect the percentage or total number of IA<sup>b</sup>/3K-specific cells capable of making IL-4 (Fig. 6A and B).

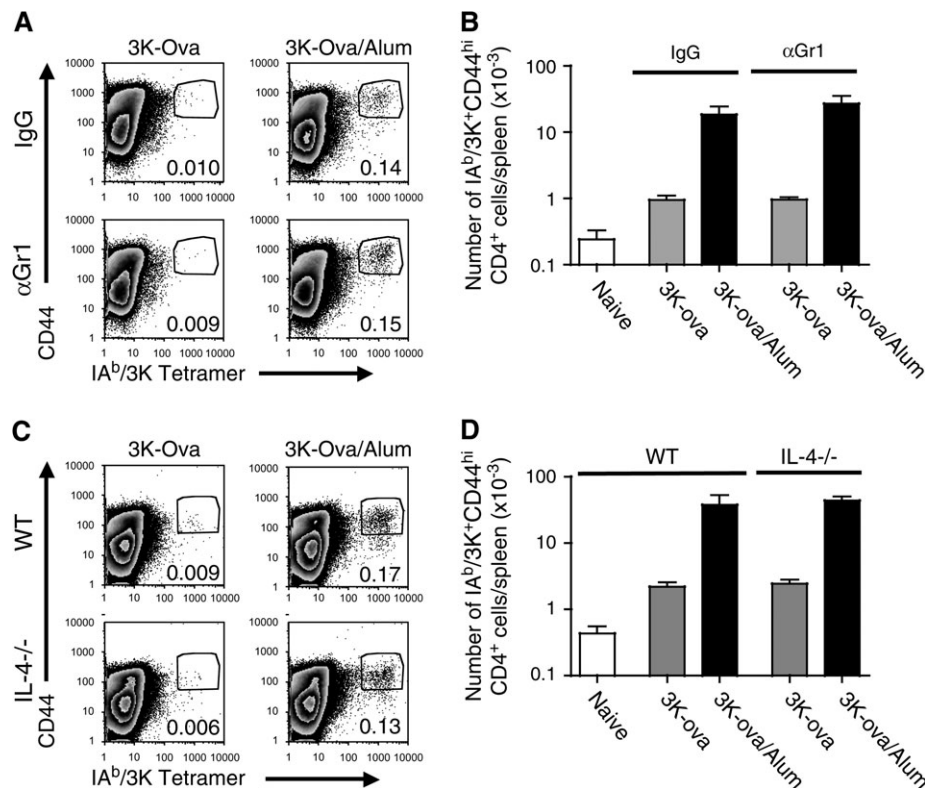
The phenotype of T cells affects the isotypes of antigen-specific antibodies produced during immune responses. T<sub>h</sub>2-driven responses are characterized by high levels of IgE and IgG1 while responses involving T<sub>h</sub>1 are characterized by elevated production of IgG2a/c isotypes. Mice injected with OVA/alum contained high levels of IgG1 anti-OVA antibodies compared with mice that received OVA alone (Fig. 6C). These titers were not affected by treatment with anti-IL-4 antibodies or removal of Gr1+ cells during the first week of the primary response. We did not observe OVA-specific IgE levels in these B6 mice at this time point.

As expected, OVA antigen given with CFA induced large amounts of IgG2c anti-OVA (Fig. 6D). Not unexpectedly, given the T<sub>h</sub>2 phenotype of the T cell response, titers of IgG2c anti-OVA antibodies were only slightly elevated in mice given OVA/alum versus OVA alone (Fig. 6D). In this case, however, as has been shown in IL-4-deficient mice (23), anti-IL-4 dramatically increased the ability of OVA to induce IgG2c antibodies, and surprisingly anti-Gr1 had a similar, although not quite so dramatic effect (Fig. 6D). We found a similar effect of Gr1 depletion in mice that had been immunized with OVA adsorbed to a commercial form of alum, Alhydrogel<sup>TM</sup>. In addition, we saw a similar effect of Gr1 depletion on antibody isotypes in BALB/c mice (Fig. 6E). While OVA-specific IgG2a levels were elevated in mice treated with Gr1-depleting antibody, OVA-specific IgG1 and IgE levels in these mice were unaffected by Gr1 depletion (Fig. 6E). In combination with the T cell results (above), these data suggest that the ability of alum to preclude T<sub>h</sub>1-like responses depends, to some extent, on Gr1+ cells and IL-4 production.

#### Discussion

It is known that helminth products and alum have overtly similar effects on the immune response. Both stimulate strong T<sub>h</sub>2 responses and elicit high levels of IgE and IgG1 against associated antigens (10, 12, 13). Others have therefore suggested that the two substances may promote T<sub>h</sub>2 responses in a similar way (34). The ability of these two quite different materials to have similar effects on the innate





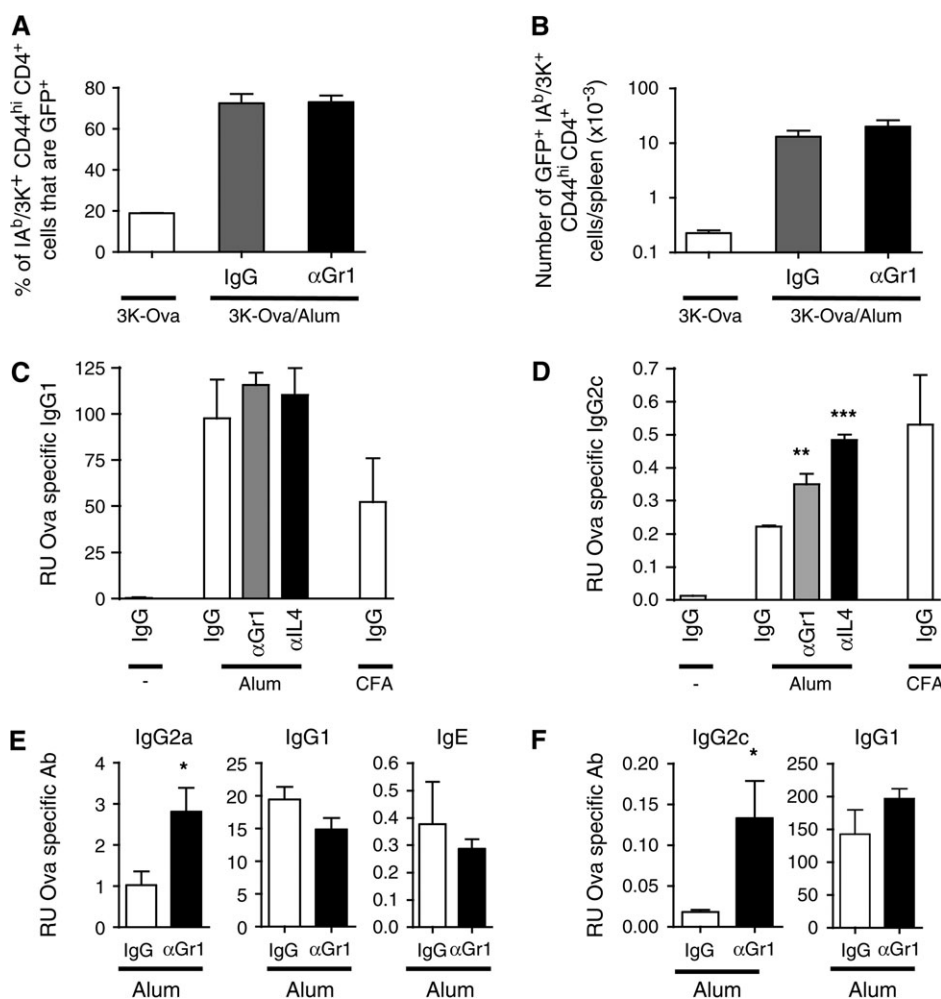
**Fig. 5.** The ability of alum to enhance expansion of antigen-specific CD4 cells is independent of Gr1-expressing cells and IL-4. (A and B) C57BL/6 mice were injected with 3K-OVA either alone or adsorbed to alum and treated with either rat IgG or anti-Gr1 as described in Methods. Nine days later, splenocytes were stained as in Fig. 4. (A) The FACS plots show the staining of F4/80<sup>-</sup> B220<sup>-</sup> CD4<sup>+</sup> cells with anti-CD44 and IA<sup>b</sup>/3K tetramer. Numbers indicate the percentages of F4/80<sup>-</sup> B220<sup>-</sup> CD4<sup>+</sup> cells that were CD44<sup>hi</sup> and IA<sup>b</sup>/3K tetramer positive and are representative of three individual samples. (B) Shown are the averages  $\pm$  standard errors of the numbers of CD44<sup>hi</sup> IA<sup>b</sup>/3K-staining CD4<sup>+</sup> cells per spleen of mice immunized as indicated. Results are representative of three independent experiments. (C and D) WT or IL-4<sup>-/-</sup> mice were injected with 3K-OVA with or without alum. Nine days later, their spleen cells were isolated and analyzed as in (A and B). Results are representative of two separate experiments.

immune system suggests that they are somehow recognized via similar pathways early after exposure. Many adjuvants activate innate immune responses via engaging TLRs; however, the effects of alum on antibody responses are independent of MyD88 and TRIF, essential signaling components downstream of TLRs (8, 9). Likewise, other studies have indicated that T<sub>H</sub>2 responses in response to *S. mansoni* are largely TLR independent (35). Therefore, both alum and *S. mansoni* eggs appear to act as T<sub>H</sub>2-driving adjuvants independently of TLRs and must act via some other sets of receptors. Alum and *S. mansoni* eggs may act via the same receptor or, alternatively, they may be first detected by different receptors that converge on the same T<sub>H</sub>2-inducing pathway. Other products that induce a similar type 2 inflammation, such as chitin (36), may converge on these same receptors and/or intracellular signaling pathways.

Here, we show that *S. mansoni* eggs and alum activate similar innate immune responses. When injected i.p., both cause the accumulation of Gr1+IL-4-producing cells in the spleen and peritoneum. Via a process that requires these cells, both substances promote changes in B cells in the spleen to respond with intense Ca<sup>2+</sup> fluxes to cross-linking of their MHC II molecules. These shared effects on the innate immune system are apparent within 24 h of administration;

therefore, responses to the two products converge quickly and are not due to later downstream effects.

The two materials do not share all properties since alum, but not *S. mansoni* eggs, acts as an adjuvant, improving T and B cell responses to co-injected antigen. At first sight, this is an unexpected result since others have shown that a Lewis<sub>x</sub>-containing oligosaccharide on *S. mansoni* eggs, lacto-*N*-fucopentaose III (LNFPIII), bound to proteins, helps B cell production of T<sub>H</sub>2-driven antibodies and *S. mansoni* eggs induce powerful antibody responses against their own antigens (37). We believe that the contradictions are related to whether or not the co-administered antigen is physically associated with the intrinsic adjuvant within *S. mansoni* eggs. Many proteins bind well to alum, and the reported adjuvant effects of LNFPIII are on responses to covalently linked antigens. In the experiments described here, in which *S. mansoni* eggs failed to act as good adjuvants, the eggs and antigen were injected at the same site, but not bound to each other. Therefore, we predict that the abilities of *S. mansoni* eggs or alum to improve immune responses probably depend on their being taken up together with the antigen by the same cell. The adjuvant effects of helminth products described elsewhere, and, by extension, alum may then be mediated by direct effects of the materials on the properties



**Fig. 6.** Gr1<sup>+</sup> cells and IL-4 inhibit production of Th1-induced antibody isotypes. (A and B) 4Get mice were injected with 3K-OVA with or without alum and were treated with either control rat IgG or anti-Gr1 mAb as described in Methods. Splenocytes were stained for the presence of IA<sup>b</sup>/3K-specific CD4 T cells and gates were drawn around CD44<sup>hi</sup> IA<sup>b</sup>/3K<sup>+</sup> CD4<sup>+</sup> cells as in Figs 4 and 5. (A) Shown are the average  $\pm$  standard errors of the percentages of IA<sup>b</sup>/3K<sup>+</sup> CD44<sup>hi</sup> CD4<sup>+</sup> spleen cells that expressed GFP (IL-4 mRNA). The results are from three identically treated mice per group. (B) Shown are the average numbers  $\pm$  standard errors of GFP<sup>+</sup> IA<sup>b</sup>/3K<sup>+</sup> CD44<sup>hi</sup> CD4<sup>+</sup> cells per spleen from mice immunized as indicated. The results are from three identically treated mice per group. (C and D) B6 mice were injected with OVA with or without alum or CFA. Mice were treated with IgG (white bars), anti-Gr1 (gray bars) or anti-IL-4 (black bars) on days 1, 3 and 5 after primary immunization. On day 9, mice were injected with OVA alone and on day 12 their sera were tested for OVA-specific antibodies by ELISA as described in Methods. The results represent the mean relative units  $\pm$  standard errors of OVA-specific antibody detected from three individual mice per group. Asterisks indicate groups in which the amount of antibody detected was significantly different from that in similarly immunized mice that received control IgG; \* $P$  < 0.05, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001. Results are representative of two independent experiments. (E and F) BALB/c mice (E) or B6 mice (F) were injected with either alum (E) or Alhydrogel (F)-adsorbed OVA and were treated with IgG (white bars) or αGr1 (black bars) antibody as above. On day 10, mice were boosted with alum-adsorbed OVA and sera were tested for OVA-specific isotypes 5 days later. Results are representative of three (E) and two (F) individual experiments.

of the antigen-presenting cells. This idea is supported by the fact that alum and extracts from *S. mansoni* eggs have similar effects on DCs, modulating the outcome of TLR engagement in the cells, inducing DCs to promote Th2 responses without elevating certain co-stimulatory proteins or promoting production of IL-12 (16–18, 38, 39).

Others have previously shown that IL-4 and IL-13 are not required for alum to prime good Th2 responses but do play a role in suppressing Th1-related cytokine production after exposure to antigen plus alum (23, 24). Here, we confirm those observations and extend them by showing that the Gr1<sup>+</sup> cells induced by the adjuvant have parallel effects.

Like IL-4, they are not required for alum to improve T cell priming and, also like IL-4, they do suppress the ability of alum to induce Th1-driven responses such as production of antibodies of the IgG2a/c isotype. These results suggest that the Gr1<sup>+</sup> cells may be an important source of the IL-4 that suppresses the type 1 responses. Strategies that prevent production of such cells during vaccination with alum might actually improve the efficacy of the vaccine, broadening the classes of antibody produced without reducing the titer.

Previously, others have shown that IL-4<sup>-/-</sup> mice injected with alum have Th2 responses that are somewhat reduced by comparison with those of WT mice (23). Here, we found

that although depletion of IL-4 on days 1, 3 and 5 of the primary response was sufficient to prevent suppression of T<sub>H</sub>1-dependent antibodies, it had no effect on the amount of IgG1 produced, a result which appears to contradict the previous observations (23). The difference may lie in the fact that the T cells in the IL-4<sup>-/-</sup> mice had never been exposed to IL-4, and IL-4 may have some effect on T cells before they respond overtly to antigen, since naive T cells can detect the cytokine (40, 41). Thus, pre-exposure to IL-4 may have some effect on the ability of T cells to differentiate into T<sub>H</sub>2, whereas acute lack of IL-4, just during response to antigen, may affect only the ability to suppress type 1 responses.

In other systems, similar effects of Gr1+ cells have been observed; for example, Gr1+ cells accumulate in secondary lymphoid organs during sepsis and depletion of these cells resulted in elevated T<sub>H</sub>1 responses (42). In other studies, Gr1+ cells were found to be accumulated after exposure to parasites and have a suppressive effect on the proliferation of CD4 cells (43). The effects of Gr1+ cells seen here are unlikely to occur by suppressing proliferation of CD4 cell, however, because total numbers of IA<sup>b</sup>/3K-specific CD4 cells were unaffected in 3K-OVA-injected mice, depleted of Gr1+ cells.

In summary, here we show that alum and *S. mansoni* eggs have similar effects on innate IL-4-producing cells. Via these cells, alum and probably also *S. mansoni* eggs suppress T<sub>H</sub>1-related immune responses, leaving the host with only T<sub>H</sub>2-related immunity. Further understanding of the mechanisms by which this occurs could lead to development of vaccines that induce a less T<sub>H</sub>2-biased immune response.

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## Abbreviations

AF	Alexa Fluor
BSS	balanced salt solution
Ca <sup>2+</sup>	calcium
DC	dendritic cell
FBS	fetal bovine serum
GFP	green fluorescence protein
i.p.	intra-peritoneally
LNFP III	lacto-N-fucopentaose III
OVA	ovalbumin
RT	room temperature
Tg	transgenic
TLR	Toll-like receptor
WT	wild type
APC	allophycocyanin
PB	pacific blue
PE	phytoerythrin
SMEM	supplemented minimum essential media
RU	relative units

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