Immunization and Infection Change the Number of Recombination Activating Gene (RAG)-expressing B Cells in the Periphery by Altering Immature Lymphocyte Production

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Abstract

Recombination activating gene (RAG) expression in peripheral B cells increases after immunization with (4-hydroxy-3-nitrophenyl) acetyl coupled to chicken gamma globulin (NP-CGG) in alum. This increase could result from reinduction of RAG expression or, alternatively, from accumulation of RAG-expressing immature B cells in the periphery. We have used mice that carry a green fluorescent protein (GFP) RAG indicator transgene (RAG2-GFP) to characterize the RAG-expressing B cells in immunized spleens. Most of the RAG2-GFP-expressing B cells in unimmunized spleen are immature B cells. Injection with NP-CGG in alum initially suppresses lymphopoiesis in the bone marrow and decreases the number of immature RAG2-GFP-expressing B cells in the spleen. Recovery of lymphopoiesis in the bone marrow coincides with accumulation of RAG-expressing immature B cells in the spleen. Most of the RAG-expressing cells that accumulate in the spleen after immunization do not proliferate and they are not germinal center cells. Neither the initial suppression of lymphopoiesis nor the subsequent accumulation of RAG-expressing cells in the spleen is antigen dependent, since similar changes are seen with alum alone. Furthermore, such changes in the numbers of developing and circulating immature lymphoid cells are seen after injection with complete Freund's adjuvant or malaria infection. Our experiments suggest that adjuvants and infectious agents cause previously unappreciated alterations in lymphopoiesis resulting in the accumulation of RAG-expressing immature B cells in the spleen.

Key words: recombination activating gene • adjuvant • malaria • lymphocyte development • green fluorescent protein indicator gene

Introduction

Recombination activating genes 1 and 2 (RAG1 and RAG2) encode a lymphocyte-specific enzyme that catalyzes V(D)J recombination (1–3). In the B lymphoid lineage, RAG expression is primarily restricted to developing B cells in the bone marrow (4, 5). However, low level RAG expression is also found in B cells in peripheral lymphoid organs, and several laboratories have reported increases in RAG expression in peripheral B cells after immunization (6–9). Two hypotheses have been put forward to account for this increase: RAG expression could be reinduced in B cells undergoing antigen-activated immune responses (6, 7); alternatively, RAG expression in the periphery might reflect accumulation of immature B cells containing residual RAG mRNA (6, 7, 10).

To examine regulation of RAG2 expression in vivo, we and others produced mice that carry RAG2 green fluorescent protein (RAG2-GFP) indicator genes (9–11). Although there were several differences in the techniques used to produce the indicator strains, the results of the experiments were similar: RAG2-GFP expression was found in spleen B cells, and in both cases it was the immature B...
cells that expressed the indicator (9, 10). However, the source of the increased RAG expression observed after immunization was not determined (9, 10).

Here we report experiments showing that transient increases in RAG expression in the spleens of immunized mice result from changes in the production of immature B cells and their export from the bone marrow.

Materials and Methods

Mice. RAG2-GFP (FVB/N or [FVB/N × C57BL/6 (B6)] F1) mice (10), TNF knockout (TNF−/−) (12), type I IFN receptor knockout (IFNR−/−) (13), and signal transducer and activator of transcription (STAT1) knockout (STAT1−/−) (14) mice and matched controls were bred and maintained under specific pathogen-free conditions. B6, RAG1 knockout (RAG1−/−), and 129S6/SvEv mice were purchased from The Jackson Laboratory or Taconic Farms. Mice were 2-5 mo old and were always age and sex matched.

Immunization. 400 μl 10% aluminum potassium sulfate with or without 100 μg of (4-hydroxy-3-nitrophenyl) acetyl (NP) coupled to chicken gamma globulin (NP-CGG; Biosearch Technologies, Inc.) was precipitated by adjusting pH to 6.2 with 1 N potassium hydroxide; the alum precipitates were washed in PBS three times and injected intraperitoneally. CFA (Sigma-Aldrich) was mixed with an equal volume of PBS, and 200 μl was injected intraperitoneally.

Malaria Infection. Plasmodium yoelii (17XNL strain) was maintained by alternating cyclic passage of the parasites in Anopheles stephensi mosquitoes and BALB/c mice. Before infecting B6 mice, parasites were passed in this strain by injecting the mice with parasite-infected BALB/c red blood cells (PRBCs). For experimental infections in B6 mice, 1 × 108 PRBCs obtained from B6 mice were injected intraperitoneally, and parasitemia was determined by microscopic examination of Giemsa-stained thin blood smears (15).

Adoptive Transfer. Adoptive transfer was performed as described (10). 2–4 × 107 splenocytes from wild-type B6 or RAG2-GFP (FVB/N × B6)F1 mice were transferred by intravenous injection into B6 RAG1−/− recipients.

Flow Cytometry and Cell Sorting. The following anti–mouse antibodies were used: biotin, PE, or allophycocyanin (APC) anti-B220 (RA3-6B2); biotin or FITC anti-CD43 (S7); biotin or PE anti–heat-stable antigen (HSA) (M1/69); biotin anti-IgM (R6-60.2); biotin anti-Igα,1,2 (R 26-46); biotin anti-Igκ (R 5-250); GL7; biotin anti-CD4 (H129.19); APC anti-CD8 (Ly-2); PE anti-Fas (Jo2); biotin anti-Ly-6G (RB6-8C5); PE DX5; FITC anti-GL7; biotin anti-CD4 (H129.19); APC anti-CD8 (Ly-2); PE anti-Fas (Jo2); biotin anti-Ly-6G (RB6-8C5); PE DX5; FITC annexin V; and FcBlock (2.4G2) (all from BD PharMingen). Biotinylated antibodies were visualized with streptavidin–Red 613 (GIBCO BRL) or streptavidin–PerCP (Becton Dickinson). GL7 was visualized using PE- or biotin-labeled mouse anti–rat IgM.

For DNA staining, cells were suspended in 20 μM Hoestch 33342 (Molecular Probes)/2% fetal bovine serum/PBS and incubated at 37°C for 40 min. Data were acquired with a FACS Lumin™ or FACS Vantage™ (Becton Dickinson) and analyzed with CellQuest™ software (Becton Dickinson) or Deltagraph™ software (DeltaPoint, Inc.). For DNA analysis, spleen cells stained with anti-B220, anti–HSA, and anti-IgM antibodies and fractions of cells (1–2 × 105) were sorted directly into TRIzol LS (GIBCO BRL).

Reverse Transcription PCR. RNA was extracted using TRIzol (GIBCO BRL), and cDNA was synthesized using Superscript II reverse transcriptase (GIBCO BRL). Reverse transcription (RT)-PCR reactions were performed on serial dilutions of cDNA template using HotStarTaq polymerase (Qiagen). PCR conditions and primers were as described (10). Specificity of PCR products was confirmed by Southern blotting as described (16). The oligonucleotide probe used for Igβ was 5'-GTGACCTGAC-CACTGAGATTTCGCAGA-3'.

Results

Half-Life of GFP Expression in Immature B Cells In Vivo. GFP+ B cells in the spleens of RAG2-GFP mice display the surface features of immature B cells (B220lowHSAhigh 493+) (10), whereas GFP− B cells are mature B cells (B220high HSAlowA43+) (10). Upon transfer to RAG1−/− recipients, GFP+ B cells become GFP− and home to lymph nodes and spleen, suggesting that they mature and enter the long-lived B cell compartment (10). To determine the kinetics of loss of GFP expression, we transferred spleen cells from RAG2-GFP indicator mice into RAG1−/− recipients (Fig. 1 A). After adoptive transfer the number of GFPhigh B cells decreased with a half-life of 52 h (Fig. 1 A), and after 4 d these cells were no longer found in RAG1−/− recipients. Similar kinetics of loss of GFP expression were found when GFP− spleen B cells were cultured in vitro (10; and not shown). Therefore, it is unlikely that GFP− cells preferentially migrate out from spleen. We conclude that RAG2-GFP is only expressed for a short time after B cells leave the bone marrow, and that GFP− B cells are not generated from spleen B cell precursors.

To confirm that loss of RAG2-GFP expression correlates with loss of endogenous RAG mRNA, we measured RAG1 mRNA by RT-PCR. Spleen cells were transferred into RAG1−/− mice, and B220−HSA− high cells were purified after 7 d. RAG1 mRNA could not be detected in the transferred B cells, whereas immature spleen B cells from a wild-type control were positive for RAG1 mRNA expres-

Figure 1. Adoptive transfer of RAG2-GFP mouse spleen cells into RAG1−/− mice. (A) Percentage of GFPhigh cells in IgM+ population recovered from RAG1−/− mouse spleen, monitored at 24-h intervals after adoptive transfer (four mice per time point). The curve was fitted using Deltagraph™ software (DeltaPoint, Inc.). (B) RAG1 mRNA expression in B cells purified from the spleens of RAG1−/− mice that had received 2–4 × 106 wild-type splenic cells 7 d earlier. Serial fivefold dilutions of the cDNA are shown. PCR for Igβ is a cDNA loading control. R1, RAG1; TRSF, B cells isolated from RAG1−/− adoptive transfer recipient; WT, unmanipulated wild-type mouse B cells.

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expression (Fig. 1 B). We conclude that RAG-2-GFP expression after transfer correlates with endogenous RAG mRNA expression and that RAG-expressing immature B cells are not of splenic origin.

The number of immature B cells is regulated during immune responses. Experiments in several laboratories have shown that B cell RAG expression increases in peripheral lymphoid organs after immunization (6–9). To determine whether immunization also increases RAG-2-GFP expression, we injected indicator mice with NP-CGG in alum and performed time course experiments (Fig. 2 A). Although the number of immature GFP+B cells in the spleen initially decreased (34 ± 15% of control [n = 5]; Fig 2 A), by day 16 their number had increased above the starting value (199 ± 38% of control [n = 6]; Fig 2 A). Both the timing and magnitude of this transient increase in RAG expression are consistent with previous observations (6, 7, 9).

The GFP+B cells in the spleen on day 16 after immunization were heterogeneous and showed an increased proportion of less mature IgMlow B cells (Fig. 2 A). In contrast, there was no shift in the surface IgM expression in GFP-B cells with immunization. To confirm that changes in RAG-2-GFP expression after immunization reflect changes in endogenous RAG mRNA expression, we compared RAG1 mRNA levels in GFP+ and GFP− B cells purified from spleens of immunized and control mice. Endogenous RAG1 mRNA was found in GFP+ but not in GFP− cells, and the level of RAG1 expression per cell increased after immunization (Fig. 2 B). The relative increase in RAG1 expression in GFP− B cells may reflect a relative increase in less mature IgMlow B cells, since these are known to express higher levels of RAGs than IgMhigh immature B cells (Fig. 2 A, middle panels [9, 10]). We conclude that the number of RAG-expressing immature B cells in the spleen increases after immunization with NP-CGG in alum. Finally, the increase in RAG-2-GFP expression in spleen at day 16 was transient and the number of GFP+B cells returned to normal by day 28 after immunization (Fig. 2 A). Interestingly, the number of immature B lineage cell in the bone marrow also varied during the response, but GFP intensity was not significantly altered by immunization (Fig. 2 A, and data not shown).

As noted above, the majority of the GFP+B cells in unimmunized spleens are not derived from splenic precursors. To determine whether immunization induces spleen B cells to express RAG-2-GFP, we performed adoptive transfer experiments. Spleen cells from RAG2-GFP indicator mice were transferred into RAG1−/− hosts that were immunized on day 1 after transfer. Although the immunized recipients showed an increase in spleen GL7+B cells consistent with an ongoing immune response, we found no increase in GFP+B cells at day 16 after adoptive transfer of spleen cells (Fig. 2 C). We conclude that the GFP+B cells found in the spleen at day 16 after immunization are not generated from splenic precursors.

To determine whether GFP+B cells in the spleen at day 16 after immunization were dividing, we measured their DNA content with Hoechst dye. In seven independent experiments, only 1.18 ± 0.52% of the GFP+B cells had S or G2/M phase DNA content. Most of these cycling cells were found in a GL7lowB220low but Fas+ subpopulation of GFP+B cells (Fig. 3, A and B; see gate G2). Therefore, most of the GFP+B cells in the spleens of immunized RAG2-GFP indicator mice are not germinal center (GC) cells, which are predominantly GL7high and Fas− (Fig. 3, A and B; see gate G1) (6, 19, 20).

Figure 2. RAG2 expression in peripheral B cells in immune responses. (A) RAG2-GFP expression in mice injected with NP-CGG in alum. The first row shows GFP intensity on B220+ spleen cells. The second and third rows show IgM intensity (bold lines) on GFP+B220+ and GFP−B220− spleen cells; dotted lines indicate B220− cell controls. The fourth row shows B220/IgM density plots of bone marrow (BM) cells. Numbers are the percentages of gated cells or absolute cell numbers × 106 (shown in parentheses). (B) Increased endogenous RAG1 mRNA expression in immunized mouse spleen. RAG1 mRNA levels were estimated by RT-PCR. Sorted GFP+B220+ (GFP+), GFP−B220− (GFP−), and B220− splenocytes from mice immunized (+) 16 d earlier or unimmunized (−) were analyzed. Serial fivefold dilutions of the cDNA are shown. PCR for IgM is a cDNA loading control. (C) RAG2-GFP and GL7 expression in adoptively transferred RAG1−/− mice 16 d after immunization with NP-CGG in alum. Histograms show GFP and GL7 expression on B220+ spleen cells. Percentage of cells found in each category is indicated. R1, RAG1; RAG2-GFP, RAG2-GFP mouse control; unimm., unimmunized control; imm., immunized mouse; TRSF imm., RAG1−/− mouse that received 3 × 107 RAG2-GFP spleen cells 1 d before immunization.
Altered B Lymphopoiesis Induced by Adjuvants or Infectious Agents. As noted above, B lymphopoiesis is initially suppressed by intraperitoneal injection with NP-CGG in alum. To determine which stages of B cell development are affected, we immunized B6 mice and counted B cell progenitors in the bone marrow (Fig. 4 A). We found that pro-B cell, pre-B cell, and immature B cell numbers are all reduced by 5–20-fold by day 4 after immunization but all of these precursors recover by day 16. In contrast, mature B cell numbers in the spleen change little with immunization (not shown). We conclude that immunization with NP-CGG in alum initially suppresses B lymphopoiesis.

To determine whether the effect of NP-CGG plus alum is antigen dependent, we injected RAG2-GFP indicator mice and controls with alum alone. Such mice were indistinguishable from mice injected with NP-CGG in alum. The number of GFP+ B cells in the spleen dropped to 36 ± 12% of control on day 7 (n = 5) and then increased to 189 ± 26% of control on day 16 after alum injection (n = 5). These changes coincided with initial suppression of B lymphopoiesis followed by recovery at day 16. Furthermore, CFA had similar effects on B lymphopoiesis (Table I). Thus, altered lymphopoiesis in NP-CGG and alum–injected mice is antigen independent and can be induced by either alum or CFA.

In addition to suppressing B lymphopoiesis, adjuvant injection also suppressed thymopoiesis. 4 d after administration of alum or CFA, the number of CD4+CD8+ thymocytes decreased 7–50-fold (Fig. 4 B, and Table I). The loss of CD4+CD8+ thymocytes was associated with an increase in annexin V staining indicative of increased programmed cell death (Fig. 4 B). In contrast, the number of myeloid cells in the bone marrow increased while the number of erythroid cells was not significantly altered (Fig. 4 C). We conclude that administration of alum or CFA initially suppresses both T and B lymphopoiesis, that this effect is cell type and developmental stage specific, and that it correlated with an increase in thymocyte apoptosis (21).

IFNs and TNF are known to suppress hematopoiesis during certain viral infections (22, 23). For example, type I IFNs are essential for suppression of hematopoiesis after lymphocytic choriomeningitis virus (LCMV) infection (22). To determine whether suppression of lymphopoiesis by adjuvants is mediated by IFNs or TNF, we injected type I IFNR−/−, TNF−/−, and STAT1−/− mice with alum and examined B and T cell development on day 4 (12–14) (Fig. 5). Although we found significant differ-

**Figure 3.** Cell cycle analysis of spleen B cells from immunized mice. RAG2-GFP mice were immunized with NP-CGG in alum and analyzed at day 16. (A) Histograms show DNA content of GFP GL7high (G1), GFP− GL7low (G2), and GFP−GL7− (G3) B cells. The gates are shown on the density plot. (B) Expression of Fas in B220+GL7+ GFP+ (G1 in A) and B220+ GL7+GFP− (G2 in A) spleen cells at day 16 of NP-CGG/ alum immunization. Numbers are percentages of cells within the marked area.

**Figure 4.** Changes in hemato-lymphopoiesis after injection with NP-CGG in alum. (A) Bone marrow pro-B, pre-B, and immature B cells. B6 mice were immunized with NP-CGG in alum, and bone marrow cells obtained from a single femur were analyzed. Cells with the B220lowCD43+ HSAhighIgM−, B220lowCD43− HSAhighIgM−, and B220lowIgM+ phenotypes were counted as pro-, pre-, and immature B cells, respectively. Mean ± SE (n = 4–5) is shown. (B) Thymocytes increased cell death in the thymus 4 d after alum injection. Density plots show B6 thymocytes stained with anti-CD4 and anti-CD8. Percentage of CD4−CD8+ cells is indicated. Histograms show annexin V staining on gated populations of CD4−CD8+ (DP) and CD4+CD8− (SP) thymocytes. Control mice were not injected with alum. (C) Myeloid and erythroid cells obtained from single femurs. Ly-6G− B220−DX5− and Ly-6G− B220+DX5+ cells were counted as myeloid or erythroid/others respectively. Mean ± SE (n = 3–4) is shown.
Table I. Effect of Adjuvant Injection on T and B Lineage Cells

| Cell               | PBS                 | Alum                | CFA                 |
|--------------------|---------------------|---------------------|---------------------|
|                    | Number of cells (×10⁶) |                     |                     |
| Bone marrow*       |                     |                     |                     |
| T total            | 106.38 ± 9.93       | 126.67 ± 3.33       | 112.67 ± 7.22       |
| Pro-B              | 1.02 ± 0.17         | 0.12 ± 0.001        | 0.12 ± 0.03         |
| Pre-B              | 5.52 ± 0.69         | 0.62 ± 0.11         | 1.05 ± 0.20         |
| Immature B         | 2.31 ± 0.41         | 0.45 ± 0.07         | 0.36 ± 0.10         |
| R recirculating B  | 7.67 ± 1.48         | 0.35 ± 0.12         | 0.20 ± 0.08         |
| M yeloid           | 37.50 ± 2.97        | 85.28 ± 4.97        | 65.92 ± 3.16        |
| Thymus             |                     |                     |                     |
| T total            | 772.50 ± 52.82      | 64.67 ± 21.36       | 136.67 ± 50.44      |
| DP                 | 440.87 ± 41.69      | 9.06 ± 4.08         | 61.46 ± 26.37       |
| Spleen             |                     |                     |                     |
| B                  | 26.01 ± 1.78        | 26.37 ± 0.34        | 28.56 ± 3.02        |
| T                  | 13.37 ± 1.37        | 10.06 ± 0.55        | 10.14 ± 0.68        |

Reagents were intraperitoneally injected 4 d before analysis. Numbers are mean ± SE (n = 3 or 4). Pro-B, B220lowCD43−HSAhighIgM+; Pre-B, B220lowCD43−HSAhighIgM−; Immature B, B220lowIgM+; R recirculating B, B220high; M yeloid, Ly-6G−/B220−/DX5−; DP, CD4+CD8−; T, CD3+.

* Number of cells/femur.

Discussion

Adjuvants were thought to impact the immune system by some of the same mechanisms as natural infections. To determine whether lymphopoiesis is also suppressed during acute infection, we exposed mice to P. yoelii. At the peak of parasitemia, day 14 after infection, the number of B220low immature B lineage cells in the bone marrow was severely reduced (Fig. 6), but the number of developing B cells recovered and even increased by day 28 when the malaria parasites had been cleared (24). We conclude that the development of immature B lineage cells is suppressed during malaria infection, as it is after administration of adjuvants.

Figure 6. B220/IgM staining of bone marrow cells from malaria-infected mice. Date after infection is shown above the density plots and parasitemia below. Percentages of B220low cells and absolute cell numbers × 10⁶ (shown in parentheses) are indicated.

Figure 5. STAT1−/−, type 1 IFNR−/−, and TNF−/− mice are susceptible to alum-mediated suppression of lymphopoiesis. Mice with null mutations of Stat1, Ifnar, or Tnf were injected with alum 4 d before analysis. B220low cells from a single femur and CD4+CD8− (DP) thymocytes were counted and compared with background-matched controls. □, un.injected wild-type mouse; ■, injected wild-type mouse; ○, un.injected knockout mouse; ●, injected knockout mouse; W, wild-type; −/−, knockout.
RAG mRNA, below the dynamic range of detection in the targeted indicator strain, and the significance of this low level of RAG expression has yet to be determined.

Several groups have shown that immunization increases RAG expression in B cells in peripheral lymphoid organs, raising the possibility that RAG could be reinduced in mature B cells (6–9). In our initial experiments, we found that RAG expression was not reinduced in mature B cells after immunization and we were unable to induce RAG expression in vitro with LPS plus IL-4 (10). Nevertheless, there is an increase in the number of RAG2-GFP-expressing B cells in spleens of mice injected with alum. Most of these cells are not of splenic origin, they are nondividing cells that carry the cell surface markers of immature B cells. Only a subset of the GFP+ B cells in the spleen is rapidly proliferating, and a small subfraction of these rapidly dividing cells express high levels of both GL7 and Fas, which are markers of GC B cells (19, 20). Therefore, most GFP+ B cells found in the spleen on day 16 after injection with alum are immature, and only a very small number of these cells have the characteristics of GC cells. It has been proposed that RAG expression in peripheral B cells might contribute to further diversification of the antibody repertoire in GCs during immune reactions (6–8, 25, 26). Our results suggest that antibody gene replacement in GCs is a rare event. However, inflammatory or pathological processes that lead to accumulation of immature B cells might increase the frequency of such events (16, 27).

What is the origin of the RAG H2-GFP-expressing cells that accumulate in the spleen after immunization? Immature B cells are thought to be produced by the bone marrow at a steady rate of 1–2 × 107/d, and homeostasis maintained by selecting a variable fraction of these cells into the long-lived B cell compartment (28–33). However, our experiments suggest that the increase in RAG2-GFP-expressing B cells found in the spleen after immunization is due to an increase in immature B cell production and export 12d after initial suppression of B lymphopoiesis by adjuvant. This rebound effect resembles the increase in immature B cell production and emigration from the bone marrow found 12–14d after sublethal irradiation (17) and differs from the augmentation of pre-B cell production observed 4d after injection with sheep RBCs (34). We conclude that, like irradiation, adjuvants and infection affect lymphopoiesis and alter the rates of immature B cell production and export from the bone marrow.

Transient suppression of B and T lymphopoiesis has been reported during infection with LCMV and after IFN injection, but these agents produced a more general suppression of hematopoiesis not found with adjuvant injection (22, 35). In LMCV infection, bone marrow suppression is mediated by type I IFNs (22), but neither type I IFNs, nor STAT1, nor TNF were essential for the effect produced by alum injection.

What is the significance of altering lymphopoiesis after administration of adjuvant or during infection? Alterations in lymphopoiesis could be limited to malaria and LCMV, and to administration of CFA or alum, and therefore have little physiological significance. However, the documented suppressive effects of inflammatory cytokines, IFNs, and TNF on lymphopoiesis (36–39) suggest that any infectious or inflammatory agent that significantly increases the production of these cytokines and possibly other such effectors may alter lymphopoiesis. Therefore, subsets of T and B cells recently emigrated from the bone marrow may resemble myeloid cells, which display well-characterized shifts during infection and inflammation. Such shifts in the composition of the lymphocyte populations might change the outcome of immune responses. In particular, immature B cells recently emigrating from the bone marrow are highly susceptible to tolerance and less likely to enter the memory pool (40–45). Thus, decreasing the number of such cells exported in acute infection may protect against tolerance and indirectly enhance B cell memory.

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