IgE receptor-positive non-B/non-T cells dominate the production of interleukin 4 and interleukin 6 in immunized mice

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ABSTRACT The phenotype and antigenic specificity of cells secreting interleukin (IL) 4, IL-6, and interferon γ was studied in mice during primary and secondary immune responses. T lymphocytes were the major source of interferon γ, whereas non-B/non-T cells were the dominant source of IL-4 and IL-6 in the spleens of immunized animals. Cytokine-secreting non-B/non-T cells expressed surface receptors for IgE and/or IgG types II/III. Exposing these cells to antigen-specific IgE or IgG in vivo (or in vitro) "armed" them to release IL-4 and IL-6 upon subsequent antigenic challenge. These findings suggest that non-B/non-T cells may represent the "natural immunity" analogue of CD4+ T helper type 2 cells and participate in a positive feedback loop involved in the perpetuation of T helper type 2 cell responses.

Immunoregulatory cytokines play an important role in determining the nature and strength of the immune system's response to pathogens, autoantigens, and tumor cells (1, 2). Cytokines are commonly classified as being either T helper type 1 (Th1) [interferon (IFN) γ and interleukin (IL) 2] or T helper type 2 (Th2) (IL-4, IL-5, IL-6, and IL-10), based on original studies involving cloned murine CD4+ T-cell subsets (3). These two classes of cytokines exert distinct and, in some cases, antagonistic effects. Th1 cytokines promote cell-mediated immunity, whereas Th2 cytokines facilitate humoral immune responses (3). Moreover, Th2 cytokines such as IL-4 and IL-10 can inhibit the production of IL-2 and IFN-γ (4–6), while IFN-γ can interfere with the maturation of naive CD4+ T cells into Th2 cells (5, 7).

The order and abundance of which Th1 and Th2 cytokines are secreted in vivo have been studied in a variety of settings. Immune responses dominated by IFN-γ confer protection to hosts challenged with agents such as Leishmania major and Candida albicans, while responses dominated by IL-4 and related cytokines are protective against infection with the helminth Trichuris muris (8–12).

Although the production of both Th1 and Th2 cytokines is commonly associated with CD4+ T lymphocytes (3, 13), B cells, macrophages, fibroblasts, monocytes, mast cells, stromal cells, epithelial cells, and neutrophils can secrete IL-6 (14–18); basophils, mast cells, and CD8+ T cells can secrete IL-4 (19–21); and natural killer cells and CD8+ T lymphocytes can secrete IFN-γ (22–25). Indeed, the phenotype of the cells that are predominantly responsible for producing IL-4, IL-6, and IFN-γ in immunized animals has not been established.

In an effort to determine which cells are the predominant producers of these cytokines after immunization, sensitive and specific cytokine ELISpot assays were developed to detect individual cells producing IL-4, IL-6, and IFN-γ in vivo. These ELISpot assays have been used to monitor the number of cells spontaneously secreting cytokines in freshly harvested bone marrow, spleen, and lymph nodes and to detect the production of cytokines by single cells after in vivo and in vitro stimulation with antigen or mitogen (26–28). In the current work, ELISpot assays were used to determine the phenotype of the cells secreting cytokines in immunized mice. Our results indicate that a population of IgE receptor-positive (FcRγ) non-B/non-T cells is a dominant producer of IL-6 and IL-4, while T cells are the major source of IFN-γ.

EXPERIMENTAL PROCEDURES

Mice. Female BALB/c mice were obtained from The Jackson Laboratory. Animals used in these experiments were 3–5 months of age. They were immunized i.p. with 25 μg of ovalbumin (OVA) in complete Freund's adjuvant (CFA) and boosted 31 days later with the same antigen in incomplete Freund's adjuvant (IFA).

Cell Purification. Immunized animals were killed by cervical dislocation 10 days after immunization or 4 days after booster injection. Spleens were removed aseptically. Erythrocytes were lysed, and a single-cell suspension was prepared by washing nucleated cells three times in PBS supplemented with 2% (vol/vol) fetal calf serum. For experiments shown in Table 1, cells were treated with anti-mouse IgG receptor (FcR) II/III antibody (2.4G2) to prevent nonspecific immunoglobulin receptor (FcR) binding and then stained with phycoerythrin (PE)-conjugated anti-mouse CD4 (Gk1.5), PE-conjugated anti-mouse CD8a (2.43), or fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD45R/B220 (6B2) (PharMingen) for 30 min at 4°C. Stained cells were washed twice, then analyzed, and sorted using a FACStar Plus flow cytometer (Becton Dickinson).

For experiments shown in Table 2, cells were treated with 2.4G2 to prevent nonspecific antibody binding and then incubated with FITC-labeled anti-CD4, anti-CD8a, anti-CD23, and anti-B220 antibodies. The cell suspension was washed twice and then mixed with anti-FITC-coated magnetic beads (Advanced Magnetics, Cambridge, MA) as described (29). FITC-stained T and B cells were depleted by two cycles of exposure to a magnetic field.

Treatment with Anti-2,4,6-trinitrophenyl (TNP) Antibodies. For in vitro experiments, single cell suspensions were prepared from the spleens of unimmunized 3-month-old BALB/c mice in RPMI 1640 medium supplemented with 5% fetal calf serum. Cells were washed three times and resuspended at 10^6 nucleated cells per ml in medium alone or medium plus monoclonal anti-TNP IgE (clone IGEI a2; American Type Culture Collection) at 10 μg/ml, monoclonal anti-TNP IgG (clone 1B7.11, American Type Culture Collection) at 10 μg/ml, or 10% (vol/vol) mouse serum. Sera were obtained by retroorbital bleeding of mice prior to or 14 days after immunization with 25 μg of TNP13–keyhole limpet

Abbreviations: IL, interleukin; IFN-γ, interferon γ; Th1 and Th2 cells, T helper cells types 1 and 2, respectively; FcR, FcR, FcR, etc., receptor for the constant region of IgE, IgG, etc.; OVA, ovalbumin; CFA and IFA, complete and incomplete Freund's adjuvant, respectively; FITC, fluorescein isothiocyanate; TNP, 2,4,6-trinitrophenyl.
hemocyanin in CFA. After a 1-h incubation, cells were washed three times in medium and assayed for cytokine production in the presence of OVA (20 μg/ml), TNP-OVA (20 μg/ml), or medium.

For in vivo experiments, unimmunized 3-month-old BALB/c mice were injected i.v. with 10 μg of monoclonal anti-TNP IgE, monoclonal anti-TNP IgG, or PBS. After 24 h, a single-cell suspension was prepared from the spleens of these mice in RPMI 1640 medium supplemented with 5% fetal calf serum and analyzed for cytokine production.

Cytokine-Specific ELISpot Assays. Ninety-six-well nitrocellulose-backed microtiter plates (Millipore) were coated with anti-IL-6 (clone MP5-20F3; PharMingen) at 10 μg/ml, anti-IL-4 (clone BVD6-24G2; Endogen, Cambridge, MA) at 10 μg/ml, or anti-IFN-γ (clone RA6a2; Lee Biomolecular Laboratories, San Diego) at 10 μg/ml in 0.1 M sodium carbonate (pH 9.6) for 3 h at room temperature as described (26, 27, 29). The plates were then blocked with PBS containing 5% (wt/vol) bovine serum albumin for 1 h and washed extensively with PBS containing 0.05% Tween 20.

Serial dilutions of a single-cell suspension, starting with 10^5–10^6 cells per well, were incubated on the plate for 5 h at 37°C in a humidified 5% CO_2/95% air incubator. In some experiments, soluble OVA or TNP-OVA (20 μg/ml) was added to the wells for the duration of the assay. Preliminary experiments showed that this concentration of antigen induced optimal cytokine production in vitro. The plates were then washed with PBS/Tween and overlaid with biotinylated anti-IL-6 (clone MP5-32c11; PharMingen) at 1 μg/ml, anti-IL-4 (clone 11B11) at 1 μg/ml, or anti-IFN-γ (clone XMG-1.2; PharMingen) at 1 μg/ml overnight at 4°C. Plates were washed again, treated with a 1:2000 dilution of avidin-conjugated alkaline phosphatase (Vector Laboratories) for 2 h, and washed again.

The cytokine secreted by single cells was visualized by the addition of a solution of BCIP/NBT (Kirkegaard & Perry Laboratories). This solution yields a purple precipitate in the presence of phosphatase. The colorimetric reaction was halted after 30 min by washing with water, and spots were enumerated under ×40 magnification. The dilution of cells yielding optimal numbers of spots per well was used to calculate the total number of cytokine-secreting cells per sample (26, 27, 29).

Statistical Analysis. Statistical significance was determined by using a two-tailed t test unless otherwise specified.

RESULTS

Phenotype of Cytokine-Producing Cells Freshly Harvested from Immunized Mice. BALB/c mice were immunized with 25 μg of OVA in CFA. B, T, and non-B/non-T cells were purified by cell sorting from the spleens of these animals either 10 days after primary stimulation or 4 days after secondary antigenic challenge. Each cell population (>98% pure) was analyzed immediately for spontaneous cytokine production by ELISpot assay. The absolute number of IL-6-producing cells was substantially greater than the number of IL-4-producing cells, which in turn exceeded the frequency of IFN-γ-producing cells. As seen in Table 1, T lymphocytes accounted for virtually all of the IFN-γ-producing cells but were only 40% of the IL-4- and 12% of the IL-6-producing cells in the spleens of animals sacrificed 10 days after immunization. The remaining cytokine-producing cells were primarily of the CD4^+ , CD8^+ , B220^- phenotype (non-B/non-T cells). An even larger fraction of the IL-4- and IL-6-producing cells from animals 4 days after booster injection had the non-B/non-T phenotype. In these populations, 85% of the IL-4-producing cells and 94% of the IL-6-producing cells were non-B/non-T cells, while the IFN-γ-producing cells remained predominantly T cells.

Effect of in Vitro Antigenic Challenge on the Frequency of Cytokine-Producing Cells. Previous experiments have shown that spleen cells from immunized mice can be triggered to release additional IL-4 and IL-6 when exposed to antigen in vitro (29, 30). This allowed us to examine whether cytokine production by non-B/non-T cells was antigen-dependent. ELISpot assays were performed on spleen cells and non-B/non-T cells in the presence or absence of soluble OVA.

The number of non-B/non-T cells secreting IL-4 and IL-6 increased 3- to 4-fold when splenocytes from OVA-immunized but not naive BALB/c mice were incubated with soluble OVA (Table 2). A similar effect was observed when non-B/non-T cells from mice immunized and given booster injections with OVA were studied. This response was antigen-specific, since spleen cells from OVA-immunized mice did not release additional cytokine when cultured with an unrelated antigen (such as TNP-keyhole limpet hemocyanin, data not shown). These experiments also demonstrate that the frequency of non-B/non-T cells spontaneously producing IL-4 and IL-6 is greater in immunized mice than naive donors.

Table 1. Production of cytokines by phenotype-positive cells in vivo

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cell type</th>
<th>10 days after</th>
<th>4 days after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1°</td>
<td>2°</td>
</tr>
<tr>
<td>IL-4</td>
<td>B</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>83</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Non-B/non-T</td>
<td>99</td>
<td>53</td>
</tr>
<tr>
<td>IL-6</td>
<td>B</td>
<td>55</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>290</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Non-B/non-T</td>
<td>1900</td>
<td>85</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>B</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Non-B/non-T</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Three-month-old BALB/c mice were immunized i.p. with 25 μg of OVA in CFA (1°) and given a booster immunization 31 days later with 25 μg of OVA in IFA (2°). Populations of B cells, T cells, and non-B/non-T cells (>98% pure) were isolated by cell sorting and analyzed for cytokine production. All data points reflect results from at least two independent experiments. The number of cells per 10^6 phenotype-positive cells producing each cytokine and the percent contribution of phenotype-positive cells to the total number of cytokine-producing cells in the spleen are shown. The percent contribution was calculated by the formula: (number of cytokine-secreting cells per 10^6 phenotype-positive cells) × (number of phenotype-positive cells per spleen × 10^-9)/(total number of cytokine-secreting cells per spleen) × 100%. In the 1° response, 43% of spleen cells were B220^- and 27% were CD4^- or CD8^- . In the 2° response, 44% of spleen cells were B220^- and 33% CD4^- or CD8^- . Remaining cells had the non-B/non-T phenotype.

Table 2. Effect of in vitro antigenic stimulation on cytokine production by non-B/non-T cells

<table>
<thead>
<tr>
<th>Donor</th>
<th>IL-4</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>− OVA + OVA</td>
<td>− OVA + OVA</td>
</tr>
<tr>
<td>Naive</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>OVA-primed</td>
<td>126</td>
<td>346</td>
</tr>
<tr>
<td>OVA-boosted</td>
<td>340</td>
<td>940</td>
</tr>
</tbody>
</table>

Spleen cells were obtained from immunologically naive mice, from mice immunized 10 days earlier with 25 μg of OVA in CFA (OVA-primed), and from mice 4 days after a booster injection with 25 μg of OVA in IFA (OVA-boosted). Non-B/non-T cells were isolated from mice in each group and analyzed for cytokine production in the presence (+) or absence (−) of soluble OVA. Results represent the number of IL-4- and IL-6-secreting cells (per 10^6 spleen cells) in medium alone (−) and with ovalbumin (20 μg/ml) (+). Data represent the average of two experiments. Responses that were significantly increased (P < 0.05) due to exposure to OVA are underlined.
Since T and B lymphocytes are the only immunologically active cells known to encode cell surface receptors for antigen, this antigen-specific release of IL-4 and IL-6 by non-B/non-T cells at first appeared paradoxical. However, cells expressing FcRs are capable of acquiring antigenic specificity by binding circulating IgG and/or IgE (19, 31–33). To examine whether non-B/non-T cells bore such receptors, spleen cells obtained from immunized mice were stained for the expression of Fc, R and Fc,RII/III. Three distinct populations were identified and isolated by flow cytometry: Fc, RI/III+, Fc, R+ (double positive) cells, Fc, RII/III+, Fc, R- cells, and double-negative cells. Cells expressing only Fc, R represented <2% of the non-B/non-T population and could not be recovered for functional studies.

As seen in Table 3, the double-positive cell population contained the highest proportion of cells secreting IL-4 and IL-6 (both spontaneously and after stimulation with OVA in vitro). A far lower fraction of Fc, RII/III+, Fc, R+ cells from OVA-immunized mice secreted IL-4 or IL-6, although in vitro antigen exposure increased the number of cytokine-producing cells from this population as well. In contrast, antigen induced no increase in the modest number of double-negative cells secreting IL-4 or IL-6 (Table 3). Prior studies indicate that the bulk of the double-positive cells are basophils or basophilic myelocytes. Those experiments demonstrated that cross-linkage of Fc, R on basophils induced the secretion of IL-4 (34–38).

Antibody-Dependent Antigen-Specific Cytokine Release by FcR+ Non-B/Non-T Cells. These findings led us to examine whether FcR+ non-B/non-T cells from immunized mice might acquire the capacity to release cytokines in response to antigenic challenge by binding circulating IgG or IgE. Spleen cells from unimmunized BALB/c mice were treated in vitro with anti-TNP antibodies and then analyzed for cytokine production in response to TNP-OVA. Pretreatment with anti-TNP IgG, anti-TNP IgE, or serum from TNP-keyhole limpet hemocyanin-immunized mice significantly increased the number of non-B/non-T cells that secreted IL-4 and IL-6 when exposed to TNP-OVA in vitro (Fig. 1). Monoclonal anti-TNP IgE was particularly effective, inducing an antigen-dependent 3–4 fold increase in the number of cells releasing IL-4 and IL-6. A series of negative controls showed that this response was specific; OVA did not induce these cells to release cytokine nor did TNP-OVA stimulation of cells pretreated with control IgG or IgE antibodies (Fig. 1).

Further studies were performed to determine whether cells exposed to circulating anti-TNP IgE and IgG in vivo could acquire specificity for TNP-OVA (as measured by cytokine release). Unimmunized BALB/c mice were injected with monoclonal anti-TNP IgG or anti-TNP IgE. As seen in Fig. 2, spleen cells from these mice acquired the ability to release IL-4 and IL-6 when exposed to TNP-OVA in vitro, while cells from mice that received control monoclonal antibodies did not. This enhancement was particularly striking in mice injected with monoclonal anti-TNP IgE. The degree of enhancement for IL-4 production was greater than that for IL-6 production.

### DISCUSSION

The experiments reported here establish several important principles concerning antigen-induced cytokine production.

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**Table 3.** FcR+ phenotype of cytokine-secreting non-B/non-T cells

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cell type</th>
<th>10 days after 1*</th>
<th>4 days after 2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>Fc+, Fc-</td>
<td>- OVA + OVA</td>
<td>- OVA + OVA</td>
</tr>
<tr>
<td></td>
<td>Fc+, Fc+</td>
<td>50 950</td>
<td>620 1,200</td>
</tr>
<tr>
<td></td>
<td>Fc+, Fc-</td>
<td>15 40</td>
<td>23 31</td>
</tr>
<tr>
<td></td>
<td>Fc-, Fc+</td>
<td>10 10</td>
<td>13 13</td>
</tr>
<tr>
<td></td>
<td>Fc-, Fc-</td>
<td>17,000 38,000</td>
<td>92,000 280,000</td>
</tr>
<tr>
<td>IL-6</td>
<td>Fc+, Fc+</td>
<td>2,600 4,500</td>
<td>3,700 7,800</td>
</tr>
<tr>
<td></td>
<td>Fc+, Fc-</td>
<td>440 480</td>
<td>810 860</td>
</tr>
</tbody>
</table>

Three-month-old BALB/c mice were immunized and given a booster injection as described in Table 1. Non-B/non-T cells were isolated, stained for FcR expression, and isolated by cell sorting. The resultant >98% pure cell populations were analyzed for cytokine production in medium alone (-) or ovalbumin at 20 μg/ml (+). Data represent the average number of IL-4- and IL-6-secreting cells (per 10⁶ phenotype-positive cells) from two experiments.

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**Fig. 1.** Antigen-specific cytokine release after in vitro exposure to IgE or IgG. Single spleen cell suspensions from unimmunized BALB/c mice were treated in vitro with monoclonal anti(a)-TNP IgE (10 μg/ml), anti-TNP IgG (10 μg/ml), or 10% mouse serum. Cells were washed and assayed for cytokine production in the presence of TNP-OVA (20 μg/ml) or unconjugated OVA (20 μg/ml) as described in Table 2. Data represent the average ± SD of two experiments. Fold increase in responding cells was calculated by the formula: number of cells secreting cytokine in the presence of TNP-OVA/number of cells secreting cytokine in the presence of OVA.

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**Fig. 2.** Antigen-specific cytokine release after in vivo exposure to IgE or IgG. Unimmunized 3-month-old BALB/c mice were injected i.v. with 10 μg of monoclonal anti-TNP IgE, 10 μg of monoclonal anti-TNP IgG, or PBS. After 24 h, a single-cell suspension prepared from the spleens of these mice was analyzed for cytokine production as described in Fig. 1. Data represent the average ± SD of two experiments.
(i) T cells were found to be the primary in vivo source of IFN-γ production during standard primary and secondary immune responses. (ii) FcR+ non-B/non-T cells were identified as the dominant source of antigen-induced IL-4 and IL-6 secretion. This was particularly true of primary and secondary IL-6 responses and secondary IL-4 responses. It is noteworthy that the profile of cytokines produced by non-B/non-T cells resembled that conventionally associated with the activation of Th2 (rather than Th1) cells. This raises the possibility that non-B/non-T cells may represent natural immunity analogues of CD4+ Th2 lymphocytes. Conceptually, CD4+ Th2 cells might be responsible for providing cognate help directly to antigen-specific lymphocytes (39), while non-B/non-T cells might help establish the cytokine milieu needed to induce and maintain a Th2-type response. The cytokines secreted by non-B/non-T cells may also play a role in recruiting other immunologically active cells to sites of antigen exposure (38).

It has been shown (19, 34, 35) that a population of non-B/non-T cells expressing FcRs could bind IgG and/or IgE in vitro, thereby acquiring specificity for antigen. Subsequently, non-B/non-T cells from mice treated with anti-IgD or chronically infected with Nippostrongylus brasiliensis or Schistosoma mansoni were found to secrete IL-4 in vivo and in some cases after restimulation with antigen in vitro (31–33). Our studies build upon these earlier observations by demonstrating that non-B/non-T cells (i) are the dominant source of both IL-6 and IL-4 in vivo, (ii) are stimulated by conventional antigens and by chronic parasitic infections or polyclonal activators, and (iii) can be "armed" with IgG or IgE in vivo to respond to antigenic challenge. Based on phenotypic studies of cytokine-producing FcR+ cells in humans, it seems likely that the IL-4- and IL-6-producing FcR+ murine non-B/non-T cells identified in this report are predominantly basophils (33, 36, 37). Unfortunately, antibodies that uniquely identify surface markers on murine basophils are not available, complicating efforts to directly analyze this issue.

When non-B/non-T cells from unimmunized mice were pretreated with antigen-specific IgG or IgE, they acquired the capacity to release IL-4 and IL-6 upon exposure to antigen in vitro. This response had several interesting characteristics. (i) IgE conferred antigen specificity more efficiently than IgG. (ii) The relative increase in IL-4 production generally exceeded that of IL-6, possibly because the background number of IL-6-producing cells was higher than that of IL-4-producing cells (Figs. 1 and 2). (iii) Perhaps most interesting, only a small fraction of available non-B/non-T cells were armed to release IL-4 or IL-6 when treated with antigen-specific antibodies. This limited acquisition of antigen responsiveness could have several explanations. (i) Non-B/non-T cells may differ in their capacity to produce cytokines depending upon their activation state, FcR density, or FcR occupancy. Indeed, it has been shown that virtually all high-affinity FcRs on non-B/non-T cells are occupied in vivo, thus limiting the capacity of non-B/non-T cells to bind additional antibody (11, 33). (ii) The ELISPOT assay is sensitive to cells secreting >100 molecules of cytokine per second. Thus, non-B/non-T cells secreting IL-4 or IL-6 at lower rates could escape detection (26, 40). (iii) Accessory immunostimulatory factors might be required to optimize cytokine production by non-B/non-T cells. For example, IL-3 has been shown to strikingly increase the secretion of IL-4 by basophils and mast cells (34).

Finally, findings in this report raise the possibility that a positive feedback loop involving Th2-associated cytokines, non-B/non-T cells, and antibody may be operating in vivo (Fig. 3). We propose that such a loop is initiated when antigen induces IL-4 and IL-6 production by Th2 cells. These Th2 cytokines could then facilitate the production of antigen-specific IgG and/or IgE, which in turn would bind to and arm FcR+ non-B/non-T cells to release additional IL-4 and IL-6 when reexposed to the same antigen. This series of events could be responsible for maintaining the cycle of cytokine/antibody production characteristic of Th2-associated immune responses.