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Novel Diversity in IL-4-Mediated Responses in Resting Human Naive B Cells Versus Germinal Center/Memory B Cells

Eric F. Wagner,* Naazeh Hanna,* Loren D. Fast,† Nicola Kouttab,‡ Peter R. Shank,§ Aime Vazquez,‖ and Surendra Sharma*‡*

Recent studies have defined several phenotypic and molecular changes associated with the maturation of naive human B cells within the milieu of germinal centers. Although naive B cells serve as natural precursors to germinal center (GC)/memory (M) subpopulations, little is known about the physiological requirements for the survival of the naive B cell pool in the absence of cell-cell contact or Ag-mediated activation. Because IL-4 induces expression of several membrane receptors such as CD23 which are uniquely present on resting human naive B lymphocytes, we hypothesized that these cells might be intrinsically programmed to respond to IL-4 in the absence of cell division. Using buoyant density-dependent isolation and further enrichment by negative/positive selection of human naive and GC/M subpopulations, we characterized cytokine receptor moieties on these cells and analyzed their survival and growth in the presence of IL-4 or IL-10. Resting naive B cells expressed significantly higher IL-4 receptor α-chain on their cell surface than the combined GC/M subpopulation. The IL-10 receptor and the IL-2 receptor γc chain were almost equally expressed on both subpopulations. When cultured in vitro, the addition of IL-4, but not IL-10, protected naive B cells from apoptosis in the absence of activation and growth. However, IL-4 exerted no such effect on resting GC/M B cells. These data support the hypothesis that IL-4 plays a pivotal role in the survival and maintenance of resting human naive B cells. The Journal of Immunology, 2000, 165: 5573–5579.

Naive B cells are small and Ag-inexperienced lymphocytes generated in the bone marrow (1–3). Upon export to the secondary lymphoid organs, these cells serve as precursors to germinal center (GC) B cells and are activated within the milieu of GCs followed by affinity maturation and somatic mutations of their Ig variable regions (4, 5). This pool of GC B cells is further subjected to positive selection and subsequent T cell-dependent differentiation into memory (M) precursors and plasma cells (6–9). Thus, regulatory processes, including B and T cell interaction through CD40, cytokine-mediated development, and elimination of autoreactive B cells through apoptosis, are intrinsically in place to program GC/M cells for their maintenance and functions (10–17). However, the mechanisms by which the maintenance of the Ag-inexperienced naive B cell pool is regulated are not well understood.

To establish in vitro models to understand intrinsic differences between naive human B lymphocytes and their GC/M counterparts, current research has focused on characterization and isolation of distinct B cell subpopulations from the secondary lymphoid organs (9, 18). It has been possible to follow the development of GC and M B cells by defining the changes in surface molecule expression (5, 19). Indeed, it has been shown that naive B cells, characterized by IgD⁺CD23⁺CD38⁻CD44⁻CD95⁻, acquire a partial GC phenotype when their CD40 and Ag receptors are triggered (14). Moreover, GC B cells, distinguished by their surface phenotype of IgD⁺CD23⁺CD38⁻CD44⁻CD95⁺, can also develop memory or plasma cell characteristics in the context of cytokine-mediated signaling and gp39 (CD154)-CD40 interactions (13, 15, 20, 21). The GC to M transition is characterized by the surface phenotype IgD⁺CD23⁻CD38⁻CD44⁻CD95⁺ (4, 15). We recently began to delineate intrinsic differences between naive human B cells and GC/M B cells and demonstrated that naive B cells, when activated by triggering CD40 and B cell Ag receptor, underwent robust DNA synthesis in response to IL-4, but to a much lesser extent to IL-2 or IL-10 (22). In contrast, activated combined GC/M subpopulation responded to all of these cytokines, albeit with variable proliferation index (22). However, the question arises about the milieu that supports the maintenance of the naive B cell pool in the absence of activation and rapid proliferation, a situation reminiscent of the primary follicles. In this regard, specific expression of CD23, the low-affinity IgE receptor, on freshly isolated naive B cells is intriguing (23). CD23 up-regulation has been shown to be transcriptionally controlled by IL-4 or EBV (24–26). In addition, IL-4 up-regulates expression of class II MHC and IL-4R on total resting B cells (27, 28). Given these observations, we propose that IL-4 uniquely influences and regulates the survival of B cells, particularly of naive human B cells, in the absence of activation or proliferation.

In the present study, we employed highly purified naive and GC/M human tonsillar B cell subpopulations to ascertain the role of IL-4 and IL-10 in regulating their in vitro survival. We report that naive B cells exhibited much slower propensity to apoptosis than GC/M B cells and that IL-4 protected unstimulated naive, but not GC/M, B cells from in vitro cell death. Curiously, IL-10 had no effect on naive B cells, irrespective of the presence of its receptor...
on these cells. The IL-4Rα chain was significantly detected on naive B cells, whereas the IL-2 common γ-chain (IL-2Rγc) was similarly expressed on both subpopulations. Thus, maintenance of the functional receptor and partial competence of IL-4-mediated signaling may be the mechanisms whereby this cytokine supports the survival of naive B cells in the absence of stimulation.

Materials and Methods

Reagents and cell culture

All cell culture experiments were performed in RPMI 1640 supplemented with penicillin (50 U/ml)/streptomycin (50 μg/ml) and 10% FCS (Life Technologies, Grand Island, NY). Cells were incubated at 37°C/5% CO₂ and seeded at a density of 1 × 10⁶ cells/ml. Activation of B cells was achieved by using anti-CD40 Ab derived from hybridoma cells secreting the anti-human CD40 Ab G28.5 (American Type Culture Collection, Manassas, VA), immunobeads covalently bound to rabbit anti-human IgM specific for μ-chains (Irvin Scientific, Santa Ana, CA) or soluble anti-IgM F(ab')₂ fragments or rabbit anti-human IgM Ab specific for μ-chains (Dako, Carpentry, CA). Anti-human CD30 Ab was further purified and characterized as described previously (22). Recombinant human IL-2 and IL-4 were obtained from Genzyme Genetics (Cambridge, MA). Recombinant human IL-10 was purchased from R&D Systems (Minneapolis, MN). Anti-human IgA (clone G20-59), anti-human IgG (clone G1 8-145), anti-human CD38 (clone HIT2), and anti-human IgD (clone IAG-2) were purchased from PharMingen (San Diego, CA). Rat anti-mouse IgG1 magnetic microbeads and CS₃ separation columns were obtained from Miltenyi Biotec (Auburn, CA).

Isolation of tonsillar B cell subpopulations

Human tonsillar B cells were isolated as described previously (22). In brief, tonsils were obtained after routine tonsillectomy and finely minced, and the resulting cell suspension was depleted of T cells by resettling with neuraminidase-treated sheep RBC and subsequent Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. After adherence to plastic for 2 h to overnight light stimulation in a humidified atmosphere of 5% CO₂ to remove non-B cells, B cells were further purified on discontinuous Percoll gradients (Pharmacia) according to density as previously described (22). Cells collected at the 70% layer represent high-density IgD⁻CD38⁻naive B cells (herein referred to as high-density B cells). Cells collected at the 60% layer represent low-density IgD⁺CD38⁻combined GC/M B cells (herein referred to as low-density B cells). Naive and GC/M subpopulations were further purified by negative and/or positive selection on magnetic microbead columns. Abs to human CD38, IgD, IgG, and IgA were used as required. Naive B cells were enriched from Percoll gradient-purified high-density cells by negatively selecting IgD⁺ cells followed by positive selection. GC/M B cells were enriched from low-density cells first by negative selection in an IgD⁻ reaction followed by positive selection by incubating these cells with Abs to CD38, IgD, and IgA. Cells were first incubated for 15 min on ice with appropriate Abs. Rat anti-mouse IgG1 microbeads (Miltenyi Biotec) were added and cells were incubated for an additional 15 min on ice. The labeled cells were magnetically separated on Miltenyi CS³ columns using the VarioMACS (Miltenyi Biotec) magnetic separator. The purified cells were then collected and assessed for respective phenotypic characteristics by FACSscan analysis (Becton Dickinson, Mountain View, CA).

FACSscan analysis

Purified human tonsillar B cell subpopulations (1 × 10⁶ cells/ml) were incubated with mouse anti-human CD95-FITC (Immunotech, Marseille, France), rat anti-human IgD-FITC (PharMingen), mouse anti-human CD38-PE (Immunotech), mouse anti-human CD19-PE (Becton Dickinson Immunocytemetry Systems, San Jose, CA), mouse anti-human IL-2Rγc-PE (PharMingen), or mouse anti-human IL-10R-PE (PharMingen) for 30 min on ice in the dark. After washing twice with PBS containing 2% FCS, cells were fixed in 0.5% parafomaldehyde and analyzed with a FACSscan flow cytometer. To detect IL-4Rα chain, a high sensitivity immunofluorescence staining was conducted as described elsewhere (29). Briefly, purified human tonsillar B cells were incubated with a biotinylated goat polyclonal Ab against IL-4Rα chain (R&D Systems) followed by the sequential addition of biotin-conjugated anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and PE-streptavadin (Becton Dickinson Immunocytemetry Systems) for 30 min each on ice in the dark. Cells were washed twice with PBS + 2% FCS, fixed in 0.5% paraformaldehyde, and analyzed by flow cytometry.

Proliferation assays

Purified human tonsillar B cells (1 × 10⁶ cells/well) were seeded in 96-well round-bottom microtiter plates in a final volume of 200 μl. Cells were grown in 10% FCS and in the presence or absence of covalently linked anti-IgM beads (1:250 dilution) and anti-CD40 (1 μg/ml) plus IL-4 (20 U/ml), IL-2 (100 U/ml), or IL-10 (10 ng/ml). After 72 h in culture, cells were pulsed with 1 μCi/well [³H]thymidine for an additional 16 h, and incorporated radioactivity was measured.

Analysis of DNA fragmentation

Analysis of apoptosis by DNA fragmentation was performed as previously described using the apoptotic DNA ladder kit obtained from Roche Diagnostics (Mannheim, Germany) (30). Briefly, after culturing with appropriate cytokine and/or activator(s) for 24 or 48 h, cells were washed once in PBS, resuspended in 0.2 ml PBS, and brought to a final volume of 0.4 ml with binding buffer (10 mM Tris-HCl (pH 8.0), 10 mM urea, 6 M guanidine-HCl, and 20% Triton X-100, pH 4.4). Samples were incubated for 10 min at room temperature where 0.1 ml isopropanol was added following vortexing. The resulting mix was passed through a filter tube and washed twice with washing buffer (20 mM NaCl, 2 mM Tris-HCl (pH 7.5), and EtOH). Purified DNA was obtained using elution buffer (10 mM Tris, pH 8.5) heated to 70°C and spun through the column at 8000 rpm for 1 min. DNA (20 μg in each lane) was separated on a 1.2% agarose gel and visualized by ethidium bromide staining.

Propidium iodide staining

The percentage of apoptotic cells was determined by analyzing the nuclear content by flow cytometry as described previously (30). Purified tonsillar human high-density and low-density B cells were incubated either alone (10% serum) or in the presence of IL-4 or IL-10 for 24 or 72 h. Cells were harvested and resuspended in 1.5 ml hypotonic fluorochrome solution (50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100) and incubated for 30 min at room temperature in the dark. DNA content was then analyzed by flow cytometry. Debris was excluded from analysis by raising the forward scatter threshold. Apoptotic cells were defined as those having hypodiploid nuclei emitting fluorescence in channels 10–200 and were enumerated as a percentage of the total population.

Results

Characterization of naive and GC/M B cells by distinct cell surface markers and size

To evaluate intrinsic phenotypic characteristics of naive and GC/M B cells, highly purified tonsillar B cells (see Materials and Methods) were stained with various cell surface markers. Flow cytometry shows that the naive B cell subpopulation (high-density fraction) predominately expressed the surface markers IgD and CD23. This subpopulation lacked expression of the GC/M surface markers CD95 and CD38 (Fig. 1A). Conversely, the GC/M B cell subpopulation (low-density fraction) displayed high levels of CD38 and CD95 on their surface (Fig. 1A). Both subpopulations represented highly purified B cells as demonstrated by >95% staining for the pan B cell marker CD19 (Fig. 1A). These results are typical of those obtained from several independent preparations and agree with published observations (31, 32).

It is known that while naive B cells are resting small sized cells, GC/M cells are a heterogeneously sized population that were in different stages of activation, cell division, and maturation (9). Using this distinction, we wished to further characterize purified B cell subpopulations by their size. Unstimulated high- and low-density B cells were subjected to forward scatter analysis (linear scale) by flow cytometry. As shown in Fig. 1B, the high-density (naive) B cell fraction was found to be a homogeneous population of small sized cells showing a single peak with a forward scatter of ~100 mean channel units. However, the low-density (GC/M) B cell fraction was a heterogeneous population representing cells of various sizes, showing a forward scatter of 140 mean channel units with a large fraction scattered >150 mean channel units. These results further confirm that isolated naive and GC/M B cells represented highly purified subpopulations.
IL-4R expression differs between naive and GC/MB cell subpopulations

It has been reported that unfractionated resting tonsillar human B cells express low levels of IL-4R (29). However, the precise nature of a subpopulation that may express this receptor is not well defined. To address this question, unstimulated high-density and low-density B cell subpopulations were stained with a polyclonal Ab against the IL-4Rα chain and analyzed by FACS. An indirect labeling technique was used to enhance the sensitivity of IL-4Rα detection as described in Materials and Methods. As shown in Fig. 2A, the high-density fraction expressed significantly more IL-4Rα on its surface (25.3%) as opposed to the low-density fraction (6.2%). Importantly, activation of these subpopulations with anti-IgM/anti-CD40 for 24 h resulted in a marked increase in IL-4Rα expression in both subpopulations, 66.2% on high-density and 45.7% on low-density B cells, respectively.

To further characterize unstimulated high-density and low-density B cell subpopulations for the expression of other cytokine receptors, we examined the presence of the IL-10R and the IL-2Rγc chain. As shown in Fig. 2B, both subpopulations exhibited significant expression of IL-10R and IL-2Rγc. These data are representative of several independent tonsil specimens and suggest that although unstimulated naive (high-density) and GC/M (low-density) B cells express IL-10R and IL-2Rγc in a similar fashion, they differ in their display of the IL-4Rα chain.

IL-4 protects naive, but not GC/M, B cells from spontaneous cell death

We hypothesized that resting naive B cells were intrinsically programmed to respond to IL-4 in the absence of activation and proliferation. Because IL-4 has been shown to act as a survival factor for resting T cells and to protect chronic lymphocytic leukemia B cells from spontaneous apoptosis (33, 34), we used the measure of apoptosis as an approach to test the ability of IL-4 as a survival factor for resting naive and GC/M B cells. Here, enriched high- and low-density tonsillar B cells were either cultured alone or in the presence of IL-4 or IL-10 for 0, 24, or 72 h, and apoptosis was measured by propidium iodide staining. At 0 h, neither high-density nor low-density B cells showed any significant cell death (Fig. 3). It is important to note that a significant portion of the low-density population was in G2-M phase of the cell cycle at time 0 h. This correlates with the forward scatter analysis shown in Fig. 1B, indicating that a number of these cells are at various stages of cell division in vivo. However, by 72 h of culture, both high- and low-density B cells underwent spontaneous apoptosis, with 44 and 46.4% cell death, respectively (Fig. 3). Interestingly, high-density B cells showed a lesser propensity for apoptosis than low-density B cells. Fig. 3A shows that at 24 h, only 9.3% of the high-density B cells were hypodiploid (apoptotic) as opposed to 40.1% of the low-density B cells (Fig. 3B). The addition of IL-10 alone did not significantly improve the viability of high-density subpopulation. However, IL-10 provided some protection for low-density B cells at 24 h, reducing the apoptotic death rate from 40.1% when cultured alone to 23.4% when treated with IL-10 (Fig. 3B). This protection however dissipated by 72 h (Fig. 3B). In contrast, treatment with IL-4 resulted in a marked protection of high-density, but not low-density, B cells from apoptosis. After 72 h, there was only 15% cell death in the comparable IL-4-treated high-density subpopulation as compared with 42.4% cell death in the low-density subpopulation (Fig. 3B). This differential ability of IL-4 to protect high-density, but not
low-density, B cells from apoptosis may be associated with the varying expression of IL-4R on their cell surface and/or partial signaling competence of naive B cells to respond to this cytokine.

IL-4 augments activation-dependent protection of GC/M B cells

We hypothesized that the inability of IL-4 to protect low-density B cells in Fig. 3B was perhaps because of a second signal requirement. We and others have shown that low-density B cells are stimulated to proliferate in response to IL-4 when activated with anti-IgM and/or anti-CD40 (11, 22). Therefore, we next aimed to evaluate 1) the dose-dependent inhibition of apoptosis in both high- and low-density B cells and 2) the conditions which result in protection of low-density B cells. Resting high- and low-density B cells were cultured for 24 or 48 h in the presence of varying concentrations of IL-4, and apoptosis was measured by DNA fragmentation analysis or propidium iodide staining (only DNA fragmentation at 48-h time point shown here). IL-4 was able to protect resting high-density B cells from apoptosis in a dose-dependent manner, with concentrations of 20 ng/ml giving maximum protection, whereas no IL-4-mediated inhibition of apoptosis in resting low-density B cells was observed even at the highest dose (Fig. 4A). To test whether low-density B cells were protected from apoptosis by IL-4 in the presence of coactivators, we conducted DNA fragmentation analysis with cells cultured for 48 h in the absence or presence of IL-4 alone or in combination with anti-IgM and/or anti-CD40. As shown in Fig. 4B, IL-4 alone was not able to protect low-density B cells from apoptosis, agreeing with the results from Figs. 3B and 4A. However, coactivation of low-density B cells with anti-IgM and anti-CD40 resulted in a significant reduction in apoptosis (Fig. 4B). This protection was further enhanced by the addition of IL-4 (Fig. 4B). These results suggest that costimulation with activators such as anti-IgM and anti-CD40 renders low-density B cells capable of responding to IL-4.

IL-4-mediated protection of resting high-density B cells occurs in the absence of cellular growth

To assess whether protection of unstimulated high-density B cells was associated with concurrent cell growth, purified high- or low-density B cells were assayed for [3H]thymidine incorporation for 72 h in the presence or absence of IL-4 or IL-10 alone or in combination with anti-IgM and/or anti-CD40. Stimulation with IL-4 or IL-10 alone did not result in any significant proliferation in high- or low-density B cells (Fig. 5). Moreover, activation with either anti-IgM or anti-CD40 alone or in combination did not result in any significant proliferation. However, in low-density B cells, maximal proliferation in response to IL-4 or IL-10 occurred when these cells were coactivated with anti-IgM and anti-CD40 (Fig. 5). This robust proliferative response to IL-4 after coactivation correlates with the reduced apoptosis observed in Fig. 4B and supports the idea that low-density B cells require a costimulatory signal to respond to IL-4. Conversely, under conditions of coactivation, IL-4, but not IL-10, was capable of inducing significant proliferation in high-density B cells (Fig. 5). It is important to point out that these experiments have been repeated several times and that these results are consistent with our published findings (22). Thus, the data presented in Figs. 3–5 suggest that IL-4 can act as a survival factor for high-density B cells without inducing cellular proliferation.

Discussion

This study demonstrates that IL-4 promotes the in vitro survival of resting naive, but not GC/M, human B cells and that the cell survival is not associated with concurrent growth stimulation. A possible mechanism for this unique IL-4 activity on resting naive B cells appears to be a combination of a significantly higher display of IL-4Rα chain and IL-4 signaling competence. This notion is supported by the observations that despite significant display of its receptor on naive B cells (Fig. 2), IL-10 did not protect these cells from apoptosis (Fig. 3). IL-10 has been shown to prevent spontaneous death in resting GC B cells (35, 36). However, our data revealed only a partial and transient protection of GC/M B cells by this cytokine (Fig. 3). It is possible that resting GC and M cells will show a distinct response to IL-10 and thus a mixture of these subpopulations will only partially respond to IL-10.

Resting naive B cells may indeed differ from GC/M counterparts in their survival, growth, and cytokine signaling. Indeed, naive B cells undergo apoptosis in a delayed manner as compared with GC/M B cells (Fig. 3). Furthermore, we recently reported that naive B cells, when activated through CD40 and IgM cross-linking, significantly responded to only IL-4 for growth stimulation.
In these experiments, IL-4 was able to induce cyclin D3 and eliminate cyclin-dependent kinase inhibitor p27. In resting naive B cells, the absence of IL-4-mediated growth stimulation could be attributed to the maintenance of p27 (37). Thus, it appears that a significant expression of the IL-4R \( \alpha \) chain on resting naive B cells may be a part of hierarchy to certain cytokines. This notion is supported by defective expression of functional IL-4R on Ag-experienced IgE-producing B cells (38). Furthermore, recent observations by Corcione et al. (39) that naive, GC, or M B cell subsets differ in their selective utilization of TNF-\( \alpha \) receptor strengthens this hypothesis.

IL-4 is a key cytokine that induces growth in activated B cells, promotes Ab responses, and polarizes CD4 T cell functions (40–43). All of these effects strictly depend on the signal transduction through binding to the IL-4R, which is composed of at least two chains, IL-4R\( \alpha \) (140-kDa) chain and IL-2R\( \gamma \) (75-kDa) chain (44, 45). The data shown here present a paradigm in that although naive B cells express a complete receptor complex, IL-4-mediated protection of these cells from apoptosis occurs in the absence of DNA synthesis (Figs. 3 and 5). In this regard, it has been recently demonstrated that distinct sequence motifs within the cytoplasmic tail of the IL-4R are involved in regulating protection from apoptosis and cell growth (46–48). Moreover, inhibition of IL-4-mediated apoptosis does not depend on STAT6 activation (46, 47). In agreement with these observations, IL-4 has been shown to protect resting T cells in a STAT6-independent manner (33). Thus, it is possible that in resting naive B cells, IL-4-mediated apoptosis inhibition involves mechanisms independent of STAT6-docking regions. These studies are currently being investigated in our laboratory.

What is the biological significance of IL-4-mediated survival of naive B cell pool? Surprisingly, IL-4 knockout mice do not exhibit any gross aberrations in B cell functions and GC architecture, except for the lack of IgE production and gut mucosal Ab responses.

**A. High Density**

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**B. Low Density**

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**FIGURE 3.** Analysis of apoptosis by propidium iodide staining in high-density B cells (A) and low-density B cells (B). Unstimulated B cells were cultured in either the presence or absence of IL-4 (20 ng/ml) or IL-10 (10 ng/ml) for 72 h. Cell nuclei were stained with propidium iodide and the cells were subjected to cell cycle analysis by FACScan. The hypodiploid cellular mass corresponding to apoptotic nuclei was quantitated. Peaks representing G1, S, and G2-M phases of the cell cycle are indicated.
isolation or culturing alone, respectively. B, [3H]thymidine for an additional 16 h. Cells were harvested and DNA synthesis was measured by [3H]thymidine incorporation. All experiments were done in triplicate and with at least three different tonsillar B cell preparations.

In contrast, chronic overproduction of IL-4 is thought to be associated with humoral autoimmunity in mice (50), suggesting increased B cell numbers and/or responses associated with the autoimmune pathology. One explanation for normal B cell survival and maintenance in the absence of IL-4 is the functional presence of IL-13. Interestingly, the expression of IL-13 receptor α1-chain was found to be the highest on naive B cells (51). Thus, simultaneous presence of IL-4 and IL-13, and other redundant B cell cytokines may result in poor B cell viability, in particular naive B cells. Another important role of IL-4 in naive B cell biology may be its unique participation in inducing their migration (52). Thus, it is possible that by protecting naive B cells from apoptosis and by inducing their migration, IL-4 increases the number of naive B cells in a site-specific manner. Indeed, a very recent report clearly suggests that IL-4 increases splenic B cell number through enhanced migration of circulating B cells and increased B cell life span (53).

In summary, our data indicate that resting naive and GC/M human B cells exhibit intrinsic differences as demonstrated by their disparate IL-4R expression as well as differential propensity for apoptosis and survival in responses to IL-4 and IL-10. Importantly, our data suggest that IL-4 may be a crucial regulator of the increased presence of resting B lymphocytes, particularly naive B cell subpopulation, in lymphoid organs.

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