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B Lymphocyte-Derived IL-16 Attracts Dendritic Cells and Th Cells

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Interaction of B lymphocytes with Th cells is a fundamental step in the establishment of humoral immunity, and recent evidence suggests that direct interaction between B lymphocytes and dendritic cells (DCs) is also an important prerequisite. Factors involved in the selective recruitment of Th cells and DCs by B lymphocytes are insufficiently defined. We set out to delineate the role of IL-16, the soluble ligand of CD4, which is expressed on Th cells and DCs. B lymphocytes express IL-16 mRNA and synthesize bioactive IL-16 protein, and IL-16 is expressed in lymph node follicles in situ. B lymphocyte supernatant efficiently induces migration of CD4+ Th cells, monocyte-derived DCs, and circulating blood DCs in nitrocellulose filter-based assays. Neutralization of IL-16 bioactivity strongly inhibits this migratory response, suggesting that IL-16 might be a major chemotactic factor derived from B cells. The present data further support the idea that IL-16 might have a role in the initiation of cellular as well as humoral immunity by mediating the cellular cross-talk among T lymphocytes, B cells, and DCs, leading to recruitment of these cell types at common anatomical sites. The Journal of Immunology, 2000, 165: 2474–2480.
pure B cells (>99%, as determined by cytofluorometry) were intensely washed and cultured at a density of 1 × 10^6 (for ELISA experiments) or 1 × 10^5 (for Northern and Western experiments)/ml in CM for the indicated period of time.

For the generation of B cell supernatant for chemotaxis assays, 1 × 10^7 purified B cells/ml were cultured in RPMI 1640/0.5% BSA as follows: 1) for 24 h without addition of activating agents; 2) for 12 h on anti-CD40 mAb-coated (2 µg/ml, overnight) 48-well plates and addition of IL-4 (1000 U/ml), subsequent intense washing, and further culture for 24 h without activating agents; and 3) for 24 h with addition of anti-CD40-coated microbeads (5 µg/ml; Irvine Scientific, Santa Ana, CA) (17).

**Purification of circulating blood cells**

After washing three times with 0.5% (w/v) Triton X-100 and 0.1% Tween 20 dissolved in 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl, Bioplex standards, and 60 µg/ml rhuIL-16. Subsequently, streptavidin-peroxidase was added according to the manufacturer’s instructions. Tetramethylbenzidine (BM Blue POD substrate, Roche) was used as substrate and measured at 450 nm in an ELISA reader. The standard curve was prepared with rhIL-16 in a concentration range from 15.63 to 2000 pg/ml.

**Cytotoxicity assay**

Tissue sections of human tonsils were acquired from excess tissue obtained during elective surgery. Serial sections were fixed in 2% paraformaldehyde in Tris buffer. For blocking of specific binding sites of the pAb, 3,3'-diaminobenzidine (Sigma). Slides were counterstained with hemalun. Identification of B lymphocytes was performed by anti-CD20 mAb according to standard protocols.

**Chemotaxis assay**

Migration of cells into collagen matrix along gradients of soluble attractants was measured using a 48-well microchemotaxis chamber (Neu probe, Bethesda, MD) in which a 5-µm (for lymphocytes) or an 8-µm (for DCs) pore size filter (Sartorius, Gottingen, Germany) separates the upper and lower chamber. Thirty-microliter aliquots of chemotactic solution or control medium (RPMI 1640/0.5% BSA) were added to the lower wells of the chamber, then the filter was carefully layered onto the wells and incubated for 4 h at 37°C. After incubation, the filter was fixed, stained with crystal violet, and analyzed under a microscope. Data analysis was performed with CellQuest software (Becton Dickinson).
ratios between the distances of directed and undirected migration of DCs and T lymphocytes into the nitrocellulose filters.

**Statistical analysis**

ELISA data and chemotaxis data are expressed as the mean ± SEM. The significance of ELISA data was determined by Student’s t test for paired samples using the SPSS software package (SPSS, Chicago, IL). Means of the CI were compared by Kruskal-Wallis ANOVA and Mann-Whitney U test. A difference with p < 0.05 was considered significant. Calculation was performed using the StatView software package (Abacus Concepts, Berkeley, CA).

**Results**

*B lymphocytes are a source of IL-16*

Highly purified B lymphocytes were obtained by positive selection of CD19+ cells employing magnetic beads. Obtained B cells were >99% pure as determined by cytofluorometry and staining for CD3, CD14, CD16, CD19, and CD56 (data not shown). B lymphocytes did not express the activation Ag CD69 either immediately after isolation or after 24 h of culture in medium alone (data not shown), thus showing that the isolation procedure does not induce cell activation. mRNA analysis revealed constitutive expression of IL-16 in freshly isolated B cells (Fig. 1). Western analysis of cell extracts derived from freshly isolated purified B lymphocytes showed a single band at ~80 kDa, representing mature IL-16, but no band at ~20 kDa as would be expected for bioactive cleaved IL-16 (Fig. 2), suggesting that cleaved IL-16 is readily secreted, rather than stored, in B lymphocytes. Corresponding to these data, intracellular FACS analysis showed preformed IL-16 in the CD19+ subpopulation (i.e., B cells) of freshly isolated PBMC (Fig. 3). Stimulation of purified B cells for 3 h with various agents showed that IL-4, IFN-γ, and SACS down-regulate IL-16 mRNA expression. IL-10, IL-6, and anti-CD40 had no effect (Fig. 1). Twenty-four-hour culture of unstimulated purified B cells at a density of 1 × 10⁶/ml resulted in IL-16 synthesis of 506 ± 61 pg/ml. Analysis of IL-16 protein synthesis after culture with various cytokines for various time periods did not show statistically significant changes compared with unstimulated B cells, thus demonstrating no correlation between IL-16 mRNA regulation and IL-16 protein synthesis (Table I). ELISA performed after depletion of supernatants from pro-IL-16 with anti-pro-IL-16 pAb by affinity chromatography yielded identical results, showing that all IL-16 detected in the supernatant is bioactive, mature IL-16 (data not shown). In addition, a dose response for IL-16 chemotaxis was not altered by the column (data not shown).

**B lymphocytes in tonsil lymphatic tissue express IL-16 protein**

Immunohistochemistry was performed on serial tissue sections of tonsils obtained during elective surgery. B lymphocytes were identified by CD20 staining (Fig. 4A). IL-16 staining was most pronounced in the mantle zone and less intense in the germinal center (GC; Fig. 4, B and C). Fig. 4D proves specificity of IL-16 staining by preincubation of the IL-16 mAb with rhuIL-16.

*IL-16 is a major constituent of chemotactic activity of B cell supernatant toward CD4+ Th lymphocytes*

Supernatants of unstimulated B lymphocytes (1 × 10⁶/ml) and IL-4/anti-CD40-stimulated B cells were tested for chemotactic activity toward CD4+ Th lymphocytes. Migration data are expressed as the depth of migration into nitrocellulose filters induced by B cell supernatant compared with migration induced by medium alone (CI). Undiluted supernatant from unstimulated B cells resulted in a CI of 1.615 ± 0.077 (Fig. 5). Even a 1000-fold dilution of this supernatant produced significant CD4+ T cell migration (CI, 1.295 ± 0.093; Fig. 5). To evaluate the relative contribution of...
IL-16 in chemotactic activity of supernatant from unstimulated B cells, we added neutralizing IL-16 mAb and found that CI was reduced almost to background levels (Fig. 5). The inhibitory activity of the mAb was specific for IL-16, because migration of Th cells toward recombinant MCP-3 was not inhibited. Because CD4+CD11c+ circulating blood DCs have been suggested as the DC population migrating into lymph node follicles subsequently constituting germinal center DCs (20), we tested B cell supernatant for its chemotactic activity on circulating blood DCs. Circulating blood DCs were obtained by depletion of CD3+, CD11b+, CD16+, and CD19+ cells employing immunomagnetic microbeads. The resulting cell population had a purity of >95% as determined by cytofluorometry for lineage markers and HLA-DR. Extensive chemotaxis analysis was impossible due to the very low frequency of this cell type in circulating blood. However, undiluted B cell supernatant efficiently induced migration of circulating blood DCs (CI, 1.64 ± 0.08), which could substantially be blocked by adding neutralizing anti-IL-16 mAb (CI, 1.26 ± 0.04; Table III). As shown in Table III, rhuIL-16 induced significant migration of DCs, which could be blocked by anti-IL-16 mAb as well.

### Table 1. IL-16 synthesis in B lymphocytes

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Mean ± SEM</th>
<th>p Value</th>
</tr>
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<tbody>
<tr>
<td>Medium alone</td>
<td>506 ± 61</td>
<td></td>
</tr>
<tr>
<td>PMA 10 ng/ml</td>
<td>552 ± 101</td>
<td>0.674</td>
</tr>
<tr>
<td>Ionomycin 500 ng/ml</td>
<td>708 ± 144</td>
<td>0.273</td>
</tr>
<tr>
<td>PMA/ionomycin</td>
<td>593 ± 98</td>
<td>0.480</td>
</tr>
<tr>
<td>LPS 10 μg/ml</td>
<td>638 ± 131</td>
<td>0.481</td>
</tr>
<tr>
<td>SACS 1:5000</td>
<td>590 ± 147</td>
<td>0.920</td>
</tr>
<tr>
<td>IL-4 1000 U/ml</td>
<td>651 ± 147</td>
<td>0.425</td>
</tr>
<tr>
<td>IL-6 100 U/ml</td>
<td>478 ± 117</td>
<td>0.427</td>
</tr>
<tr>
<td>IL-10 100 U/ml</td>
<td>533 ± 60</td>
<td>0.779</td>
</tr>
<tr>
<td>IFN-γ 100 U/ml</td>
<td>769 ± 231</td>
<td>0.301</td>
</tr>
<tr>
<td>Anti-CD40 1 μg/ml</td>
<td>711 ± 119</td>
<td>0.148</td>
</tr>
<tr>
<td>Anti-CD40 + IL-4</td>
<td>1171 ± 345</td>
<td>0.127</td>
</tr>
<tr>
<td>Anti-CD40 + IFN-γ</td>
<td>826 ± 164</td>
<td>0.998</td>
</tr>
</tbody>
</table>

* Purified B cells (1 × 10⁶ per ml) were cultured for 24 h with addition of indicated agents. Supernatants were assayed for total IL-16 (pg/ml) by ELISA. Pro-IL-16 was not detected in the same samples, suggesting that all IL-16 detected is cleaved IL-16. Significance was calculated by Student’s t test for paired samples for the comparison of B cells stimulated with indicated agents compared to B cells cultured in medium alone. Various other time points (2, 4, 6, 12, 48, and 72 h) were evaluated, but not statistically significant regulation was observed (n = 7).

**IL-16 is a major constituent of chemotactic activity of B cell supernatant toward DCs**

To gain clues as to whether B cells secrete factors attracting DCs, cell supernatant was tested for chemotactic activity on highly purified monocyte-derived DCs. Supernatant from unstimulated B cells efficiently attracted monocyte-derived DCs, with a maximum CI of 1.634 ± 0.060 (Fig. 5). Significant migration was even noted at a 100-fold dilution (CI, 1.325 ± 0.058). To identify the role of IL-16, we added neutralizing anti-IL-16 mAb and found that migration was almost completely inhibited (CI, 1.099 ± 0.037 with undiluted supernatant). As shown in Fig. 5, the inhibitory effect of the mAb was specific for IL-16, because migration toward recombinant MCP-3 was not inhibited.

Furthermore, we tested supernatant from anti-CD40/IL-4-stimulated B cells as shown in Table II. The relative contribution of IL-16 to the chemotactic response was 61% as determined by neutralization experiments. Similar results were obtained with supernatant from B lymphocytes stimulated with anti-IgM-coated microbeads (data not shown).

**FIGURE 4.** IL-16 immunohistochemistry. IL-16 synthesis was studied in serial tissue sections of human tonsil lymphatic tissue obtained routinely during elective surgery. A, Identification of B lymphocytes within lymph follicles by CD20 staining. Magnification, ×200. B, IL-16 reactivity in lymphatic tissue detected by anti-IL-16 pAb. IL-16 staining is most pronounced in the mantle zone and less intense in the GC. Magnification, ×200. C, IL-16 reactivity in the GC. Magnification, ×400. D, Blocking of IL-16 reactivity by preincubation of IL-16 pAb with rhuIL-16. Magnification, ×200.
B cell supernatant is chemotactic for CD8⁺ T cells independently of IL-16

To provide further evidence of the specificity of IL-16 we tested B cell supernatant for its chemotactic properties for CD8⁺ T lymphocytes (e.g., a CD4⁻ cell type). As shown in Table II, supernatant derived from unstimulated B cells as well as from anti-CD40/IL-4-stimulated B cells was chemotactic for CD8⁺ T lymphocytes. However, addition of neutralizing IL-16 mAb did not inhibit the migratory response (Table II).

Discussion

Herein we report that human B cells are a source of IL-16. We provide evidence that freshly isolated B cells contain IL-16 mRNA as determined by Northern analysis. Western analysis of cellular extracts derived from freshly isolated B cells revealed an ~80-kDa band, representing pro-IL-16. In accordance with previous reports (2, 3, 22), we suggest that bioactive 20-kDa IL-16 is readily released into supernatant rather than stored in B lymphocytes. Intracellular FACS staining of PBMC confirmed that the CD19⁺ B cell population stains positively for IL-16. Culture of highly purified B cells for various time periods revealed constitutive synthesis and release into supernatant of cleaved, biologically active IL-16. This was not due to cellular activation during the isolation procedure, because B cells did not express the activation Ag CD69 immediately after isolation or after 24 h in culture in medium alone, while addition of PMA/ionomycin efficiently up-regulated CD69 (data not shown). Investigating modulation of IL-16 mRNA expression and protein synthesis by various cytokines, we noted that some regulation occurs at the mRNA level, while no significant changes in IL-16 release into supernatant was noted. This underscores the importance of post-transcriptional and post-translational mechanisms in regulating IL-16 release (3).

Immunohistochemistry of tonsil lymphatic tissue demonstrated IL-16 staining in CD20⁺ B lymphocytes in the mantle zone as well

Table II. Chemotaxis with supernatant derived from activated B cells

<table>
<thead>
<tr>
<th>Migrating Cell Type</th>
<th>B Cell Stimulation</th>
<th>CI ± SEM</th>
<th>p</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4⁺ T cells</td>
<td>Anti-CD40/IL-4-stimulated plus IL-16 mAb</td>
<td>1.584 ± 0.070</td>
<td>&lt;0.05</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Unstimulated</td>
<td>1.222 ± 0.043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocyte-derived DC</td>
<td>Anti-CD40/IL-4-stimulated plus IL-16 mAb</td>
<td>1.540 ± 0.082</td>
<td>&lt;0.05</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Unstimulated</td>
<td>1.212 ± 0.075</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8⁺ T cells</td>
<td>Anti-CD40/IL-4-stimulated plus rhuRANTES</td>
<td>1.442 ± 0.048</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unstimulated</td>
<td>1.280 ± 0.057</td>
<td>NS</td>
<td>No inhibition</td>
</tr>
<tr>
<td></td>
<td>Anti-CD40/IL-4-stimulated plus IL-16 mAb</td>
<td>1.345 ± 0.040</td>
<td></td>
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</tbody>
</table>

* Purified monocyte-derived DCs, CD4⁺, and CD8⁺ T lymphocytes were subjected to chemotaxis into nitrocellulose filters toward undiluted supernatant derived from anti-CD40/IL-4-stimulated B lymphocytes as described in Materials and Methods. For CD8⁺ T lymphocytes, migration toward unstimulated B cell supernatant and rhuRANTES (20 ng/ml) is additionally included. Results are expressed as CI ± SEM, and the effect of IL-16 neutralization by mAbs (10 µg/ml) is expressed as percent inhibition of the migratory response (n = 3). Statistical analysis evaluates the significance of addition of neutralizing anti-IL-16 mAb. A value of p < 0.05 was considered statistically significant. Similar results were obtained with supernatant derived from anti-μlgM-coated microbead-stimulated B cells.
as in the GC. The weaker IL-16 staining in the GC compared with that in the mantle zone might reflect enhanced release of IL-16 from B cells and consecutive depletion of intracellular stores. Although we did not observe up-regulation of the activation marker CD69 on unstimulated B cells cultured for 24 h as described in the previous paragraph, we cannot definitely exclude the possibility that B cells release IL-16 spontaneously only in the in vitro culture system and not in quiescent state in vivo. This might explain the absence of CD4^+ T lymphocytes and DCs in the mantle zone, in contrast to the GC, where both cell types are found and probably recruited by IL-16 derived from B lymphocytes.

To determine factors involved in the selective attraction of Th cells and DCs toward B cells, we tested supernatant derived by 24-h culture of highly purified B lymphocytes for its ability to induce the migration of Th cells and DCs into nitrocellulose filters. This assay has previously been employed in the evaluation of IL-16 as a chemotactic factor for DCs (2). For reasons of comparison, we demonstrated IL-16-induced migration of DCs through polycarbonate filters, showing similar results and confirming the reliability of our method (2). We furthermore provided evidence that the migratory activity of IL-16 toward monocyte-derived DCs is chemotactic rather than chemokinetic (2). Herein we demonstrate that B cell supernatant efficiently attracts CD4^+ T lymphocytes, monocyte-derived DCs, and circulating blood DCs. To delineate the role of IL-16 in chemotactic activity of B cell supernatant, we neutralized IL-16 bioactivity by adding anti-IL-16 mAb and found the migratory response almost completely abrogated with supernatant derived from unstimulated B cells. This was true for CD4^+ T lymphocytes, monocyte-derived DCs, and circulating blood DCs. To evaluate the influence of cellular activation of B lymphocytes and the consecutive up-regulation of various chemokines, we tested supernatant of anti-CD40/IL-4- and B cell Ag receptor-stimulated B cells as well. Neutralization of IL-16 by mAbs resulted in a decrease in migration of 62% for CD4^+ T lymphocytes and 61% for monocyte-derived DCs. This provides strong evidence that IL-16 remains the predominant chemotactic cytokine derived from B cells despite the activation-induced up-regulation of other chemokines (17, 23–25). Together, these data provide evidence that IL-16 derived from B cells might be a major factor in the cellular interactions required for the initiation of humoral immune responses.

In the primary immune response, T cells form small clusters with DCs in the T cell areas of lymphoid organs upon encountering of Ag (26–28). At this time, B cells are still randomly distributed throughout the follicles (26). B cells responding to Ag undergo arrest in the outer T cell zone and proliferate in response to ligation of a critical number of B cell Ag receptors (29). In a next step, T and B lymphocytes leave their respective compartments, migrate to the edge of the B cell-rich follicles and establish cognate T-B cell interactions (26), leading to the formation of GCs (29–31). Therefore, maturation of the B cell response depends on the availability of primed T cells and the ability of Ag-specific B cells to selectively recruit them. Recent evidence suggests that direct B cell-DC interactions are also a prerequisite for establishing humoral immunity (10–16, 32, 33). However, the accurate sequence of events in DC-B cell-T cell cooperation in vivo has yet to be established. This includes another distinct cell population implicated in B cell maturation, namely follicular DCs (34–37). The presence of GC DCs within primary follicles and the fact that they display a phenotype similar to CD4^+CD11c^+ blood DCs (20) suggest that DCs originating from blood colonize primary follicles. In line with this model are recent data showing the progressive localization of DCs to B cell follicles during a primary immune response (32). However, in that study migrating DCs where characterized as CD4^+ by immunohistochemistry, but this could be due to the low sensitivity of this method compared with that of cytofluorometric analysis. The elucidation of factors involved in the migratory pathway of these cell populations is of critical interest (38). Our in vitro migration data are further corroborated by demonstrating IL-16 expression in B cells in lymph follicles by immunohistochemistry, suggesting an important role in vivo.

Although it has been long established that Th/B cell interaction is required for the efficientelicitation of a humoral immune response, few reports have focused on the discovery of factors derived from B cells mediating the selective attraction of T cells (9). Schaniel et al. reported a novel chemokine, ABCD-1, derived from activated murine B lymphocytes and DCs that selectively acts on activated T cells (39). The human orthologue, STCP-1 (MDC), acts specifically on chronically activated Th2 lymphocytes (23, 24), ABCD-2, which acts via CCR4, as does ABCD-1, has also been proposed as a factor attracting T lymphocytes toward activated B cells (25). Macrophage inflammatory protein-1β has been reported to be derived from Ag receptor-triggered B lymphocytes and to partly account for T cell chemotaxis toward activated B cells (17). IL-16 is theoretically a well-suited cytokine to meet the biological requirement of selectively attracting CD4^+ Th cells, because it acts via CD4. Regarding the recently discovered direct interaction between DCs and B cells, no factors involved in DC attraction by B cells besides the herein reported IL-16 have been identified to date to our knowledge. We conclude that IL-16 could orchestrate the interaction of Th cells, B cells, and DCs during the induction phase of an immune response.

### References


### Table III. Circulating DC chemotaxis

<table>
<thead>
<tr>
<th>Chemotactrant</th>
<th>Mean</th>
<th>SEM</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>1.000</td>
<td>0.000</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td>+ mAb (10 μg/ml)</td>
<td>1.096</td>
<td>0.038</td>
<td></td>
</tr>
<tr>
<td>IL-16 (10 ng/ml)</td>
<td>1.612</td>
<td>0.079</td>
<td>0.029</td>
</tr>
<tr>
<td>+ mAb (10 μg/ml)</td>
<td>1.149</td>
<td>0.051</td>
<td></td>
</tr>
<tr>
<td>Supernatant (1:1)</td>
<td>1.637</td>
<td>0.082</td>
<td>0.029</td>
</tr>
<tr>
<td>+ mAb (10 μg/ml)</td>
<td>1.256</td>
<td>0.038</td>
<td></td>
</tr>
</tbody>
</table>

*Circulating blood DCs were purified and subjected to chemotaxis assay as described in Materials and Methods. Migration of DCs into nitrocellulose filters toward thiol-16 and B cell supernatant is compared to migration induced by medium and expressed as CT ± SEM. Statistical analysis evaluates the significance of addition of neutralizing anti-IL-16 mAb. A value of p < 0.05 was considered statistically significant.*


