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IL-7–Dependent B Lymphocytes Are Essential for the Anti-polysaccharide Response and Protective Immunity to Streptococcus pneumoniae

Anne K. Shriner,*1 Hongqi Liu,*1 Guizhi Sun,* Martin Guimond,† and Kishore R. Alugupalli*

Young children are impaired in their response to T cell-independent (TI) Ags, such as pneumococcal polysaccharide (PPS). B lymphopoiesis early in life is IL-7 independent, whereas in adults it is IL-7 dependent. Therefore, we hypothesized that IL-7–driven B lymphopoiesis plays a critical role in promoting Ab responses to TI Ags. Young but not adult mice are impaired in responses to PPS vaccination and to 4-hydroxy-3-nitrophenyl-acetyl-Ficoll, a widely studied model TI Ag, and B1b cells generate Ab responses to these Ags. In this paper, we show that, despite having B1b, B1a, and MZ B cells—all of which are involved in TI responses—young wild-type or adult mice deficient either in IL-7 or in IL-7R are severely impaired in anti-PPS responses and do not survive Streptococcus pneumoniae challenge, indicating IL-7–dependent B cells are required for TI immunity. Consistent with this, PPS immunization induced a robust TI response in young IL-7 transgenic mice that was comparable to adult wild-type responses. Moreover, immunized young or adult IL-7 transgenic mice were completely resistant to S. pneumoniae challenge. Our data indicate that activating the IL-7 signaling pathway could restore impaired TI responses in the young. The Journal of Immunology, 2010, 185: 525–531.

Moreover, immunized young or adult IL-7 transgenic mice were completely resistant to S. pneumoniae challenge. Our data indicate that activating the IL-7 signaling pathway could restore impaired TI responses in the young. The Journal of Immunology, 2010, 185: 525–531.
The unique, age-specific roles of Flt3L and IL-7 in B lymphopoiesis and the impaired TI Ab response of young mice despite the presence of B1b, B1a, and MZ B cells led us to hypothesize that IL-7–dependent B cells are required for the response to PPS in adults. In the current study, we show that B lymphopoiesis driven by IL-7 is essential for TI immunity.

Materials and Methods

Mice

The Institutional Animal Care and Use Committee have approved these studies. Mice were housed in microisolator cages with free access to food and water and were maintained in a specific pathogen-free facility. C57BL/6J (wild-type) and B6.129S7-IL-7rtm1Imx/J (IL-7Rα−/−) were originally generated by Dr. R. Ceredig (Institut National de la Sante et de la Recherche Medicale, Strasbourg, France) and distributed to the lack of MZ B cells (8, 14). However, 3 to 4-wk-old mice were harvested from individual mice and the cell concentration adjusted to 2.5 × 10^7/ml in staining medium (deficient RPMI 1640 medium [Irvine Scientific, Santa Ana, CA] with 3% new calf serum, 1 mM EDTA). After blocking FcRs with 2.4G2 Ab (1 µg/10^6 cells), an aliquot of 25 µl peritoneal cavity cells was incubated in a microtiter plate with appropriately diluted Ab. To determine the frequency of FO and MZ B cells, 25 µl spleen cells was stained with appropriate Abs. The Abs anti–IgM-FITC (clone B4.B1), anti–Mac1-allophycocyanin (clone M1/70), and anti–CD5-PerCP (clone 53-7.3) were purchased from eBioscience (San Diego, CA); anti–CD23-PE (clone B3B4) and anti–CD21-FITC (clone 7G6) were from BD Biosciences. After staining, cells were washed twice with staining medium and the preparations were analyzed on a FACScalibur (BD Biosciences, San Jose, CA) using the CellQuest software (BD Biosciences). Data were analyzed using the FlowJo software program (Tree Star, San Carlos, CA).

Statistical analysis

Statistics were performed using the Prizm 5 software program (GraphPad, La Jolla, CA). To determine statistically significant differences, the Student’s unpaired t test was performed and two-tailed p values were given.

Results

B1b, B1a, and MZ B cells are present in young mice

Impaired responses in neonates (9–12 d old) to TI-2 Ags can be attributed to the lack of MZ B cells (8, 14). However, 3 to 4-wk-old infants. Flow cytometry

To determine the frequency of B1a and B1b cells, peritoneal cavity cells were harvested from individual mice and the cell concentration adjusted to 2.5 × 10^7/ml in staining medium (deficient RPMI 1640 medium [Irvine Scientific, Santa Ana, CA] with 3% new calf serum, 1 mM EDTA). After

Table I. Mice deficient in IL-7 signaling are sufficient in B1b, B1a, and MZ B cell subsets

<table>
<thead>
<tr>
<th></th>
<th>B1b (×10^6)</th>
<th>B1a (×10^6)</th>
<th>MZ B (×10^6)</th>
<th>FO B (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>23.8 ± 3.7</td>
<td>39.4 ± 3.5</td>
<td>0.13 ± 0.055</td>
<td>11.58 ± 2.57</td>
</tr>
<tr>
<td>IL-7−/−</td>
<td>39.1 ± 21.9</td>
<td>17.7 ± 8.2</td>
<td>0.058 ± 0.019</td>
<td>0.11 ± 0.033</td>
</tr>
<tr>
<td>IL-7Ra−/−</td>
<td>1.6 ± 0.6</td>
<td>0.35 ± 0.14</td>
<td>0.008 ± 0.001</td>
<td>0.02 ± 0.012</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>142.2 ± 40.9</td>
<td>111.9 ± 75.6</td>
<td>1.74 ± 0.56</td>
<td>24.21 ± 7.3</td>
</tr>
<tr>
<td>IL-7−/−</td>
<td>66.5 ± 36.3*</td>
<td>17.9 ± 8.5*</td>
<td>0.96 ± 0.39*</td>
<td>1.03 ± 0.67*</td>
</tr>
<tr>
<td>IL-7Ra−/−</td>
<td>60.2 ± 27.3*</td>
<td>13.6 ± 9.9*</td>
<td>0.06 ± 0.034*</td>
<td>0.11 ± 0.082*</td>
</tr>
</tbody>
</table>

Cell counts of B1b and B1a in the peritoneal cavity and MZ B and FO B cells in the spleen of young (3 wk old) or adult (8–14 wk old) mice were determined. Statistically significant differences between Wt and mutant mice are indicated.

*p < 0.05; †p < 0.01; ‡p < 0.001; ns, not statistically significant.

Wt, wild-type.
mice are still unable to respond to TI Ags (7), despite having a developed MZ B cell compartment (Table I). Because B1b cells mount anti-PPS and anti–NP-Ficoll Ab responses (5, 21), we examined the peritoneal cavity of young (3 wk old) and adult (8–14 wk old) mice for B1b cells. Young C57BL6 mice had all the mature B cell subsets, including B1b cells, involved in TI responses, although the numbers were reduced compared with adult mice (Table I). The reduction in B1b, B1a, and MZ B cell numbers in young mice can be attributed to the physically smaller coelomic cavity and spleen, respectively.

**Mice deficient in IL-7 signaling are sufficient in B1b, B1a, and MZ B cell subsets**

B1b cells generated early in life are derived from IL-7–independent fetal liver B lymphopoiesis (28, 36, 37). B1b cells are also generated efficiently from adult bone marrow B lymphopoiesis (36, 37) that is presumably driven by IL-7 (28, 29). To examine the role of IL-7 signaling in the ontogeny of all B cell subsets involved in TI responses, we analyzed mice deficient either in IL-7 or in IL-7Rα.

IL-7−/− mice generated B1b cells with a higher frequency than wild-type mice (Fig. 1), although the absolute numbers were ∼2-fold less than wild-type levels (Table I). These results demonstrate that efficient B1b lymphopoiesis occurs in the absence of IL-7.

**Figure 2.** Pneumovax23 immunization neither elicits an Ag-specific Ab response nor confers protection against *S. pneumoniae*. 

A. Adult or young wild-type, IL-7−/−, and IL-7Rα−/− mice were immunized i.p. with 10 μg Pneumovax23. Blood samples were obtained on 0, 7, and 14 d postimmunization, and Pneumovax23-specific IgM or IgG3, PPS3-, or PPS14-specific IgM levels were determined by ELISA. Each dot represents a mouse, and the mean Ab levels are indicated with a solid line. Statistically significant differences between preimmune and immune mice are indicated.

B. Four weeks following Pneumovax23 immunization, mice were challenged with 5000 CFU *S. pneumoniae* WU2 (serotype 3), and survival was monitored. Survival statistics were performed using log-rank test, and *p* values are given.
or to PPS3 or PPS14, two of the PPS serotypes present in the vaccine. We found that young but not adult wild-type mice are impaired in responding to Pneumovax23, PPS3, or PPS14 (Fig. 2A) and die upon challenge with *S. pneumoniae* strain WU2 (PPS3 serotype) (Fig. 2B). To examine whether developmentally distinct B cells contribute to TI responses in adults, we immunized adult mice deficient in IL-7 or IL-7Rα signaling with Pneumovax23. Despite the presence of all TI B cell subsets including B1b cells (Fig. 1, Table I), IL-7− or IL-7Rα−deficient mice were incapable of mounting specific responses to Pneumovax23, PPS3, or PPS14 (Fig. 2A). These results demonstrate that IL-7−dependent B cells are crucial for generating an anti-PPS response.

IL-7Rα−deficient mice had higher preimmune levels of Pneumovax23- and PPS3-reactive IgM than the wild-type mice, but the increase in specific Abs upon immunization in these mice was not statistically significant (Fig. 2A). Furthermore, the increase in the PPS-specific IgM response in IL-7−/− mice upon immunization was minimal. IgG3 is also a major Ig isotype generated during TI responses in mice (40). Immunized adult wild-type mice but not adult IL-7−/− or IL-7Rα−/− or young wild-type mice generated a robust anti-Pneumovax 23-specific IgG3 response (Fig. 2A). To test whether this increase and/or the higher baseline reactivity (wild-type versus IL-7Rα−/−) (Fig. 2A) was optimal for protection or not, we challenged mice with *S. pneumoniae* 4 wk postimmunization. All the immunized adult wild-type mice that were able to generate a response survived the challenge (Fig. 2B). However, a majority of the immunized adult IL-7−/− or IL-7Rα−/− mice succumbed to infection (Fig. 2B), suggesting that a functional and PPS-specific IgM and IgG3 dependent on IL-7−mediated B lymphopoiesis was critical for protective immunity.

**B cells generated in the absence of IL-7 signaling are not efficient in producing an anti-NP-Ficoll response.**

To test whether IL-7−dependent B cells also mediate a specific response to another TI Ag, we immunized IL-7−/− or IL-7Rα−/− mice with NP-Ficoll. The binding to NP by preimmune IgM of adult IL-7Rα−/− mice was significantly greater than that of wild-type mice (p > 0.016) (Fig. 3). Because of the variable preimmune baseline-binding values, the postimmunization data of mutant mice were interpreted as the fold increase compared with their preimmune levels. In adult wild-type mice, NP-specific IgM levels were more than an order of magnitude higher than their preimmune IgM levels (Fig. 3). In contrast, immunized adult IL-7−/− or IL-7Rα−/− mice generated only a 2-fold increase in Ag-specific IgM responses, suggesting that IL-7−dependent B cells play a major role in generating NP-specific Abs.

**Transgenic expression of IL-7 restores a protective anti-PPS response in young mice.**

The above results demonstrated that IL-7−driven B lymphopoiesis is critical for TI responses. Although at present it is not clear how IL-7−driven B lymphopoiesis is contributing to TI responses, it is known that IL-7Rα−mediated signaling is involved in generating BCR diversity, proliferation, and differentiation during B cell development (41, 42). B cells in the young are known to have limited BCR diversity (43–45) because they are presumably generated in an IL-7−independent mechanism and therefore may have a restricted BCR repertoire that could limit the ability of young mice to recognize a wide range of Ags. To examine whether expression of IL-7 can restore the impaired responses to TI Ags in the young, we immunized young IL-7Tg mice with Pneumovax23. We found that young IL-7Tg mice generate robust IgM responses to Pneumovax23 that are comparable to adult wild-type mice (Fig. 4A). Moreover, unlike young wild-type mice (Fig. 2), Pneumovax23-immunized young IL-7Tg mice survived *S. pneumoniae* challenge (Fig. 4B). These data demonstrate that enforced expression of IL-7 can restore TI responses and protective immunity to *S. pneumoniae* in the young. Because the frequency of MZ B (46), B1a, and B1b cells (Table II) in young IL-7Tg were comparable to young wild-type mice, it is possible that overexpression of IL-7 may have induced qualitative changes in the B cells of young mice.

A majority of the NP-specific B cells in adult wild-type mice belong to B1b subset

Rag1−/− mice reconstituted with adult naive B1b cells survive if immunized with PPS3 prior to *S. pneumoniae* challenge (21).

**FIGURE 3.** IL-7−dependent *B. lymphopoeisis* is required for anti-NP-Ficoll responses in adult mice. Adult wild-type, IL-7−/−, and IL-7Rα−/− mice were immunized i.p. with 50 μg hapten NP-Ficoll. Blood samples were obtained on 0 d (preimmune) or 7 d (immune) following the immunization, and NP-specific IgM levels were determined by ELISA. Each dot represents a mouse, and the mean Ab levels are indicated with a solid line. Statistically significant differences between preimmune and immune mice are indicated. *p < 0.05; **p < 0.01.

**FIGURE 4.** Transgenic expression of IL-7 restores anti-PPS response and protective immunity in young mice. A. Wild-type adult mice and young or adult IL-7Tg mice were immunized i.p. with 10 μg Pneumovax23. Blood samples were obtained on 0, 7, and 14 d postimmunization, and Pneumovax23- or PPS3-specific IgM levels were determined by ELISA. Each dot represents a mouse and the mean Ab levels are indicated with a solid line. Statistically significant differences between preimmune and immune mice are indicated. ***p < 0.001. B. Four weeks following Pneumovax23 immunization, mice were challenged with 5000 CFU *S. pneumoniae* WU2 (serotype 3), and survival was monitored. Survival statistics were performed using log-rank test, and p values are given.
Protection of Pneumovax23-immunized adult wild-type mice upon *S. pneumoniae* challenge can be attributed to an expansion of Ag-specific B cells. The NP hapten system is widely used for studying Ag-specific B cells in a variety of murine models (47–49). Adoptive transfer of purified B cells into Rag1−/− mice implicated that peritoneal cell preparations containing B1b cells mount an anti-NP Ab response upon NP-Ficoll immunization (5). To directly identify which B cell subset contributes to the majority of the TI response, wild-type adult mice were immunized (5). To directly identify which B cell subset contributes to the majority of the TI response, wild-type adult mice were immunized with NP-Ficoll and analyzed for NP binding B cells by flow cytometry using NP-conjugated PE (NP-PE). In adult wild-type mice, ∼65% of the B cells recognizing NP were of the B1b phenotype, whereas the remaining ∼20 and ∼15% were of B2 and B1a phenotype, respectively (Fig. 5). This trend was observed in naive as well as in immunized mice (Fig. 5). The binding of NP-PE by B cells of NP-immunized mice was specific because NP conjugated to BSA (NP-BSA) competitively inhibited this binding (Fig. 5). These data support the notion that the resistance of immunized but not naive mice to *S. pneumoniae* infection is likely to be attributable to an expansion of Ag-specific B cell subsets, predominantly including B1b. The ability of purified naive B1b cells to generate anti-Pneumovax23-specific IgM upon immunization indicates that Ag-specific B1b cells do not remain quiescent but expand and differentiate into Ab-secreting cells (Supplemental Fig. 1).

**Discussion**

TI responses are an integral part of humoral immune defense against a variety of pathogenic microorganisms (1, 2). TI responses are impaired in the young but not adults. B lymphopoiesis late but not early in life is IL-7 dependent. In the current study, we show that the Ab responses to TI Ags are dependent on B cells generated by IL-7–dependent B lymphopoiesis. Importantly, we also show that TI responses in the young can be restored by IL-7.

Recent studies have revealed that B1b cells play a critical role in TI responses (14). Despite the presence of B1b cells, TI responses in the young are impaired. B1b cells are generated efficiently from precursors in fetal liver as well as in adult bone marrow (50). Because B1b cells generated during the perinatal period persist throughout life by self-renewal (36, 37), the B1b cell pool in adults is developmentally heterogeneous. B1b cells generated perinatally from fetal liver progenitors share a number of characteristics with B1a cells, including self-replenishment and feedback regulation of development (36, 37). The Abs produced by B1a and B1b cells generated early in life have limited repertoire of antigenic specificities. Such Abs are referred as natural Abs because they are generated spontaneously in naive mice and in normal individuals in the absence of apparent Ag stimulation (51). Although natural Abs are generally of low affinity to self or evolutionarily conserved Ags (52), they have been shown to play a role in the early control of pathogen burden in bacterial and viral infection models (51, 53–55). However, natural Abs do not play a critical role in clearance of *Borrelia hermsii*, a bacterial pathogen, whose elimination requires B1b cells (22). Moreover, natural Abs do not recognize Fhba, a *B. hermsii* Ag target for B1b cells (22), suggesting that B1b cells produced early in life do not contribute significantly to the anti-*B. hermsii* response (18, 56). Natural Abs alone are not sufficient to clear *S. pneumoniae* (21). Instead, a division of labor between the two B1 cell subsets is required to control *S. pneumoniae* (21, 57); natural Abs produced by B1a cells limit infection of *S. pneumoniae* by virtue of recognizing evolutionarily conserved Ags, such as phosphorylcholine (17, 58), whereas the anti–PPS-specific response generated by B1b cells are critical for protection against serotype–specific pneumococcal infection (21, 57).

The strength of BCR signaling is known to be critical for the development of B1a and B1b cells (59). Xid mice are defective in

### Table II. Frequency of mature B cell subsets in the peritoneal cavity of young IL-7Tg mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>B Cells (×10⁵)</th>
<th>%B1b</th>
<th>%B1a</th>
<th>%B2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult wild-type</td>
<td>3</td>
<td>411.1 ± 121.9</td>
<td>28.3 ± 4.1</td>
<td>17.1 ± 2.4</td>
<td>22.7 ± 2.9</td>
</tr>
<tr>
<td>Young wild-type</td>
<td>3</td>
<td>41.2 ± 16.9^o</td>
<td>35.3 ± 3.3^m</td>
<td>17.5 ± 4.0^m</td>
<td>13.3 ± 3.2^*</td>
</tr>
<tr>
<td>Young IL-7Tg</td>
<td>3</td>
<td>44.1 ± 20.9^m</td>
<td>23.0 ± 5.5^*</td>
<td>9.5 ± 1.1^*</td>
<td>26.6 ± 3.9^*</td>
</tr>
</tbody>
</table>

The phenotype of lymphocyte populations was determined by flow cytometry as described in the legend of Fig. 1 and in experimental procedures. The absolute numbers of total B cells (IgM+) in the peritoneal cavity were calculated by microscopic counting and FACS. The frequencies of B1b, B1a, and B2 cells among the total B cells were given. Significance values were determined by Student t test (two-tailed p value). The values given next to IL-7Tg were the differences between young wild-type and young IL-7Tg mice. The values given next to young wild-type mice were the differences between young wild-type and adult wild-type mice.

\*p < 0.05; /p < 0.01; ns, not statistically significant.

**FIGURE 5.** The majority of NP-specific cells in the peritoneal cavity of adult wild-type mice belong to B1b subset. Naive (*n* = 3) or NP-Ficoll–immunized (*n* = 3; 7 d postimmunization) mouse peritoneal cavity cells were stained (with anti-IgM-FITC, anti–Mac1-APC, anti–CD5-PerCP, and NP-PE) to determine Ag-specific B1b cells (IgM+, Mac1+, and CD5−), B1a cells (IgM+, Mac1−, and CD5+), and B2 cells (IgM+, Mac1+, and CD5−). Peritoneal cavity cells of NP-Ficoll immunized mice were also stained in the presence of 100-fold excess of NP-BSA. Mac-1 and CD5 gates are not shown. Frequencies NP-specific cells within the indicated B cell subsets are shown.
BCR signaling and are therefore deficient in B1a and B1b cell subsets and have reduced levels of serum IgM (60). Defective BCR signaling is also responsible for the impaired TI responses in xid mice (61). The fact that IL-7–/– or IL-7Rα–/– mice generate B1a and B1b cell subsets (Fig. 1, Table I) suggests that the impaired TI responses in these mice are not because of a defect in BCR signaling strength. Moreover, the B cells generated in the absence of IL-7Rα signaling are not deficient in generating spontaneous IgM or IgG. In fact, the basal levels of serum IgM and IgG Abs in naive IL-7–/– or IL-7Rα–/– mice are significantly higher than in wild-type mice (Supplemental Table 1). These results suggest that the impaired responses to specific TI Ags (e.g., PPS or NP-Ficoll) are not because of a defective BCR signaling.

The reason(s) for the lack of specific Ab responses to TI Ags in mice deficient in IL-7 signaling is currently under investigation. B1 (Table II) and MZ B (46) cell numbers in young IL-7Tg mice are comparable to young wild-type control mice, yet they generated a significantly more Ab response to PPS (Fig. 4). Although the B1b cells in IL-7–/– mice were reduced only by 2-fold (Table I), they did not mount anti-PPS response even after 14 d postimmunization (Fig. 2). Therefore, the inability of IL-7–/– adult mice to mount a TI response is likely to be attributable to a qualitative rather than quantitative difference in the B cells. Failure to express an extensive and suitable BCR repertoire by B cells could be a potential reason for the lack of Ag-specific Ab response not only in IL-7–/– mice but also in young wild-type mice. The B cell repertoire in adult wild-type mice represents all VH genes, even more so those, that are distal to the DJ segments (43–45). In contrast, B cells of young wild-type mice preferentially express VH genes proximal to DJ but not the major families of VH genes that are distal to DJ and known to be critical for extensive BCR diversity (43–45). Similarly, in the absence of IL-7-mediated signaling, the usage of several large families of VH genes distal to DJ in the IgH locus does not occur because of a constraint in the accessibility of distal VH genes for VDJ recombination events (41, 62). It is known that TdT, an enzyme necessary for the addition of nontemplate nucleotides between V-D and D-J segments during VDJ recombination, is not expressed early in life (63–65), and the B1a and B1b cells generated at this time of life have limited junctional diversity in their BCRs (63–65). IL-7 also appears to play a role in the expression of TdT (66). Thus, IL-7 signaling plays a critical role in generating a wide range of BCR diversity. It is possible that the B cells generated early in life or in the absence of IL-7Rα signaling might have a “hole in the BCR repertoire” that can account for the lack of recognition to a wide range of TI Ags, such as NP-Ficoll and PPS. Besides a role in generating BCR diversity, IL-7Rα is also known to transmit distinct signals for proliferation and differentiation during B lymphopoiesis (42). Therefore, IL-7–dependent B cells that develop in the bone marrow could be qualitatively distinct from the B cells generated independent of IL-7 early in life.

The TNF family of receptors, namely B lymphocyte-activating factor receptor, transmembrane activator and calcium modulator and cyclophilin ligand interactor, and B cell maturation Ag play an important role in TI humoral responses (67–69). It has been reported that decreased expression of these receptors on B cells is associated with the lack of humoral responses in neonates (15, 16). TLR stimulation can upregulate the expression of TACI on neonatal B cells. TLR agonists, such as CpG oligonucleotides, have been shown to restore anti–NP-Ficoll response in the young (15, 70). These findings suggest that in addition to IL-7–dependent B lymphopoiesis other immunostimulatory pathways, such as TLR and TACI signaling, play an important role in augmenting TI responses.

Analysis of TI responses in humans and mice revealed striking similarities in both species (7). An identical reactivity of serum from B. hermantis-infected individuals (71) and murine B1b cells (22) to FhbA indicates that humans have functional equivalents of B1b cells. The PPS vaccine generates a protective response in humans, and in the murine system, the B1b subset generates the bulk of the protective Ab response to PPS (21, 57). It is possible that the young might lack sufficient protection from a developmentally distinct B1b cell repertoire driven by IL-7–driven B lymphopoiesis.

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Disclosures
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