Brief Definitive Report

Inhibition of Interleukin 7 Receptor Signaling by Antigen Receptor Assembly

By Fiona M. Smart and Ashok R. Venkitaraman

From The Wellcome Trust Centre for the Study of Molecular Mechanisms in Disease and the Cancer Research Campaign Department of Oncology, University of Cambridge, The Cambridge Institute for Medical Research, Hills Road, Cambridge CB2 2XY, United Kingdom

Abstract

After the productive rearrangement of immunoglobulin (Ig) heavy chain genes, precursor (pre-)B lymphocytes undergo a limited number of cell divisions in response to interleukin (IL)-7. Here, we present evidence that this phase of IL-7-dependent expansion is constrained by an inhibitory signal initiated by antigen receptor assembly. A line of pre-B cells from normal murine bone marrow that expresses a μ heavy chain with a D-proximal V_H7183.2 region divides continuously in IL-7. IL-7 responsiveness ceases upon differentiation to the μ^+ , κ^+ stage, despite continuing expression of the IL-7 receptor (IL-7R), suggesting that antigen receptor assembly inhibits IL-7 responsiveness. This is confirmed by introduction of a rearranged λ light chain gene, which inhibits proliferative signaling through the IL-7R. Inhibition is specific to the IL-7R, because it is overcome by replacement of the IL-7R cytoplasmic domain with corresponding sequences from the closely related IL-2R β chain. Alteration of a single tyrosine residue, Tyr410, in the IL-7R cytoplasmic domain to phenylalanine also prevents the inhibition of proliferation after antigen receptor assembly. Thus, the loss of IL-7 responsiveness after antigen receptor assembly may be mediated through the recruitment of an inhibitory molecule to this residue. Our findings identify a novel mechanism that limits cytokine-dependent proliferation during B lymphopoiesis. This mechanism may be essential for the proper regulation of peripheral B lymphocyte numbers.

Key words: interleukin 7 receptor • B lymphocyte differentiation • signal transduction • antigen receptor • immunoglobulin

Introduction

A potent stimulus for the proliferation of immature B lymphocytes is provided by IL-7 (1), a cytokine secreted by stromal cells in the bone marrow. IL-7 triggers cell division by engagement of a heterodimeric receptor (IL-7R) that contains a ligand-specific α chain (2), and a common γ chain shared with the receptors for IL-2, IL-4, IL-9, IL-13, and IL-15 (3–7). We have shown elsewhere that proliferative signaling through the IL-7R is critically dependent on the integrity of a single tyrosine residue in the cytoplasmic domain of the α chain (8). This tyrosine residue is essential for the ligand-induced recruitment of the effector enzyme phosphatidylinositol 3-kinase (PI 3-k), through the Src homology 2 (SH2) domains in its regulatory subunit, p85 (9).

In mice, the ability of immature B cells to divide in response to IL-7 changes with their developmental progression. Progenitor (pre-)B cells, which have yet to complete Ig heavy chain (IgH) gene rearrangement, require stromal cell contact as well as IL-7 to divide (10, 11). Productive IgH rearrangement triggers progression to the precursor (pre-)B cell stage, at which IL-7 alone is sufficient to provoke proliferation (1, 12, 13). Pre-B cells undergo only a limited number of cell divisions before they differentiate into IL-7-unresponsive, mature B lymphocytes, which express a functional antigen receptor (14, 15). It remains unclear how IL-7-dependent proliferation can be limited during these developmental transitions to regulate the number of mature cells emerging to the periphery. Here, we present evidence that the IL-7-dependent proliferation of pre-B cells is constrained by an inhibitory signal initiated through antigen receptor assembly.
Materials and Methods

Cells. IL-7–dependent cells derived from normal bone marrow (16) were maintained in growth medium with 10 ng/ml of recombinant IL-7 (Genzyme Diagnostics). The retroviral packaging cells C.R.E (17), AM12 GP + env (18), and ßnix (19) were used, and were maintained in DMEM with 10% FCS.

Immunofluorescence Staining and Flow Cytometric Analysis. Cells were washed in ice-cold PBS. Staining for CD4 and for CD25, with an FITC-conjugated rat anti–mouse CD25, was carried out using the BLAST search algorithms (available at the web site of the intron downstream of the J<sub>H</sub> and nested primers corresponding to the V<sub>H</sub>5183 and V<sub>H</sub>558 V region families have already been described (20). Southern blotting of PCR products using a V<sub>H</sub>5183 family–specific probe (20) confirmed that the rearrangement utilized a member of this family. The V<sub>H</sub> product was then gel purified and sequenced using a nested primer corresponding to the 5<sup>′</sup> end of V<sub>H</sub>5183 (20). Sequence comparisons were carried out using the BLAST search algorithms (available at http://www.ncbi.nlm.nih.gov/BLAST).

Pre-B Cell Cloning Assay. The pre-B cell line was cloned by limiting dilution in the presence of 10 ng/ml recombinant IL-7 (Genzyme Diagnostics). The pre-B cells were plated at 0.3 cells per well in 3 ml of growth medium. The cells were then incubated at 10<sup>4</sup> cells/ml, on a 20% confluence monolayer of 2,000-rad γ-irradiated retrovirus-secreting packaging cells, in RPMI 1640 medium containing 10% FCS, 5 × 10<sup>−3</sup> β-mercaptoethanol (growth medium), and 10 ng/ml recombinant IL-7 (Genzyme Diagnostics) at 37°C in a 5% CO<sub>2</sub> atmosphere. After 2 d, cultures were washed and transferred to growth medium containing 10 ng/ml IL-4 (Genzyme Diagnostics) and 5 × 10<sup>−3</sup> β-mercaptoethanol (growth medium) and then fed every 3–4 d by removal of spent medium and replacement with new.

Proliferation Assays. Cells (5 × 10<sup>5</sup>) were washed and resuspended in 3 ml of growth medium. The cells were then incubated for 4 d at 37°C in 96-well microtiter plates in 100 μl of growth medium containing the indicated quantities of recombinant human IL-4 or recombinant mouse IL-7 (Genzyme Diagnostics). Proliferation was quantified using the MTT assay (21).

Results and Discussion

A Line of Pre-B 1 Cells from Normal Mouse Bone Marrow Derived Continuously in IL-7. We used a line of pre-B cells isolated from normal murine bone marrow (16) to study the mechanisms that limit IL-7 responsiveness during B lymphopoiesis. These cells express markers characteristic of the pre-B I stage in development (Fig. 1 A). Thus, in ad-
Figure 1. (A) Shows that markers characteristic of the pre-B I stage in differentiation are expressed by the pre-B cell line used in these studies. The panels show the results of flow cytometric analyses of 10,000 cells after staining with antibodies against the indicated markers. Forward and side light scatter profiles were used to exclude dead cells. (B) Demonstrates the dose-dependent proliferation of the pre-B cells in response to IL-7. Proliferation quantified by the MTT assay is plotted on the y-axis against cytokine dose on the x-axis. The solid and dotted lines compare the proliferation of a pre-B cell culture tested at an interval of >1 yr, showing that IL-7-dependent proliferation is not lost after prolonged passage in culture. Results are typical of at least three independent experiments.

Table 1. $\kappa^+$ cells responsive to IL-7. Cannot be cloned out

| Cell dilution | No. of clones analyzed | No. of $\kappa^+$ clones ($\geq 5\%$) | No. of $\kappa^+$ clones |
|--------------|------------------------|--------------------------------------|--------------------------|
| 0.3 cells/well | 100                    | 100                                  | 0                        |

Pre-B cells were cloned at limiting dilution (0.3 cells/well) in 96-well plates using culture medium containing 10 ng/ml of recombinant IL-7. Cell surface expression of $\kappa^+$ and $\kappa^-$ in individual clones was determined by flow cytometry as described above.
Specific to the IL-7R Cytoplasmic Domain. Accordingly, we inhibition of proliferative signaling through the IL-7R.

Figure 3. (A) Shows that $\kappa_1$ and $\kappa_2$ pre-B cells stain with equal frequency and intensity for the murine IL-7 (MIL7) receptor. (B and C) The results of experiments with pre-B cells transduced with retrovirally encoded huIL-4R/IL-7R wild-type (WT) or huIL-4R/IL-2R $\beta$ chimeric receptors. The top panel in each case shows that expression of the transduced receptors is equivalent, whereas the bottom panels demonstrate the marked difference in the capacity of the two chimeric receptors to support the outgrowth of $\kappa_2$ cells. Results are typical of at least three independent experiments.

Table II. Ability of Chimeric Receptors to Support the Outgrowth of Cells Expressing a $\mu_1$ Antigen Receptor

| Construct | n | Outgrowth of $\mu_1$, $\kappa$ cells |
|-----------|---|-----------------------------------|
| $\lambda_1$IR ES-huIL-4R/IL-7R | 5 | – |
| $\lambda_1$IR ES-huIL-4R/IL-2R $\beta$ | 3 | + |
| $\lambda_1$IR ES-huIL-4R/IL-7R Y410F | 3 | + |

Pre-B cells were transduced with bicistronic retroviruses encoding a $\lambda_1$ light chain cDNA linked through an IR ES to chimeric cytokine receptors. Transduced cells were selected in human IL-4 (10 ng/ml) for 7 d, and the surface expression of $\mu_1$ and $\kappa$ was determined by flow cytometry (see Materials and Methods). n, the number of independent experiments.

Tested if replacement of the IL-7R cytoplasmic domain with sequences from the related IL-2R $\beta$ chain (27) could overcome this inhibition. Pre-B I cells transduced with a huIL-4R/IL-2R $\beta$ chimeric receptor were selected in human IL-4 before analysis for $\kappa_1$ expression. As shown in Fig. 3 C, the huIL-4R/IL-2R $\beta$ supports the outgrowth not only of $\kappa_1$ pre-B I cells but also of cells that have differentiated to the $\mu_1$, $\kappa$ stage. This is in marked contrast to the huIL-4R/IL-7R (Fig. 3 B). Thus, the loss of proliferative signaling at this developmental stage is specific to the IL-7R cytoplasmic domain. Taken together with the results in Table I, this finding confirms that the differentiation of pre-B I cells to the $\mu_1$, $\kappa$ stage triggers the inhibition of proliferative signaling through the IL-7R, despite continuing expression of the receptor.

A antigen receptor assembly initiates the inhibition of Proliferative Signaling through the IL-7R. Our results suggest that the assembly of a functional antigen receptor initiates the inhibition of proliferative signaling through the IL-7R. To test this hypothesis directly, we created bicistronic gene constructs in which a rearranged $\lambda_1$ light chain gene was linked through an IR ES to a cDNA encoding either the huIL-4R/IL-7R or huIL-4R/IL-2R $\beta$ chimeric receptors (Table II). These constructs were introduced into pre-B I cells by retroviral transduction, and $\lambda_1$-expressing cells were selected in human IL-4.

As shown in Table II, $\lambda_1$-expressing cells were readily detected in cultures transduced with the $\lambda_1$IR ES-huIL-4R/IL-2R $\beta$ construct. These $\kappa$ cells could be maintained in IL-4-dependent culture for >1 mo without loss of $\lambda_1$ expression (data not shown). In contrast, $\lambda$ cells could not be isolated from cultures transduced with the $\lambda_1$IR ES-huIL-4R/IL-7R construct in multiple independent experiments, suggesting that their outgrowth was not supported by signaling through the huIL-4R/IL-7R. Taken together, our results indicate that assembly of a $\mu_1$, $\lambda_1$ antigen receptor results in the loss of responsiveness to proliferative signaling through the huIL-4R/IL-7R, and that this inhibition is specific to the cytoplasmic sequences of the IL-7R but not the IL-2R $\beta$.

Inhibition of Proliferative Signaling is Dependent on Tyr410. To
investigate the mechanism by which this inhibitory signal might act, we constructed a series of mutant huL-4R/IL-7R constructs in which cytosolic Tyr residues were altered to Phe. The cytoplasmic domain of the IL-7R contains three Tyr residues (Tyr410, Tyr449, and Tyr456) conserved in evolution between the murine and human receptors, suggesting an important function. The Tyr410Phe and Tyr456Phe mutants were tested in these experiments because we have already shown that Tyr449, which recruits the effector enzyme PI 3-k, is essential for proliferative signaling through the IL-7R (8, 9).

Fig. 4A shows that both the huL-4R/IL-7R Tyr 410Phe and huL-4R/IL-7R Tyr456Phe mutants are equally proficient at supporting the proliferation of pre-B1 cells. Thus, neither residue is essential for proliferative signaling. However, in contrast to both the wild-type receptor (Fig. 3B) and the Tyr456Phe mutant (Fig. 4C), the huL-4R/IL-7R Tyr410Phe mutant alone could support the outgrowth of μ+κ+ cells (Fig. 4B). This implies that mutation of a single Tyr residue can overcome the inhibition of proliferative signaling through the IL-7R.

Accordingly, we performed a further experiment in which a rearranged l1 light chain gene was linked in a bicistronic expression vector to the huL-4R/IL-7R Tyr410Phe mutant receptor (Table II). In contrast to the huL-4R/IL-7R, but like the huL-4R/IL-2Rβ, the Tyr410Phe mutant was competent to support the outgrowth of l1-expressing cells. Taken together, these results confirm that the inhibitory signal initiated by antigen receptor assembly is critically dependent on the integrity of Tyr410 in the cytoplasmic domain of the IL-7R α chain. Therefore, we propose that the inhibitory signal may work through the recruitment of an inhibitory molecule to this residue.

In summary then, the results we have presented in this paper identify a novel mechanism that limits the expansion of pre-B lymphocytes in the murine bone marrow. By suppressing proliferative signaling through the IL-7R cytoplasmic domain upon antigen receptor assembly, this mechanism provides a feedback loop that may regulate the throughput of cells reaching the mature B lymphocyte stage. The assembly of a functional pre-B cell receptor may itself conceivably be sufficient to trigger this growth inhibitory process (28, 29), restricting the number of divisions pre-B cells can undergo before commencing Ig rearrangement. Our findings demonstrate for the first time a homeostatic mechanism that limits cytokine-dependent cell expansion during hematopoiesis.

We thank Dr. J. Sims (Immunex Corp., Seattle, WA), Prof. S.I. Nishikawa (Kyoto University, Kyoto, Japan), Dr. A. Corcoran (The Cambridge Institute for Medical Research), and Dr. G. Nolan (Stanford University, Stanford, CA) for the generous gift of reagents used in this work, Dr. A. Corcoran and Dr. K.J. Patel for much helpful discussion, and Andrew Riddell for assistance with flow cytometry.

F.M. Smart received a Ph.D. Studentship from the Medical Research Council, U.K. A.R. Venkitaraman holds a Professorship generously endowed by the late Dr. F.A. Zoellner. Work in A.R. Venkitaraman’s laboratory is supported by the Medical Research Council, U.K.

Submitted: 21 O dober 1999
Acepted: 3 N ovember 1999

References

1. Namen, A.E., A.E. Schmierer, C.J. March, R.W. Overell, L.S. Park, D.L. Urdal, and D.Y. Mochizuki. 1988. B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors J. Exp. Med. 167:988–1002.

2. Goodwin, R.G., D. Friend, S.F. Ziegler, R. Jerzy, B.A. Falk, S. Gimpel, D. Cosman, S.K. Dower, C.J. March, and A.E. Namen. 1990. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. J. Exp. Med. 167:988–1002.

3. Takeshita, T., H. Asao, K. Ohnishi, N. Ishii, S. Kumaki, N. Tanaka, H. Murakata, M. Nakamura, and K. Sugamura. 1992. Cloning of the gamma chain of the human IL-2 recep-
14. Decker, D.J., N.E. Boyle, J.A. Koziol, and N.R. Klinman. 1993. Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. Science 262:1877–1880.

15. Suda, T., S. Okada, J. Suda, Y. Miura, M. Ito, T. Sudo, S. Hayashi, N. Ishikawa, and H. Nakamura. 1990. Stepwise progression of B lineage differentiation supported by interleukin-7 and other stromal cell molecules. J. Exp. Med. 171:1073–1089.

16. Danos, O., and R.C. Mulligan. 1988. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc Natl. Acad. Sci. USA 85:6460–6464.

17. Markowitz, D., S. Goff, and A. Bank. 1988. Construction and use of a safe and efficient amphotropic packaging cell line. Virology. 167:400–406.

18. Kinsella, T.M., and G.P. Nolan. 1996. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. Hum. Gene Ther. 1:1405–1413.

19. Corcoran, A.E., A. Riddell, D. Krooshoop, and A.R. Venkitaraman. 1998. Impaired immunoglobulin gene rearrangement in mice lacking the IL-7 receptor. Nat. Immunol. 23:904–907.

20. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 66:55–65.

21. Zhou, L.J., D.C. Ord, A.L. Hughes, and T.F. Tedder. 1991. Structure and domain organization of the CD19 antigen of human, mouse, and guinea pig B lymphocytes. Conservation of the extensive cytoplasmic domain. J. Immunol. 147:1424–1432.

22. Rolkina, A., U. Grawunder, T.H. Winkler, H. Karayumak, and F. Melchers. 1994. IL-7 receptor alpha chain transmits distinct signals for proliferation and differentiation during B lymphopoiesis. EMBO J. 13:2822–2830.

23. Rolink, A., U. Grawunder, T.H. Winkler, H. Karayumak, and F. Melchers. 1994. IL-7 receptor induces the association of phosphatidylinositol 3-kinase with the alpha chain of the interleukin-7 receptor. Eur. J. Immunol. 24:2168–2174.

24. Chen, J., A. Ma, F. Young, and F.W. Alt. 1994. IL-2 receptor alpha chain expression during early B lymphocyte differentiation. Int. Immunol. 6:1265–1268.

25. Yamamoto, G., S.V. Desiderio, M. Paskind, J.F. Kearney, D. Baltimore, and F.W. Alt. 1984. Preferential utilization of the most JH-proximal VH gene segments in pre-B-cell lines. Nature 317:727–733.

26. Idzerda, R.L., C.J. March, B. Mosley, S.D. Lyman, T. Vanden Boss, S.D. Gimpel, W.S. Din, K.R. Grabstein, M.B. Widmer, L.S. Park, et al. 1990. Human interleukin 4 receptor confers biological responsiveness and defines a novel receptor superfamily. J. Exp. Med. 171:861–873.

27. Hatakeyama, M., H. Mori, T. Doi, and T. Taniguchi. 1989. Changes in the VH repertoire of developing precursor B lymphocytes in mouse bone marrow mediated by the pre-B-cell receptor. Int. Immunol. 1:1405–1413.

28.十日博, E., F. Mächers, and A.G. Rokin. 1997. Changes in the VH repertoire of developing precursor B lymphocytes in mouse bone marrow mediated by the pre-B-cell receptor. Immunity. 7:357–368.

29. Wasserman, R., Y.S. Li, S.A. Shinton, C.E. Carmack, T. Manser, D.L. Wiest, K. Hayakawa, and R.R. Hardy. 1998. A novel mechanism for B cell repertoire maturation based on response by B cell precursors to pre-B receptor assembly. J. Exp. Med. 187:259–264.