Inhibition of T Cell and Promotion of Natural Killer Cell Development by the Dominant Negative Helix Loop Helix Factor Id3

By Mirjam H. M. Heemskerk,* Bianca Blom,* Garry Nolan,‡ Alexander P. A. Stegmann,* Arjen Q. Bakker,* Kees Weijer,* Pieter C. M. Res,* and Hergen Spits*

From the *Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands; and ‡Department of Pharmacology, Stanford University, Palo Alto, California

Summary

Bipotential T/natural killer (NK) progenitor cells are present in the human thymus. Despite their bipotential capacity, these progenitors develop predominantly to T cells in the thymus. The mechanisms controlling this developmental choice are unknown. Here we present evidence that a member(s) of the family of basic helix loop helix (bHLH) transcription factors determines lineage specification of NK/T cell progenitors. The natural dominant negative HLH factor Id3, which blocks transcriptional activity of a number of known bHLH factors, was expressed in CD34+ progenitor cells by retrovirus-mediated gene transfer. Constitutive expression of Id3 completely blocks development of CD34+ cells into T cells in a fetal thymic organ culture (FTOC). In contrast, development into NK cells in an FTOC is enhanced. Thus, the activity of a bHLH transcription factor is necessary for T lineage differentiation of bipotential precursors, in the absence of which a default pathway leading to NK cell development is chosen. Our results identify a molecular switch for lineage specification in early lymphoid precursors of humans.

The earliest progenitor cells in the thymus have the capacity of developing into multiple hematopoietic lineages. Upon further development within the thymus, these progenitor cells progressively lose their multipotentiality (1). There is considerable evidence that the human (2) and the mouse thymuses (3, 4) contain bipotential T/NK progenitors. However, given the paucity of mature NK cells in the thymus, the thymic environment strongly favors development of these bipotential T/NK progenitor cells into T cells. The molecular mechanisms controlling T/NK lineage specification in the thymus are not yet elucidated, but it seems fair to assume that this developmental choice is under transcriptional control.

Several transcription factors that orchestrate lymphoid development have now been identified in the mouse. Ikaros is a key factor that affects development of all lymphoid (T, NK, and B) cells (5). Several other factors appear to be critical for development of specific lymphoid lineages like GATA-3 (6) and TCF-1 (7) for T cells and Pax5 (8), EBF (9), Sox4 (10), and the products of the E2A gene (11, 12) for B cells. The E2A proteins E12 and E47 probably regulate B cell development by controlling expression of immunoglobulin, RAG, and a number of B cell-specific genes including mb-1, λ5, CD19, and Pax5 (11, 12, 14). Transcriptional activity of bHLH factors is controlled by the inhibitor of DNA binding (Id) proteins. This family of HLH factors comprises four members, Id1, 2, 3, and 4, which are highly homologous in their HLH domains and have distinct tissue distributions (15, 16). Id factors can heterodimerize with bHLH factors but lack a basic DNA binding domain, and therefore they block transcriptional activity by bHLH factors. Constitutive overexpression of Id1 under control of a B cell-specific mb-1 promoter leads to inhibition of B cell development (17), similar to the one observed in E2A-deficient animals (11, 12).

Recent evidence has suggested a role for bHLH factors in development of T cells as well. E2A-deficient mice were initially reported to have no gross abnormalities in T cell development, but the size of the thymus is smaller than that of wild-type animals (12). Mice deficient for another bHLH factor, HEB, displayed a partial block in T cell development at an early stage of development (18). Whether HEB and E2A deficiencies affect NK cell development is not known.
In this paper we have investigated the role of bHLH factors in human T and NK cell development. We have made use of retrovirus-mediated gene transfer to enhance expression of one of the Id proteins, Id3, in CD34+ progenitors using a bicistronic vector harboring Id3 and the marker gene green fluorescent protein (GFP). Id3 can interact with a wide range of bHLH factors and blocks transcriptional activity of E12, E47, and HEB (19). We found that enforced expression of Id3 strongly inhibits development of CD34+ progenitors to CD3+ T cells in an in vitro fetal thymic organ culture (FTOC), but promotes development of NK cells from the FTOC cultures using a feeder cell mixture as described previously (24).

NK Cell Assays. 10,000 CD34+ sorted cells were cultured in Yssel's medium in the presence of 10 ng/ml SCF, 10 ng/ml IL-7, and 100 U/ml IL-2 (Eurocetus, Amsterdam, Netherlands). After 1 wk, cells were harvested, counted, and analyzed by FACS®, using anti-CD3 and anti-CD56 (provided by Dr. J.H. Phillips, DNAx, Palo Alto, CA). The cytotoxic activity of NK cells was determined with a standard 51Cr-release assay using cells of NK-sensitive (K562) and insensitive (the EBV-transformed B cell line EBV225) cell lines as target cells.

Analysis of D-ßR rearrangements. D-ßR rearrangements were determined by PCR as described previously (25). Primers and probes used for TCR-ß rearrangement were: 5-TGGTGTTCTCTCCCAGGCTCT-3' (Dß1.1), 5'-CCAGCTGTCAGCCTTGACTT-3' (ßß1.3-1.4) and 5'-AAAAAGTGAACATGTGGGAC-3' (Dßpar probe). To control for the amount of DNA in the PCR, genomic amplification of the RAG-2 gene was performed. Primers and probe were used: 5'-TGGTAATTGCACTGCTTGCAAG-3' (RAG-2 sense), 5'-GGGTTGTTGTAAC-3' (RAG-2 antisense), and 5'-CAAGATATGTTTGGAGAAGACATGGGAAA-3' (RAG-2 probe).

Materials and Methods

Construction of the Vectors and Transduction of Target Cells. Previously we have successfully used bicistronic vectors with a gene of interest linked to a downstream internal ribosomal entry site (IRE) and a marker gene that allow independent translation of the products of both genes in the transduced target cells (20). The IRE-GFP sequence was ligated into the LZR vector (21), and a polylinker was placed downstream of the gag and upstream of the IRES sequences. The Id3 coding sequence was cloned from a polylinker was placed downstream of the gag and upstream of the (IRES) and a marker gene that allow independent translation of interest linked to a downstream internal ribosomal entry site previously we have successfully used bicistronic vectors with a gene of interest linked to a downstream internal ribosomal entry site (IRE) and a marker gene that allow independent translation of the products of both genes in the transduced target cells (20). The IRE-GFP sequence was ligated into the LZR vector (21), and a polylinker was placed downstream of the gag and upstream of the IRES sequences. The Id3 coding sequence was cloned from

Thymocyte, but Promotes NK Development in an FTOC. To study the role of bHLH factors in T cell development, we overexpressed the dominant negative HLH protein Id3 in T cell progenitors and monitored the fate of the transduced cells. Id3 has a very broad tissue distribution and CD34+ fetal liver and thymic progenitor cells express Id3 messenger RNA as determined by reverse transcription PCR (results not shown). Purified CD34+ fetal liver cells were cultured with a combination of SCF and IL-7 for 24 h. The cells were then transduced by overnight coculture with supernatants of packaging cells producing recombinant viruses harboring either Id3-IRES-GFP or IR6-GFP, and cultured in an FTOC for 4 wk (22). The flow cytometric analysis shown in Fig. 1 demonstrates that the GFP marker was transferred to 23% of the progeny of the transduced progenitor cells. The patterns of CD3, CD4, and CD5 stainings of the GFP+ cells from the IR6-GFP–transduced cells are identical to that of the nontransduced GFP+ cells (Fig. 1). The great majority of the GFP+ cells express CD1a, and very few CD56+ cells were present in these samples. By contrast, cells harvested from the FTOC with Id3-IRE-GFP–transduced fetal liver cells did not express CD1a, and 32% of these cells were positive for the NK cell marker CD56. Almost no CD3+ cells were observed (Fig. 1). Not only the proportions of CD56+ cells were different, but also the absolute numbers since >30-fold more GFP+ CD56+ cells were found in the FTOC, populated with Id3-transduced CD34+ cells. These data indicate that CD34+ fetal liver cells, overexpressing Id3, develop preferentially into CD3+CD56+ cells in the FTOC. It was important to ensure that these GFP+CD3+CD56+ cells represent functional NK cells. To obtain enough cells for testing their cy
The observation that the proportion of CD56+ cells in the Id3-GFP+ progeny is much higher than in the control GFP progeny and in the Id3-GFP− cells in the FTOC is consistent with the notion that Id3 overexpression switches the cell fate of the bipotential thymic T/NK progenitors. An alternative possibility is that overexpression of Id3 confers a growth or survival advantage to developing NK cells resulting in an overrepresentation of these cells in the FTOC. To investigate this, we studied the effect of Id3 transduction on development of CD34+ fetal liver cells into NK cells in a mixture of SCF, IL-7, and IL-2 (2). All cells generated from transduced CD34+ fetal liver cells and cultured with the cytokines for 2 wk were CD3− (not shown). In both Id3-IRES-GFP and the IRES-GFP cultures, we observed the same proportions of GFP+ cells (35%) and an identical distribution of CD56 in the IRES-GFP+ and the Id3-IRES-GFP+ cells (Fig. 3). Thus, constitutive expression of Id3 in the CD34+ fetal liver cells does not significantly enhance the percentage of NK cells that develop under the influence of SCF, IL-7, and IL-2. This result makes it unlikely that the increase of NK cell numbers observed in the FTOC with Id3+ progenitor cells is caused by improved survival or proliferation by Id3 overexpression.

TCR-β rearrangements are initiated in the CD1a+ CD3−CD4−CD8−CD34+ cells (25, 26). Moreover, CD1a+ cells are unable to develop into NK cells (2). This suggests that upregulation of CD1a is correlated with T cell commitment. If this is correct and if overexpression of Id3 inhibits T cell commitment, we should expect that CD1a+CD34+ cells transduced with Id3 fail to develop into T cells in an FTOC. Purified CD1a+ CD34+ cells were cultured with SCF and IL-7, transduced, and incubated in an FTOC for 3 wk. Fig. 4 demonstrates that the control-transduced cells developed normally. By contrast, the proportion of CD3+ cells that developed from Id3-transduced CD1a−
CD34+ cells is strongly reduced, whereas the proportion of NK cells is dramatically increased. The absolute numbers of GFP+ NK cells in the Id3-transduced samples was 10-fold higher than in the GFP+ cells in the control-transduced samples. The inhibition of generation of CD3+ by Id3 is underscored by the almost complete absence of CD4+ and CD4+CD8+ in the cells expressing Id3-GFP. The CD8+ cells observed in these cultures are CD3-CD56+ cells. The Id3-IRES-GFP+CD3-CD56+ could be expanded with a feeder cell mixture and displayed cytolytic activity against K562 cells (results not shown).

Enforced Expression of Id3 Inhibits Induction of D-Jβ Rearrangements by IL-7 and SCF. T cells are defined by TCR gene rearrangements in NK cells, these genes are in the germ line configuration (1). The fact that Id3 inhibits generation of CD3+ cells and stimulates that of NK cells, raises the possibility that bHLH factors are required for processes that result in TCR rearrangements. To test this, we cultured transduced CD1a-CD34+ and CD1a+CD34+ cells for 7 d in IL-7 and SCF, sorted GFP+ and GFP- cells, and analyzed for the presence of D-Jβ rearrangements with a sensitive PCR technique (25). Fig. 5 shows that Id3 completely blocked IL-7 and SCF-mediated induction of D-Jβ rearrangement in CD1a+CD34+ cells. One trivial explanation for this observation is that SCF and IL-7 induce growth of a very small population of contaminating CD1a-CD34+ cells that have already undergone D-Jβ rearrangements; overexpression of Id3 could inhibit the growth of these contaminating cells. This is unlikely because D-Jβ rearrangements were still detectable in Id3-transduced CD1a+CD34+ cells recovered after 7 d of culture (Fig. 5). The ratios of the intensities of the bands hybridized with the D-Jβ and genomic DNA control (RAG-2) probes in the starting CD1a-CD34+ (lane 4) and in the Id3-CD1a+CD34+ (lane 6) populations were the same. This ratio is increased in the untransduced CD1a+CD34+ cells. These data indicate that

**Figure 3.** Overexpression of Id3 does not affect NK development and expansion of CD34+ fetal liver cells incubated in IL-2, IL-7, and SCF. CD34+ fetal liver cells were isolated, transduced, and incubated with the cytokines for 14 d.

**Figure 4.** Enforced expression of Id3 in CD1a+CD34+ thymocytes blocks T cell development and promotes NK development in an FTOC. CD1a+CD34+ thymocytes were isolated from a thymic fragment of a 6-mo-old child, transduced and incubated in an FTOC for 4 wk at 10^4 cells/lobe. Cell recoveries were 5 x 10^4 and 6 x 10^4 cells/lobe for the Id3- and the control-transduced cells, respectively. Percentages of GFP+ cells 2 d after transduction were 9% in both samples. Percentages of GFP+ cells harvested from the FTOC were 7.2% in the control and 2.4% in the Id3-transduced samples.
Id3 inhibits the IL-7 and SCF-mediated increase D-Jβ rearrangements in CD1a-CD34+ cells. It appears, therefore, that Id3 overexpression inhibits induction of D-Jβ rearrangement. It remains to be determined whether the rearrangement process itself is blocked or whether an earlier commitment is blocked unresolved. It is possible that HEB, E2A, and perhaps other bHLH factors collaborate in inducing T cell development, and that a complete inhibition ensues when these factors are simultaneously inhibited by overexpression of Id3. Thus, T cell development may be dictated by a combined dosage of several bHLH factors. It is in this respect noteworthy that B cell development appears to be regulated by the combined dosage of E2A, HEB, and E2-2 proteins (18). Future studies should provide more exact information about which bHLH factors are critical for T cell development and which genes are controlled by these factors.

We thank the staff of the Bloemenhovenkliniek in Heemstede (Netherlands) for their cooperation in obtaining fetal tissue. We thank Dr. Cees M urre for providing us with the pCDNAId3 plasmid. Dr. A. Venkitaraman is acknowledged for reviewing the manuscript.

This work was supported by grants from the Dutch Cancer Foundation and the Netherlands Organization for Scientific Research (NWO).

Address correspondence to Dr. Hergen Spits, Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. Phone: 31-20-5122003; FAX: 31-20-5122257; E-mail: hergen@nki.nl

Received for publication 7 July 1997 and in revised form 21 August 1997.

References
1. Spits, H., L. Lanier, and J.H. Phillips. 1995. Development of human T and natural killer cells. Blood. 85:2654-2670.
2. Sánchez, M.-J., M.O. Muench, M.G. Roncarolo, L. Lanier, and J.H. Phillips. 1994. Identification of a common T/NK cell progenitor in human fetal thymus. J. Exp. Med. 180:569-576.
3. Rodewald, H.R., P. Moinen, J.L. Lucich, C. Dosiou, P. Lopez, and E.L. Reinherz. 1992. A population of early fetal thymocytes expressing Fc gamma RII/III contains precursors of T lymphocytes and natural killer cells. Cell. 69:139-150.
4. Matsuzaki, Y., J.-I. Gyohtoku, M. Ogawa, G.-I. Nishikawa, Y. Katsura, G. Gachelin, and H. Nakauchi. 1993. Characterization of c-kit positive intrathymic stem cells that are restricted to lymphoid differentiation. J. Exp. Med. 178:1283-1292.
5. Georgopoulos, K., M. Bigby, J.-H. Wang, A. Molnar, P. Wu, S. Winandy, and A. Sharpe. 1994. The Ikaros gene is
required for the development of all lymphoid lineages. Cell. 79:143–156.

6. Ting, C.-N., M.C. Olson, K.P. Barton, and J.M. Leiden. 1997. Transcription factor GATA-3 is required for development of the T cell lineage. Nature (Lond.). 384:474–479.

7. Verbeek, S., D. Izen, F. Hofhuis, E. Robanus M aandag, H. te Riele, M. van de Wetering, M. Oosterwegel, A. Wilson, H.R. MacDonald, and H. Clevers. 1995. An HMG-box-containing T-cell factor required for thymocyte differentiation. Nature (Lond.). 374:70–74.

8. Urbanek, P., Z.Q. Wang, I. Fetka, E.F. Wagner, and M. Busslinger. 1994. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. Cell. 79:901–912.

9. Lin, H., and R. Groschedl. 1995. Failure of B cell differentiation in mice lacking the transcription factor EBF. Nature (Lond.). 376:263–267.

10. Schilham, M.W., M.A. Oosterwegel, P. Moerer, J.Y. P. de Boer, M. van de Wetering, S. Verbeek, W.H. Lamers, A.M. Kruisbeek, A. Cumano, and H. Clevers. 1996. Defects in cardiac outflow tract formation and pro-B-lymphocyte expansion in mice lacking Sox-4. Nature (Lond.). 380:711–714.

11. Zhuang, Y., P. Soriano, and H. Weintraub. 1994. The helix-loop-helix gene E2A is required for B cell formation. Cell. 79:875–884.

12. Bain, G., E. Robanus M aandag, D.J. Izen, D. Amsen, A.M. Kruisbeek, B.C. Wintraub, L. Krop, L. Schlesser, A.J. Feeney, M. van Roon, and C. Murre. 1994. E2A proteins are required for proper B cell development and initiation of Ig gene rearrangements. Cell. 79:885–892.

13. Murre, C., G. Bain, M.K. van Dijk, I. Engel, B.A. Furnari, M.E. Massari, J.R. Matthews, M.W. Qong, R.R. Rivera, and M.H. Stuiver. 1994. Structure and function of helix-loop-helix proteins. Biochim. Biophys. Acta. 1218:129–135.

14. Bain, G., E. Robanus M aandag, H. te Riele, A.J. Feeney, A. Sheehy, M. Schlessel, S.A. Shinton, R.R. Hardy, and C. Murre. 1997. Both E12 and E47 allow commitment to the B cell lineage. Immunity. 6:145–154.

15. Benezra, R., R.L. Davis, D. Lockshon, D.L. Turner, and H. Weintraub. 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell. 61:49–59.

16. Reichmann, V., I. van Cruchten, and F. Sablitzky. 1994. The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. Nucletic Acids Res. 22:749–755.

17. Sun, X.H. 1994. Constitutive expression of the Id1 gene impairs mouse B cell development. Cell. 79:893–900.

18. Zhuang, Y., P. Cheng, and H. Weintraub. 1996. B lymphocyte development is regulated by the combined dosage of three basic-helix-loop-helix genes, E2A, E2-2 and HEB. Mol. Cell. Biol. 16:2898–2905.

19. Loveys, D.A., M.B. Streiff, and G.J. Kato. 1996. E2A basic-helix-loop-helix transcription factors are negatively regulated by serum growth factors and by the Id3 protein. Nucletic Acids Res. 24:2813–2820.

20. Staal, F.J.T., A.Q. Bakker, M. Verkuylen, E. van Oort, and H. Spits. 1996. Use of bicistronic retroviral vectors encoding the LacZ gene together with a gene of interest: a method to select producer cells and follow transduced target cells. Cancer Gene Ther. 3:345–351.

21. Kinsella, T.M., and G.P. Nolan. 1996. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. Hum. Gene Therapy. 7:1405–1413.

22. Res, P., E. Martinez Cáceres, A.C. Jaleco, E. Noteboom, K. Weijer, and H. Spits. 1996. CD34+CD38dim cells in the human thymus can differentiate into T, natural killer and dendritic cells but are distinct from stem cells. Blood. 87:5196–5206.

23. Yssel, H., J.E. De Vries, M. Koken, W. van Blitterswijk, and H. Spits. 1984. Serum-free medium for the generation and the propagation of functional human cytotoxic and helper T cell clones. J. Immunol. Methods. 72:219–227.

24. Res, P., B. Blom, T. Hori, E. Noteboom, K. Weijer, and H. Spits. 1997. Downregulation of CD1a marks acquisition of functional competence by human thymocytes and marks a novel control point in a late stage of human T cell development. J. Exp. Med. 185:141–152.

25. Blom, B., P. Res, E. Noteboom, K. Weijer and H. Spits. 1997. Prethymic CD34+ progenitors capable of developing into T cells are not committed to the T cell lineage. J. Immunol. 158:3571–3577.

26. Raimiro, A.R., C. Trigueros, C. Marquez, J.L. San Millan, and M.L. Toribio. 1996. Regulation of pre-T cell receptor α(1-TCRβ) gene expression during human thymic development. J. Exp. Med. 184:519–530.

27. Borst, J., H. Jacobs, and G. Brouns. 1996. Composition and function of T-cell and B-cell receptor complexes on precursor lymphocytes. Curr. Opin. Immunol. 8:181–190.

28. Bain, G., I. Engel, E.C. Robanus M aandag, H. P.J. te Riele, J.R. Voland, L.L. Sharp, J. Ghun, B. Huey, D. Pinkel, and C. Murre. 1997. E2A deficiency leads to abnormalities in CD19 T-cell development and to rapid development of T-cell lymphomas. Mol. Cell. Biol. 17:4782–4791.