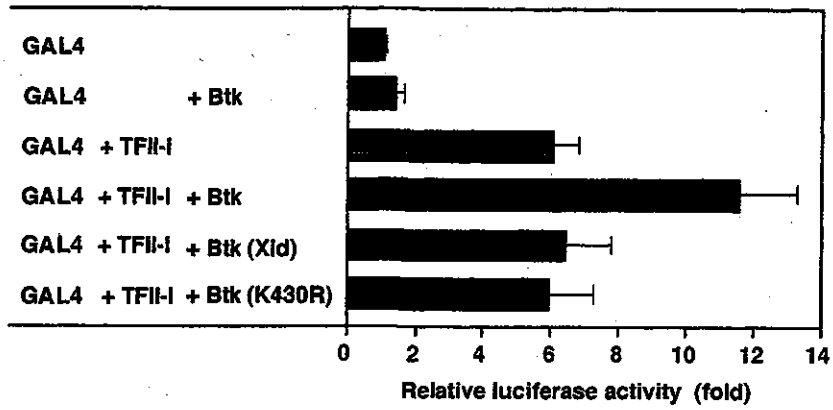

Fig. 5. Effects of Btk on TFII-I transcriptional activity. COS7 cells were co-transfected with the pG5*luc* reporter plasmid, pBIND and p146, with or without pME-Btk (Xid) or pME-Btk (K430R). After 48 h of incubation, luciferase activities from cell lysates were measured and indicated as in Fig. 1. Data are given as means of three independent experiments and SD.

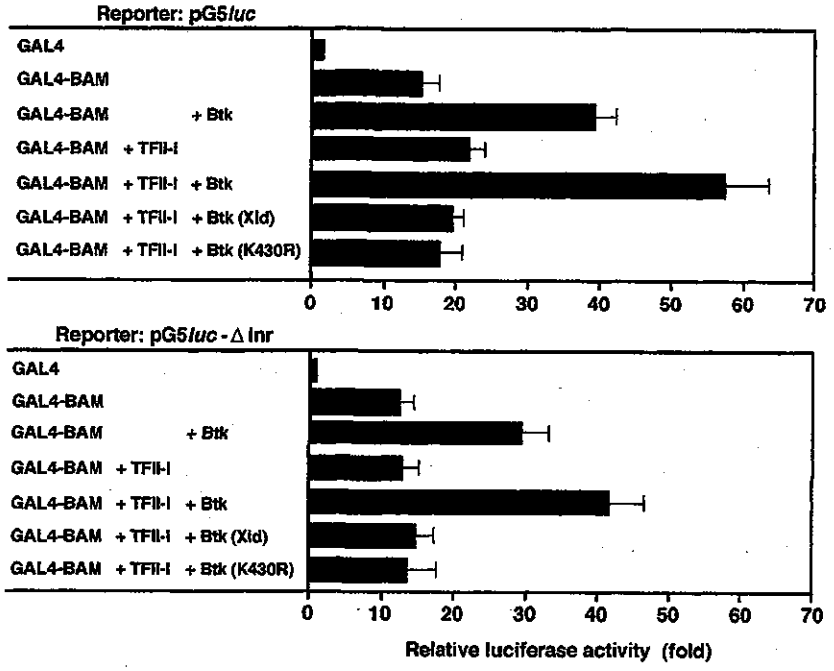

Fig. 6. Assay to determine BAM transcriptional co-activation activity using an Inr-deleted reporter plasmid. COS7 cells were co-transfected with reporter plasmid, pG5*luc* (upper panel) or pG5*luc*-ΔInr (lower panel) and pBIND, with or without p146 or pME-Btk. After 48 h of incubation, luciferase activities from cell lysates were measured and indicated as in Fig. 1. Data are given as means of three independent experiments and SD.

antigen NLS (four out of five NLS), and (ii) a bipartite NLS composed of 2 basic amino acids and a spacer region of 10–12 amino acids, that is typical nucleoplasmin (one out of five NLS). More interestingly, BAM also contains a consensus sequence for a nuclear export signal (NES) defined as a set of critically spaced hydrophobic residues, usually leucines (LXXLXXLXL, where X indicates *any residue*) (40).

BAM does not appear to contain a DNA-binding consensus sequence such as a helix–loop–helix motif or zinc finger motif. Search of an EST library for BAM homologs revealed that BAM does not belong to a known family of transcriptional factors. We were also unable to find BAM DNA-binding activity (data not shown). Rubnitz *et al.* (46) reported that LTG19/ENL/MLLT1 could activate transcription from synthetic reporter genes in both lymphoid and myeloid cells. Their results support the notion that BAM is a transcriptional regulator that binds *transcriptional* factor(s) and regulates expression of certain gene(s) indirectly.

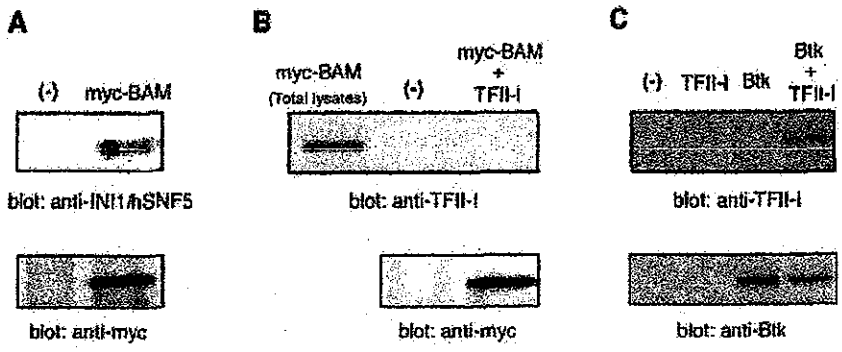

Fig. 7. Association of BAM with INI1/SNF5. COS7 cells were transfected with pMEmyc-BAM (A), pMEmyc-BAM and p146 (B) by electroporation. After 48 h, nuclear extracts were prepared and immunoprecipitated with an anti-myc antibody. Precipitates were subjected to western blotting using anti-INI1/hSNF5 (A) or anti-TFII-I (B) antibodies. The blot was stripped and reprobed with an anti-myc antibody (lower panel). (C) COS7 cells were transfected with pME-Btk and p146. After 48 h, cells were lysed and immunoprecipitated with an anti-Btk antibody. Immune complexes were subjected to western blotting using an anti-TFII-I antibody. The same blot was stripped and reprobed with an anti-Btk antibody (lower panel).

In this study, we showed BAM transcriptional co-activation activity in a transient expression system using the pG5*luc* reporter gene as a substrate (Figs 2–6). Experiments using truncated forms of BAM showed that the C-terminus is necessary and sufficient for the transcriptional co-activation activity (Fig. 2B). This is in sharp contrast to the fact that amino acids 186–363 of BAM are indispensable for Btk binding (Fig. 2A). BAM seems to have at least two functional domains—a domain for interacting with the PH domain of Btk and C-terminus for transcriptional co-activation. The C-terminus of BAM is a serine-rich region. This is reminiscent of the Sox family of proteins which require both serine-rich and C-terminus regions for transactivation (51). We do not yet know which molecules interact with the C-terminus or serine-rich regions of BAM. As reported by Garcia-Cuellar *et al.* (52), it is interesting that the C-terminal hydrophobic domain of human LTG19/ENL/MLLT1 protein interacts with Polycomb 3. Together with the trithorax group of proteins, the polycomb group of proteins maintains a stable transcriptional state despite changing chromatin architecture (53). We need further molecular analyses to clarify the role of the C-terminus region and/or serine-rich regions of BAM in BAM activity.

Several studies have reported that other molecules which bind to Btk control its activation. Full Btk activation appears to depend on transphosphorylation of Tyr551 by protein tyrosine kinases (54). Yao *et al.* (18) provided evidence that multiple isoforms of PKC interact with Btk and that PKC-mediated phosphorylation down-regulates the enzymatic activity of Btk. In our previous study, forced expression of the Btk-binding region of BAM in an IL-5-dependent early B cell line, Y16, suppressed IL-5-induced Btk activation and cell proliferation while full-length BAM showed little suppressive effect, if any (25). We carefully re-examined the effect of full-length BAM on IL-5-induced proliferation and Btk activation using other IL-5-dependent mouse early B cell lines, and found that enforced expression of full-length of BAM in T88-M significantly suppressed IL-5-induced Btk activation and cell proliferation (unpublished observation). We think that full-length BAM is capable of suppressing Btk activity. As we clearly demonstrated in Fig. 3, Btk enhances BAM transcriptional co-activation activity in COS7 cells. Activation of kinase activity of Btk is induced by transfection of Btk expression plasmid into COS7 cells (30). This enhancing effect was not observed in Btk(ΔPH), Btk(Xid) and Btk(K430R), indicating an essential role for the functional interaction between Btk and BAM as well as Btk's kinase activity for the Btk-mediated enhancement of BAM activity. Thus, mutations impairing the physical and/or functional association between BAM and Btk may result in diminished BAM-dependent transcription. BAM contains three possible tyrosine phosphorylation sites; however, *in vitro* tyrosine phosphorylation of BAM was not induced by Btk (data not shown). These results may suggest that BAM is not a major substrate of Btk and the Btk-mediated phosphorylation of BAM is not required for the enhancement of BAM activity. We speculate that Btk regulates the BAM activity indirectly, because Btk(Xid) can associate with BAM. To examine the functional interaction between BAM and Btk in mature B cells, we transfected siRNA matching a 22-nucleotide sequence of BAM (BAMsiRNA) into a mouse mature B cell line, A20/2J, and examined anti-IgM-induced calcium mobilization. Transfection of BAMsiRNA into A20/2J induced a marked reduction of endogenous expression of BAM mRNA and a 15–20% reduction of BCR-mediated calcium mobilization (data not shown), suggesting that BAM is involved to a certain extent in BCR-mediated signaling in mature B cells.

TFII-I is a multifunctional transcription initiation factor that is expressed ubiquitously and is tyrosine phosphorylated shortly after BCR stimulation (30). TFII-I can function both as a basal factor through interaction with the Inr element and as an activator in the absence of a functional Inr element. TFII-I functionally interacts with Btk and Btk(K430R), but not with Btk(Xid). Reported data indicate that in a heterologous expression system Btk, but not Btk(Xid) or Btk(K430R), augments the transcriptional activity of ectopically expressed TFII-I, suggesting the necessity of both Btk kinase activity and its ability to directly associate with TFII-I to function as a positive regulator of TFII-I transcriptional activity. It is evident that a Btk enhancing effect was observed in COS7 transfec-

tants expressing GAL4–BAM, Btk and pG5*luc*-ΔInr as well as pG5*luc* (Fig. 6). Co-expression of Btk(Xid) and Btk(K430R) with GAL4–BAM and pG5*luc*-ΔInr or pG5*luc* was not effective. Importantly, TFII-I is able to augment the transcriptional co-activation activity of BAM together with Btk in the absence of the Inr element (Fig. 6, lower panel). These results indicate that TFII-I is indispensable for Btk-mediated enhancement of BAM activity and further suggest that TFII-I functions as an activator of transcription, but not as a basal factor, in our system. BAM may regulate gene expression in B cells during their development or triggering through the SWI/SNF complex that is enhanced by Btk and TFII-I. Another possibility is that BAM may regulate TFII-I-mediated transcription together with Btk.

SWI/SNF chromatin remodeling complexes are large multi-subunit enzymes with a molecular mass of 1–2 MDa. In mammals, SWI/SNF complexes have been shown to participate in the transcriptional regulation of many genes, some of which are critical for the normal growth of organisms (39). In mice, heterozygous mutations of some SWI/SNF components result in an increased risk of cancer, whereas homozygous mutations cause embryonic lethality (41,55). Genetic and biochemical data indicate that the SWI/SNF complex can increase transcription factor as well as histone-modifying complex access to DNA (56). Mutations in SWI/SNF component genes could be suppressed by mutations that alter histone gene expression, histone structure or non-histone chromatin proteins. This leads to the suggestion that these gene products facilitate transcriptional activation by altering chromatin structure (57–59). Genome-wide expression studies have shown that SWI/SNF is involved in both transcriptional activation and repression (57,58,60,61). Several members of the human ATP-dependent chromatin-remodeling SWI/SNF complex family, which consist of 9–12 subunits, have previously been described (41–44). Recently, LTG19/ENL/MLLT1 was found to be a new member of SWI/SNF complex (45). SNF5 is reported to bind to TFG3/TAF30/ANC1, a component of the yeast SWI/SNF complex (47). It is intriguing that BAM can be co-immunoprecipitated with INI1 when overexpressed in COS7 cells. INI1/SNF5 has a NES that mediates hCRM1/exportin1-dependent nuclear export (62). This result and our data suggest that BAM and INI1/SNF5 cooperate to export Btk to the cytosol. The BAM-INI1/SNF5 association and the high sequence identify between BAM and TFG3/TAF30/ANC1 support the idea that BAM is the mouse homolog of yeast TFG3/TAF30/ANC1 and a component of the mouse SWI/SNF complex.

In conclusion, by using transient transcription assays, we demonstrate that BAM contains transcriptional co-activation activity that is enhanced by Btk. The functional interaction between BAM and Btk as well as the kinase activity of Btk are essential for Btk-mediated enhancement of BAM activity. BAM's transcriptional co-activation activity can be regulated by TFII-I and the SWI/SNF complex of transcription. Btk may participate in the transcriptional regulation of many genes in B cells either by TFII-I activation together with BAM or SWI/SNF complex activation together with TFII-I. The experimental system described in this study should provide a useful tool for delineating the Btk-mediated BCR and IL-5 receptor signaling pathways; however, it remains elusive which gene(s) and their respective patterns of expression are regulated by BAM.

Supplementary data

Supplementary data are available at *International Immunology* Online.

Acknowledgements

We are grateful to Satoshi Takaki and R. S. Davis for valuable suggestions and for reviewing the manuscript. We thank Tadashi Yamamoto and A. L. Roy for providing the pME18S-myc vector and the p146 vector respectively. This work was supported in part by a Research Grant from the Human Frontier Science Program (K. T.), by Special Coordination Funds for Promoting Science and Technology (K. T.), and by Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture, Japan.

Abbreviations

BAM	Btk-associated protein
Btk	Bruton's tyrosine kinase
GFP	green fluorescent protein
NES	nuclear export signal
NLS	nuclear localization signal
PH	pleckstrin homology
PIP_3	phosphatidylinositol-3,4,5-triphosphate
PKC	protein kinase C
TBS	Tris-buffered saline
TBS-T	TBS containing 0.05% Tween 20
XLA	X-linked agammaglobulinemia
Xid	X-linked immunodeficiency

References

1 Kurosaki, T. 1999. Genetic analysis of B cell antigen receptor signaling. *Annu. Rev. Immunol.* 17:555.
2 Yang, W. C., Ghiotto, M., Castellano, R., Collette, Y., Auphan, N., Nunes, J. A. and Olive, D. 2000. Role of Tec kinase in nuclear factor of activated T cells signaling. *Int. Immunol.* 12:1547.
3 Kurosaki, T. 2000. Functional dissection of BCR signaling pathways. *Curr. Opin. Immunol.* 12:276.
4 Campbell, K. S. 1999. Signal transduction from the B cell antigen-receptor. *Curr. Opin. Immunol.* 11:256.
5 Kurosaki, T. and Tsukada, S. 2000. BLNK: connecting Syk and Btk to calcium signals. *Immunity* 12:1.
6 Marshall, A. J., Niiro, H., Yun, T. J. and Clark, E. A. 2000. Regulation of B-cell activation and differentiation by the phosphatidylinositol 3-kinase and phospholipase Cγ pathway. *Immunol. Rev.* 176:30.
7 Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J. and Waterfield, M. D. 2001. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu. Rev. Cell. Dev. Biol.* 17:615.
8 Li, Z., Wahl, M. I., Eguinoa, A., Stephens, L. R., Hawkins, P. T. and Witte, O. N. 1997. Phosphatidylinositol 3-kinase-γ activates Bruton's tyrosine kinase in concert with Src family kinases. *Proc. Natl Acad. Sci. USA* 94:13820.
9 Salim, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Gout, I., Scaife, R., Margolis, R. L., Gigg, R., Smith, C. I., Driscoll, P. C., Waterfield, M. D. and Panayotou, G. 1996. Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J.* 15:6241.
10 Tsukada, S., Baba, Y. and Watanabe, D. 2001. Btk and BLNK in B cell development. *Adv. Immunol.* 77:123.
11 Kurosaki, T., Maeda, A., Ishiai, M., Hashimoto, A., Inabe, K. and Takata, M. 2000. Regulation of the phospholipase C-γ2 pathway in B cells. *Immunol. Rev.* 176:19.
12 Rawlings, D. J., Scharenberg, A. M., Park, H., Wahl, M. I., Lin, S., Kato, R. M., Fluckiger, A. C., Witte, O. N. and Kinet, J. P. 1996. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science* 271:822.

13 Bajpai, U. D., Zhang, K., Teutsch, M., Sen, R. and Wortis, H. H. 2000. Bruton's tyrosine kinase links the B cell receptor to nuclear factor κB activation. *J. Exp. Med.* 191:1735.
14 Petro, J. B., Rahman, S. M., Ballard, D. W. and Khan, W. N. 2000. Bruton's tyrosine kinase is required for activation of IκB kinase and nuclear factor κB in response to B cell receptor engagement. *J. Exp. Med.* 191:1745.
15 Kaku, H., Horikawa, K., Obata, Y., Kato, I., Okamoto, H., Sakaguchi, N., Gerondakis, S. and Takatsu, K. 2002. NF-κB is required for CD38-mediated induction of C(γ)1 germline transcripts in murine B lymphocytes. *Int. Immunol.* 14:1055.
16 Mizuno, T. and Rothstein, T. L. 2003. Cutting edge: CD40 engagement eliminates the need for Bruton's tyrosine kinase in B cell receptor signaling for NF-κB. *J. Immunol.* 170:2806.
17 Tsukada, S., Simon, M. I., Witte, O. N. and Katz, A. 1994. Binding of βγ subunits of heterotrimeric G proteins to the PH domain of Bruton tyrosine kinase. *Proc. Natl Acad. Sci. USA* 91:11256.
18 Yao, L., Kawakami, Y. and Kawakami, T. 1994. The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. *Proc. Natl Acad. Sci. USA* 91:9175.
19 Tsukada, S., Saffran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Klisak, I., Sparkes, R. S., Kubagawa, H., Mohandas, T., Quan, S., *et al.* 1993. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X- linked agammaglobulinemia. *Cell* 72:279.
20 Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarstrom, L., Kinnon, C., Levinsky, R., Bobrow, M., *et al.* 1993. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature* 361:226.
21 Satterthwaite, A. B., Li, Z. and Witte, O. N. 1998. Btk function in B cell development and response. *Semin. Immunol.* 10:309.
22 Rawlings, D. J., Saffran, D. C., Tsukada, S., Largaespada, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., Bazan, J. F., Howard, M., Copeland, N. G., *et al.* 1993. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science* 261:358.
23 Thomas, J. D., Sideras, P., Smith, C. I., Vorechovsky, I., Chapman, V. and Paul, W. E. 1993. Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. *Science* 261:355.
24 Tarakhovsky, A. 1997. Xid and Xid-like immunodeficiencies from a signaling point of view. *Curr. Opin. Immunol.* 9:319.
25 Kikuchi, Y., Hirano, M., Seto, M. and Takatsu, K. 2000. Identification and characterization of a molecule, BAM11, that associates with the pleckstrin homology domain of mouse Btk. *Int. Immunol.* 12:1397.
26 Tkachuk, D. C., Kohler, S. and Cleary, M. L. 1992. Involvement of a homolog of *Drosophila* trithorax by 11q23 chromosomal translocations in acute leukemias. *Cell* 71:691.
27 Yamamoto, K., Seto, M., Komatsu, H., Iida, S., Akao, Y., Kojima, S., Kodera, Y., Nakazawa, S., Ariyoshi, Y., Takahashi, T., *et al.* 1993. Two distinct portions of LTG19/ENL at 19p13 are involved in t(11;19) leukemia. *Oncogene* 8:2617.
28 Mohamed, A. J., Vargas, L., Nore, B. F., Backesjo, C. M., Christensson, B. and Smith, C. I. 2000. Nucleocytoplasmic shuttling of Bruton's tyrosine kinase. *J. Biol. Chem.* 275:40614.
29 Yang, W. and Desiderio, S. 1997. BAP-135, a target for Bruton's tyrosine kinase in response to B cell receptor engagement. *Proc. Natl Acad. Sci. USA* 94:604.
30 Novina, C. D., Kumar, S., Bajpai, U., Cheriyath, V., Zhang, K., Pillai, S., Wortis, H. H. and Roy, A. L. 1999. Regulation of nuclear localization and transcriptional activity of TFII-I by Bruton's tyrosine kinase. *Mol. Cell Biol.* 19:5014.
31 Cheriyath, V., Desgranges, Z. P. and Roy, A. L. 2002. c-Src-dependent transcriptional activation of TFII-I. *J. Biol. Chem.* 277:22798.
32 Perez Jurado, L. A., Wang, Y. K., Peoples, R., Coloma, A., Cruces, J. and Francke, U. 1998. A duplicated gene in the breakpoint regions of the 7q11.23 Williams–Beuren syndrome deletion encodes the initiator binding protein TFII-I and BAP-135, a phosphorylation target of BTK. *Hum. Mol. Genet.* 7:325.
33 Roy, A. L., Du, H., Gregor, P. D., Novina, C. D., Martinez, E. and Roeder, R. G. 1997. Cloning of an Inr- and E-box-binding protein, TFII-I, that interacts physically and functionally with USF1. *EMBO J.* 16:7091.
34 Grueneberg, D. A., Henry, R. W., Brauer, A., Novina, C. D., Cheriyath, V., Roy, A. L. and Gilman, M. 1997. A multifunctional DNA-binding protein that promotes the formation of serum response factor/homeodomain complexes: identity to TFII-I. *Genes Dev.* 11:2482.
35 Roy, A. L., Carruthers, C., Gutjahr, T. and Roeder, R. G. 1993. Direct role for Myc in transcription initiation mediated by interactions with TFII-I. *Nature* 365:359.
36 Roy, A. L., Meisterernst, M., Pognonec, P. and Roeder, R. G. 1991. Cooperative interaction of an initiator-binding transcription initiation factor and the helix–loop–helix activator USF. *Nature* 354:245.
37 Roy, A. L., Malik, S., Meisterernst, M. and Roeder, R. G. 1993. An alternative pathway for transcription initiation involving TFII-I. *Nature* 365:355.
38 Egloff, A. M. and Desiderio, S. 2001. Identification of phosphorylation sites for Bruton's tyrosine kinase within the transcriptional regulator BAP/TFII-I. *J. Biol. Chem.* 76:27806.
39 Klochendler-Yeivin, A., Muchardt, C. and Yaniv, M. 2002. SWI/SNF chromatin remodeling and cancer. *Curr. Opin. Genet. Dev.* 12:73.
40 Nigg, E. A. 1997. Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature* 386:779.
41 Bultman, S., Gebuhr, T., Yee, D., La Mantia, C., Nicholson, J., Gilliam, A., Randazzo, F., Metzger, D., Chambon, P., Crabtree, G. and Magnuson, T. 2000. A *Brg1* null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes. *Mol. Cell.* 6:1287.
42 Wang, W., Cote, J., Xue, Y., Zhou, S., Khavari, P. A., Biggar, S. R., Muchardt, C., Kalpana, G. V., Goff, S. P., Yaniv, M., Workman, J. L. and Crabtree, G. R. 1996. Purification and biochemical heterogeneity of the mammalian SWI–SNF complex. *EMBO J.* 15:5370.
43 Wong, A. K., Shanahan, F., Chen, Y., Lian, L., Ha, P., Hendricks, K., Ghaffari, S., Iliev, D., Penn, B., Woodland, A. M., Smith, R., Salada, G., Carillo, A., Laity, K., Gupte, J., Swedlund, B., Tavtigian, S. V., Teng, D. H. and Lees, E. 2000. BRG1, a component of the SWI–SNF complex, is mutated in multiple human tumor cell lines. *Cancer Res.* 60:6171.
44 Klochendler-Yeivin, A., Fiette, L., Barra, J., Muchardt, C., Babinet, C. and Yaniv, M. 2000. The murine SNF5/INI1 chromatin remodeling factor is essential for embryonic development and tumor suppression. *EMBO Rep.* 1:500.
45 Nie, Z., Yan, Z., Chen, E. H., Sechi, S., Ling, C., Zhou, S., Xue, Y., Yang, D., Murray, D., Kanakubo, E., Cleary, M. L. and Wang, W. 2003. Novel SWI/SNF chromatin-remodeling complexes contain a mixed-lineage leukemia chromosomal translocation partner. *Mol. Cell Biol.* 23:2942.
46 Rubnitz, J. E., Morrissey, J., Savage, P. A. and Cleary, M. L. 1994. ENL, the gene fused with HRX in t(11;19) leukemias, encodes a nuclear protein with transcriptional activation potential in lymphoid and myeloid cells. *Blood* 84:1747.
47 Cairns, B. R., Henry, N. L. and Kornberg, R. D. 1996. TFG/TAF30/ANC1, a component of the yeast SWI/SNF complex that is similar to the leukemogenic proteins ENL and AF-9. *Mol. Cell Biol.* 16:3308.
48 Slany, R. K., Lavau, C. and Cleary, M. L. 1998. The oncogenic capacity of HRX-ENL requires the transcriptional transactivation activity of ENL and the DNA binding motifs of HRX. *Mol. Cell Biol.* 18:122.
49 Schreiner, S. A., Garcia-Cuellar, M. P., Fey, G. H. and Slany, R. K. 1999. The leukemogenic fusion of MLL with ENL creates a novel transcriptional transactivator. *Leukemia* 13:1525.
50 Doty, R. T., Vanasse, G. J., Disteche, C. M. and Willerford, D. M. 2002. The leukemia-associated gene *Mllt1*/ENL: characterization of a murine homolog and demonstration of an essential role in embryonic development. *Blood Cells Mol. Dis.* 28:407.
51 Nowling, T. K., Johnson, L. R., Wiebe, M. S. and Rizzino, A. 2000. Identification of the transactivation domain of the transcription factor Sox-2 and an associated co-activator. *J. Biol. Chem.* 275:3810.

52 Garcia-Cuellar, M. P., Zilles, O., Schreiner, S. A., Birke, M., Winkler, T. H. and Slany, R. K. 2001. The ENL moiety of the childhood leukemia-associated MLL-ENL oncoprotein recruits human Polycomb 3. *Oncogene* 20:411.
53 van Lohuizen, M. 1999. The trithorax-group and polycomb-group chromatin modifiers: implications for disease. *Curr. Opin. Genet. Dev.* 9:355.
54 Wahl, M. I., Fluckiger, A. C., Kato, R. M., Park, H., Witte, O. N. and Rawlings, D. J. 1997. Phosphorylation of two regulatory tyrosine residues in the activation of Bruton's tyrosine kinase via alternative receptors. *Proc. Natl Acad. Sci. USA* 94:11526.
55 Versteege, I., Sevenet, N., Lange, J., Rousseau-Merck, M. F., Ambros, P., Handgretinger, R., Aurias, A. and Delattre, O. 1998. Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature* 394:203.
56 Kingston, R. E. and Narlikar, G. J. 1999. ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes Dev.* 13:2339.
57 Cote, J., Quinn, J., Workman, J. L. and Peterson, C. L. 1994. Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. *Science* 265:53.
58 Imbalzano, A. N., Kwon, H., Green, M. R. and Kingston, R. E. 1994. Facilitated binding of TATA-binding protein to nucleosomal DNA. *Nature* 370:481.
59 Cairns, B. R., Lorch, Y., Li, Y., Zhang, M., Lacomis, L., Erdjument-Bromage, H., Tempst, P., Du, J., Laurent, B. and Kornberg, R. D. 1996. RSC, an essential, abundant chromatin-remodeling complex. *Cell* 87:1249.
60 Kwon, H., Imbalzano, A. N., Khavari, P. A., Kingston, R. E. and Green, M. R. 1994. Nucleosome disruption and enhancement of activator binding by a human SW1/SNF complex. *Nature* 370:477.
61 Owen-Hughes, T. and Workman, J. L. 1996. Remodeling the chromatin structure of a nucleosome array by transcription factor-targeted trans-displacement of histones. *EMBO J.* 15:4702.
62 Craig, E., Zhang, Z. K., Davies, K. P. and Kalpana, G. V. 2002. A masked NES in INI1/hSNF5 mediates hCRM1-dependent nuclear export: implications for tumorigenesis. *EMBO J.* 21:31.

Transgene-mediated hyper-expression of IL-5 inhibits autoimmune disease but increases the risk of B cell chronic lymphocytic leukemia in a model of murine lupus

Xiangshu Wen[1], Danqing Zhang[1], Yuji Kikuchi[2], Yi Jiang[1,3], Kazuhiro Nakamura[1], Yan Xiu[1], Hiromichi Tsurui[1], Kazuko Takahashi[1], Masaaki Abe[1], Mareki Ohtsuji[1], Hiroyuki Nishimura[4], Kiyoshi Takatsu[5], Toshikazu Shirai[1] and Sachiko Hirose[1]

[1] Department of Pathology, Juntendo University School of Medicine, Tokyo, Japan
[2] Laboratory of Immunoregulation, Kitasato Institute for Life Science, Kitasato University, Tokyo, Japan
[3] Central Laboratory of First Clinical College, China Medical University, Shenyang, China
[4] Department of Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan
[5] Toin Human Science and Technology Center, Department of Biomedical Engineering, Toin University of Yokohama, Yokohama, Japan

IL-5 preferentially activates B1 cells to produce natural antibodies cross-reactive to self antigens. To determine the role of IL-5 in antibody-mediated autoimmune disease, we generated systemic lupus erythematosus (SLE)-prone (NZB×NZW)F1 mice congenic for IL-5 transgene (TG-F1). The transgene unexpectedly reduced the incidence of lupus nephritis. Anti-DNA antibodies in sera and those produced by splenic B cells *in vitro* were markedly decreased in TG-F1 mice, while total polyclonal Ig levels were comparable to those in IL-5 transgene-negative (NZB×NZW)F1 (non-TG-F1) littermates. Flow cytometry-sorted splenic B1 cells showed a significant reduction of anti-DNA antibody synthesis in response to IL-5, while proliferative responses to IL-5 did not significantly differ between TG-F1 and non-TG-F1 mice. As TG-F1 mice aged, frequencies of peripheral B1 cells progressively increased, and the mice frequently developed B cell chronic lymphocytic leukemia (B-CLL). Our results suggest that dysregulated, continuous high expression of IL-5 in SLE-prone mice may directly or indirectly mediate a skewed signaling of proliferation/differentiation of self-antigen-activated B1 cells, leading to suppression of autoimmune disease, but instead to aberrant expansion of B1 cells, giving rise to B-CLL. Thus, this model may provide a clue to the pathogenesis of both SLE and B-CLL.

Received	30/4/04
Revised	23/6/04
Accepted	13/7/04

Key words: SLE / B cell chronic lymphocytic leukemia / B1 cells / IL-5 / Transgenic mice

1 Introduction

Currently, at least two subpopulations of mouse B cells, B1 and B2 cells, can be divided on the basis of differences in phenotype, physiology and antibody repertoire. The majority of B1 cells express CD5 on their cell surface (B1a cells), while there is a minor subset of B1 cells (B1b cells) that lack the CD5 phenotype [1, 2]. Functionally, B1 cells mainly participate in innate immunity, in contrast to conventional B2 cells, which participate in acquired immunity. B1 cells produce IgM natural antibodies of a low-avidity nature, which are polyreactive and cross-react with a variety of self antigens [3, 4].

IL-5 is a growth and differentiation factor of eosinophils and B cells [5]. Takatsu and collaborators [6, 7] found that the IL-5 receptor is preferentially expressed on $CD5^+$ B1 cells [6], and that IL-5 selectively maintains B1 cells in an *in vitro* bone marrow culture [7]. Furthermore, IL-5-transgenic C3H mice showed a massive eosinophilia and an expansion of B1 cells associated with increased production of IgM antibodies to DNA [8]. Thus, even in normal healthy mice, B1 cells continuously exposed to IL-5 have the potential to produce large amounts of IgM autoantibodies. However, IL-5-transgenic C3H mice do not develop autoimmune disease, probably because B1 cells in IL-5-transgenic mice on a C3H genetic

[DOI 10.1002/eji.200425267]

Abbreviations: B-CLL: B cell chronic lymphocytic leukemia **TG-F1:** IL-5 transgene-congenic (NZB×NZW)F1 **non-TG-F1:** IL-5 transgene-negative (NZB×NZW)F1 **PAS:** Periodic acid-Schiff

background do not show IgM-to-IgG class switching and affinity maturation.

(NZB×NZW)F1 mice spontaneously develop a severe autoimmune disease closely resembling human SLE [9]. Young F1 mice before the onset of SLE show IgM hypergammaglobulinemia, including anti-DNA autoantibodies. We earlier found that $CD5^+$ B1 cells are virtually entirely responsible for these events [10, 11]. Unlike the case in normal healthy strains of mice, B1 cells in (NZB×NZW)F1 mice are hyper-responsive to IL-5, and produce large amounts of IgM *in vitro* in the presence of IL-5 [11–13]. Of note was the finding that in response to IL-5, these B1 cells change their phenotype with the Mott cell, *i. e.* a pathological state of plasma cells containing large numbers of intracellular inclusions of Ig [14]. Whereas Mott cells are rarely found in healthy individuals, they do appear in large numbers in lymphoid organs and other inflammatory tissues in autoimmune diseases including SLE [15, 16]. Thus, it is suggested that IL-5 contributes to aberrant B1 cell activation and differentiation found in early life of (NZB×NZW)F1 mice, which mediate IgM hypergammaglobulinemia and a high frequency of Mott cell formation.

Development of SLE in (NZB×NZW)F1 mice, mainly characterized by lupus nephritis, is significantly associated with IgM-to-IgG class switching and affinity maturation of anti-DNA antibodies, which occurs when the animals are 5–6 months of age [9]. However, characterization of cell surface phenotypes of anti-DNA antibody-producing precursors showed that while anti-DNA IgM was produced *in vitro* by $CD5^+$ B1 cells, anti-DNA IgG was mainly produced by cells in the pre-plasma stage with the $CD5^-$ phenotype. Using hybridoma technology and Ig V_H repertoire analysis, however, we [17] and others [18] showed evidence that precursors of affinity-selected pathogenic IgG anti-DNA clones in aged F1 mice with overt SLE originate from expanded low-affinity IgM anti-DNA clones. Thus, we postulated that IL-5-mediated hyperactivation of B1 cells in young (NZB×NZW)F1 mice may be a prerequisite for later production of pathogenic high-affinity IgG autoantibodies, leading to an early onset of severe SLE. To validate this hypothesis, we established IL-5 transgene-congenic (NZB×NZW) F1 (TG-F1) mice, and examined effects of IL-5 on SLE.

2 Results

2.1 Establishment of IL-5 transgene-congenic (NZB×NZW) F1 mice

We established IL-5 transgene-congenic NZB and NZW strains by backcrossing (NZB×IL-5-transgenic C3H) F1 and (NZW×IL-5-transgenic C3H) F1 mice to NZB and NZW, respectively. Mice with over 15% of peripheral eosinophils were selected as transgene-positive. These congenic NZB and NZW mice were crossed to obtain (NZB×NZW) F1 hybrids, and the final screening for IL-5 transgene in F1 hybrids was done using Southern blot analysis (Fig. 1). The frequency of peripheral eosinophils per total leukocytes (mean and SE) was higher in TG-F1 (24.6+2.0%) than found in transgene-negative F1 (non-TG-F1) littermates (2.4+0.4%) at 2 months of age ($p<0.001$). While serum IL-5 was undetectable in non-TG-F1 mice, significant amounts were detected in TG-F1 mice (94.3+19.4 U/ml).

2.2 Effect of IL-5 transgene on SLE features

We first examined serum levels of IgM and IgG anti-DNA antibodies and total IgM and IgG. Compared to non-TG-F1 mice, TG-F1 mice unexpectedly showed a significant reduction in both IgM and IgG anti-DNA antibodies at 5 and 10 months of age (Fig. 2A), while amounts of total serum IgM and IgG did not significantly differ between the two groups of F1 mice (Fig. 2B).

The development of lupus nephritis was in parallel with the age-associated increase in titers of IgG anti-DNA antibodies, and the cumulative incidence of proteinuria in TG-F1 mice was much lower than that found in non-TG-F1 mice (Fig. 2C). While all of the 12 non-TG-F1 mice died of lupus nephritis by 15 months of age, 12 of 15 (80%) TG-F1 mice were still alive at this age (Fig. 2C). Histological and immunofluorescence examinations of

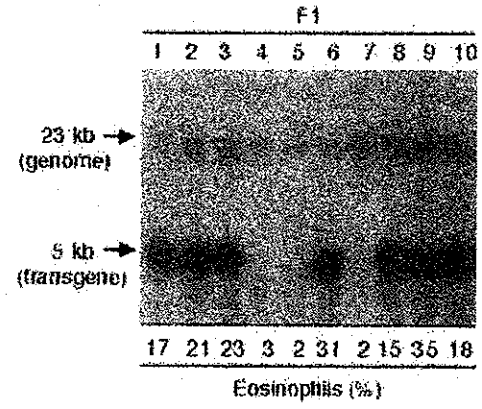

Fig. 1. Detection of the IL-5 transgene in F1 hybrids of IL-5-transgenic NZB and NZW mice by Southern blot analysis. Among ten F1 mice, numbers 1, 2, 3, 6, 8, 9 and 10 were estimated to be transgene-positive with 5-kb-long transgene. Percentage of eosinophils in peripheral blood of the individual mouse is also shown.

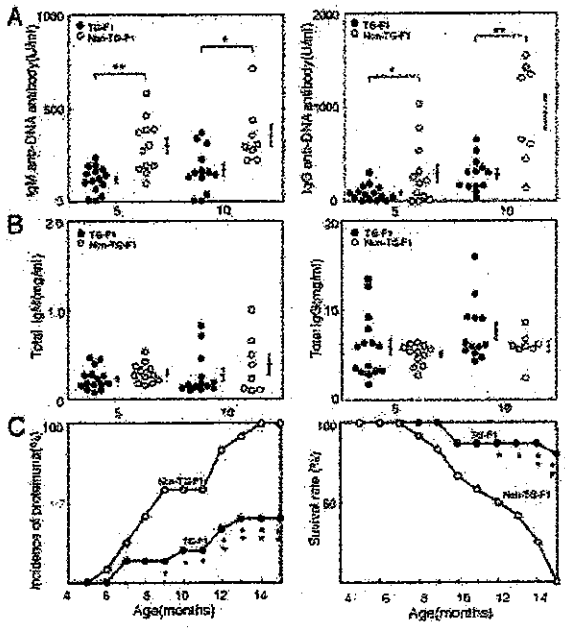

Fig. 2. Comparisons of (A) serum levels of IgM and IgG class anti-DNA antibodies, (B) serum levels of total IgM and IgG, and (C) cumulative incidence of proteinuria and survival rate between TG-F1 and non-TG-F1 mice. Mean and SE of antibody levels are shown on the right side of plots for an individual mouse. Asterisks indicate significant differences between TG-F1 and non-TG-F1 mice (**$p<0.001$, *$p<0.05$).

renal glomeruli showed that glomeruli in non-TG-F1 mice were markedly enlarged with massive deposition of immune complexes positive for periodic acid-Schiff (PAS) stain, with IgG and C3 both in mesangial areas and along capillary walls. In contrast, glomeruli in the TG-F1 mice were of normal size and limited amounts of IgG and C3 deposits were observed only in mesangial areas (Fig. 3).

2.3 Effect of IL-5 transgene on B1 cell frequencies

We then examined age-associated changes in frequencies of $CD5^+$ B1 cells per total $B220^+$ B cells in peripheral blood, using flow cytometry (FCM) (Fig. 4A). Frequencies of B1 cells progressively increased with aging in TG-F1 mice and were significantly higher than those found in non-TG-F1 mice at 5 months of age onward (Fig. 4B). Absolute B1 cell counts/μl (mean and SE) in the peripheral blood of TG-F1 and non-TG-F1 mice aged 12 months were 10,299+2,019 and 2,605+306, respectively, and the difference was statistically significant ($p<0.02$). There was no significant difference in absolute numbers of T cells, B2 cells and a subset of $CD5^-B220^{dull}$

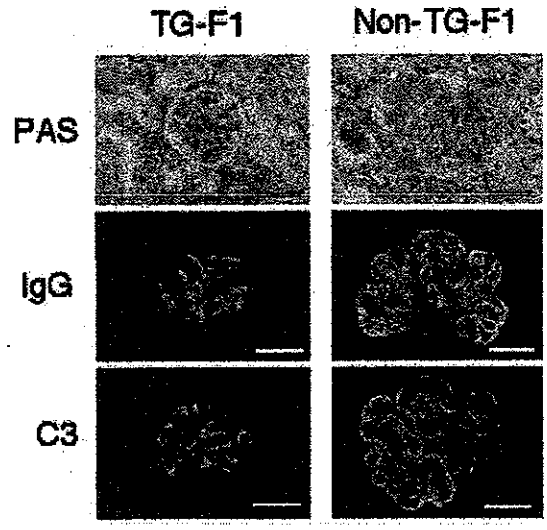

Fig. 3. Representative histopathological findings of renal glomeruli in 9-month-old TG-F1 and non-TG-F1 mice. Sections were stained with PAS solution or with fluorescence-conjugated anti-IgG and anti-C3 antibodies. Scale bar, 50 μm.

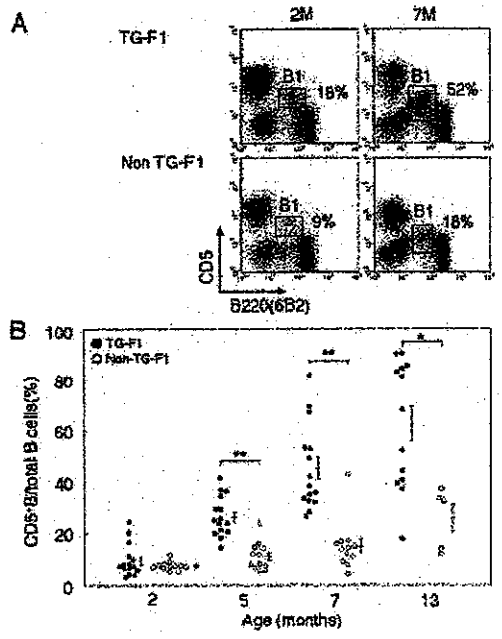

Fig. 4. (A) Representative FCM profiles of peripheral lymphocytes stained with mAb to CD5 and B220 (6B2) in TG-F1 and non-TG-F1 mice at 2 and 7 months of age. B1 cell subset is boxed and B1 cell frequencies per total $B220^+$ B cells are indicated. (B) Sequential changes in frequencies of $CD5^+$ B1 cells per total B cells in peripheral blood in TG-F1 and non-TG-F1 mice. Mean and SE are shown on the right side of plots for an individual mouse. Asterisks indicate significant differences in B1 cell frequencies between TG-F1 and non-TG-F1 mice (**$p<0.001$, *$p<0.05$).

B1 cells (B1b cells) [1, 2] between TG-F1 and non-TG-F1 mice (data not shown).

In TG-F1 mice, 6 out of 15 mice (40%) showed B1 cell frequencies over 80% at 13 months of age. Using criteria that mice with total peripheral leukocyte counts/μl over 25,000 (mean + 2SD in non-TG-F1 mice over 12 months of age) are diagnosed as having B cell chronic lymphocytic leukemia (B-CLL), these 6 TG-F1 mice eventually developed B-CLL by the age of 22 months. Fig. 5A shows a representative FCM profile of peripheral blood lymphocytes in these mice, in which $CD5^{dull}B220^{dull}$ B-CLL cells are markedly expanded. Blood film presented numerous B-CLL cells having a basophilic cytoplasm and an atypical large cleaved nucleus with a course granular chromatin (Fig. 5B). These B-CLL cells show massive infiltration into interstitial tissues of the liver, kidney and lung (Fig. 5C–E). Fig. 5F shows a representative Southern blot analysis of genomic DNA from T cell-depleted spleen cells for clonal Ig V_H region gene rearrangements, using an Ig H chain joining region (J_H) probe. While DNA from a 5-month-old TG-F1 mouse showed smear pattern, a single rearranged band was observed with DNA from a 22-month-old TG-F1 mouse with B-CLL.

2.4 Changes in cell surface phenotypes of splenic B cells

Fig. 6 shows expression levels of cell surface molecules that can potentially affect proliferation and differentiation of B cells. Compared to B2 cells, B1 cells preferentially expressed IL-5Rα and CD80 on their cell surfaces; however, there was no difference between TG-F1 and non-TG-F1 mice. MHC class II I-A levels were almost identical between B1 and B2 cells, and there was no difference between TG-F1 and non-TG-F1 mice. Of note was the finding that IL-2Rα levels were markedly up-regulated on the majority of B1 but not B2 cells in TG-F1 mice, compared to those found in non-TG-F1 mice.

2.5 *In vitro* potential of splenic B cells to produce antibodies

Table 1 shows *in vitro* potential of T cell-depleted spleen cells from 7-month-old TG-F1 and non-TG-F1 mice to produce anti-DNA antibodies. We earlier found that splenic B cells obtained from aged (NZB×NZW) F1 mice spontaneously produce significant amounts of IgM and

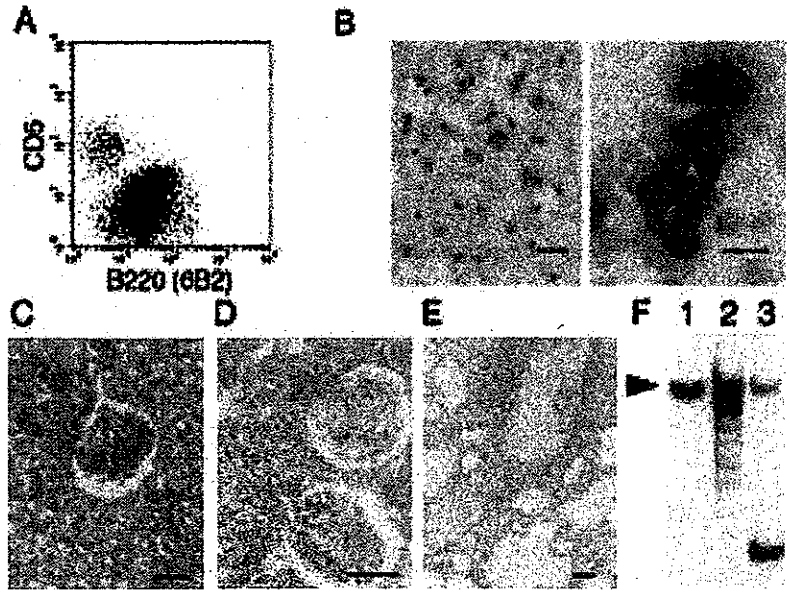

Fig. 5. Development of B-CLL in a 22-month-old TG-F1 mouse. (A) FCM profile of peripheral blood lymphocytes stained with mAb to CD5 and B220 (6B2). Frequencies of $CD5^{dull}B220^{dull}$ B-CLL cells are markedly increased. (B) Blood film (Giemsa stain). Many atypical lymphocytes with a cleaved large nucleus are shown (total peripheral leukocyte count, 41,000/μl). Scale bar, 50 μm (left), 10 μm (right). (C–E) Infiltration of leukemic cells (C) into parenchymal tissues around central vein of the liver (bar, 50 μm), (D) around renal glomeruli (bar, 50 μm) and (E) in perivascular regions in the lung (bar; 50 μm). (F) Southern blot analysis for clonal Ig V_H region gene rearrangements. DNA from the liver (lane 1) and T cell-depleted splenic cells from a 5-month-old TG-F1 mouse (lane 2) and from a 22-month-old TG-F1 mouse with B-CLL (lane 3) were digested with EcoRI and subjected to Southern blot analysis, using the J_H probe. Arrow indicates germ-line bands.

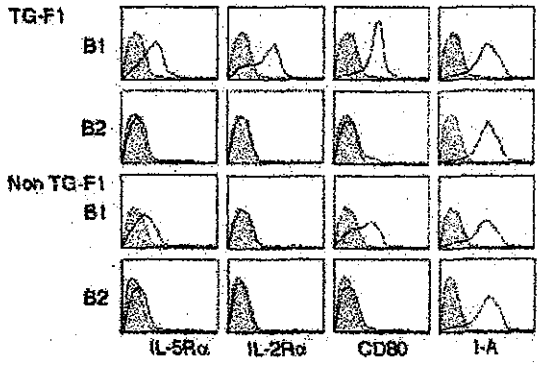

Fig. 6. Comparisons of IL-5Rα, IL-2Rα, CD80 and I-A expression levels on the cell surface of splenic B1 and B2 cells between 4-month-old TG-F1 and non-TG-F1 mice. Spleen cells were triple-stained with mAb to CD5 and B220 (6B2) and mAb to each IL-5Rα, IL-2Rα, CD80 or I-A. Expression levels of each molecule on gated $CD5^+B220^{dull}$ B1 cells and $CD5^-B220^+$ B2 cells were compared by the histogram. The shaded histogram indicates the background staining.

IgG anti-DNA antibodies *in vitro*, and that the antibody syntheses are up-regulated in the presence of IL-5 and IL-6, respectively [13]. In keeping with these findings, spleen cells from non-TG-F1 mice spontaneously produced significant amounts of IgM and IgG anti-DNA antibodies, and the levels were significantly up-regulated in the presence of IL-5 and IL-6, respectively. In the same experiments using spleen cells from TG-F1 mice, both spontaneous and IL-5-induced productions of anti-DNA IgM were significantly lower than those found in non-TG-F1 mice. Levels of spontaneous anti-DNA IgG production in TG-F1 mice were much lower than in non-TG-F1 mice, and IL-6 did not significantly promote the antibody synthesis. In contrast, when we measured amounts of polyclonal IgM and IgG spontaneously produced in the same cultures, there were no significant differences between TG-F1 and non-TG-F1 mice. IL-5 up-regulated polyclonal IgM and IL-6 up-regulated IgG syntheses; however, amounts produced were almost identical between TG-F1 and non-TG-F1 mice (Table 1).

We then examined *in vitro* potential of FCM-sorted splenic $CD5^+B220^{dull}$ B1 cells to produce IgM antibodies. Irrespective of the presence or absence of IL-5, amounts of anti-DNA IgM produced in TG-F1 mice were much smaller than those in non-TG-F1 mice, while there were no significant changes in total amounts of IgM in supernatants between TG-F1 and non-TG-F1 mice (Table 2).

2.6 *In vitro* proliferative response of B cells

Table 3 shows *in vitro* proliferative responses of splenic $CD5^+B220^{dull}$ B1 cells to IL-5. In keeping with earlier findings [12], B1 cells from non-TG-F1 mice were hyper-responsive to IL-5 and showed high proliferative responses. B1 cells from TG-F1 mice also showed comparable levels of IL-5 hyper-responsiveness, indicating that this property of B1 cells in (NZB×NZW) F1 mice remains unchanged in TG-F1 mice. B2 cells from both TG-F1 and non-TG-F1 mice were negative for proliferative response to IL-5 (data not shown). Consistent

Table 1. Reduction in anti-DNA, but not polyclonal, antibody synthesis by T cell-depleted splenic B cells from IL-5-transgenic (NZB×NZW)F1 mice[a)]

Mice	IgM class		IgG class	
	Medium	IL-5	Medium	IL-6
Anti-DNA antibodies (U/ml)				
TG-F1	0.16±0.01[b)]	1.16±0.03[b)]	0.07±0.03[b)]	0.09±0.05[b)]
Non-TG-F1	0.93±0.07	2.84±0.16	1.52±0.04	4.48±0.36
Total Ig (μg/ml)				
TG-F1	4.61±0.18	8.74±1.26	0.88±0.04	3.22±0.07
Non-TG-F1	4.73±2.45	7.90±1.57	1.02±0.08	3.77±0.32

a) *In vitro* antibody production by T cell-depleted spleen cells from 7-month-old TG-F1 and non-TG-F1 mice. A total of 2×10^5 cells were cultured in the absence or the presence of IL-5 (50 U/ml) or IL-6 (500 U/ml) for 7 days, and amounts of antibodies in culture supernatants were compared. Results represent means and SE in triple cultures. Representative results of three independent experiments are shown.

b) Differences between TG-F1 and non-TG-F1 mice were statistically significant ($p<0.05$). IL-5 up-regulated anti-DNA IgM and polyclonal IgM in both TG-F1 and non-TG-F1 mice ($p<0.05$). IL-6 up-regulated anti-DNA IgG only in non-TG-F1 mice ($p<0.05$), and polyclonal IgG in both TG-F1and non-TG-F1 mice ($p<0.05$).

Table 2. Reduction in anti-DNA, but not polyclonal, IgM synthesis by FCM-sorted splenic B1 cells from IL-5-transgenic (NZB×NZW) F1 mice[a]

Mice	Medium	IL-5
IgM anti-DNA antibodies ($\times10^{-1}$ U/ml)		
TG-F1	0.09±0.04[b]	3.50±0.59[b]
Non-TG-F1	1.61±0.03	15.33±1.90
Total IgM (μg/ml)		
TG-F1	1.91±0.10	5.64±0.73
Non-TG-F1	1.79±0.15	7.32±0.45

[a] *In vitro* antibody production by FACS-sorted splenic $CD5^+B220^{dull}$ B1 cells from 4-month-old TG-F1 and non-TG-F1 mice. A total of 2×10^5 sorted cells were cultured in the absence or the presence of IL-5 (50 U/ml) for 7 days, and amounts of antibodies in culture supernatants were compared. Results represent means and SE in triple cultures. Representative results of three independent experiments are shown.

[b] Differences between TG-F1 and non-TG-F1 mice were statistically significant ($p<0.001$). IL-5 up-regulated anti-DNA IgM and polyclonal IgM in both TG-F1 and non-TG-F1 mice ($p<0.005$).

with the finding that IL-2Rα expression was markedly up-regulated on the majority of B1 cells from TG-F1 mice (Fig. 6), proliferative responses to IL-2 of TG-F1 B1 cells were significantly higher than those of non-TG-F1 B1 cells.

3 Discussion

The transgene-mediated hyper-expression of IL-5 unexpectedly suppressed autoimmune disease in TG-F1 mice. The transgene, in some cases, is known to reduce mature B cells and to suppress antibody responses of B cells [19, 20]. Here, however, this may not be the case, because there is evidence that the original IL-5-transgenic C3H mice, a donor of the transgene, showed a significant expansion of B1 cells in association with production of large amounts of IgM anti-DNA antibodies [8]. As is the case in IL-5-transgenic C3H mice, our TG-F1 mice showed an eosinophilia and a marked expansion of B1 cells; nevertheless, autoantibody production was strikingly down-regulated. It is also unlikely that certain suppressive genetic elements for autoantibody synthesis were introduced from the C3H strain, because B1 cells from the non-TG-F1 littermates responded well to IL-5 *in vitro*, proliferated even in the absence of prior activation signals and produced large amounts of IgM anti-DNA antibodies, as was evident in wild-type (NZB×NZW) F1 mice [13].

Table 3. Comparisons of *in vitro* proliferative responses of splenic B1 cells to IL-5 and IL-2 between TG-F1 and non-TG-F1 mice[a]

Mice	$[^3H]$Thymidine incorporation ($\times10^3$ cpm)		
	Medium	IL-5	IL-2
TG-F1	1,121±85	4,884±196	7,691±584[b]
Non-TG-F1	1,688±6	5,295±122	4,089±12

[a] *In vitro* proliferative responses of FACS-sorted splenic $CD5^+B220^{dull}$ B1 cells from 4-month-old TG-F1 and non-TG-F1 mice. A total of 2×10^5 sorted cells were cultured in the absence or the presence of IL-5 (50 U/ml) or IL-2 (5,000 U/ml) for 3 days. During the last 24 h of culture, $[^3H]$thymidine was added and thymidine uptake was counted. Results represent means and SE in triple cultures. Representative results of three independent experiments are shown.

[b] Difference between TG-F1 and non-TG-F1 mice was statistically significant ($p<0.05$).

Our data clearly showed that the reduction of anti-DNA antibody synthesis is not due to the suppressive effect of IL-5 transgene on polyclonal B cells, because total amounts of IgM and IgG in sera or those produced *in vitro* in B cell cultures were almost identical between TG-F1 and non-TG-F1 mice. There are several possibilities to account for these findings. TG-F1 mice may have skewed B1 cell repertoires. As IL-5 transduces signals for proliferation to polyclonal B1 cells in an antigen-independent manner, relative frequencies of anti-DNA clones in TG-F1 mice may become low in expanded polyclonal B1 cell repertoires, resulting in low serum levels of both IgM and IgG anti-DNA antibodies even after self antigen stimulation. Alternatively, self-reactive B1 cells in wild-type (NZB×NZW) F1 mice are relatively resistant to Fas-mediated apoptosis [21]. Dysregulated, continuous stimulation by IL-5 may render such activated anti-DNA clones susceptible for Fas-mediated elimination or unresponsive to signals for antibody synthesis in TG-F1 mice. To our knowledge, however, there have been no reports suggesting the involvement of IL-5 in the induction of immunological tolerance.

Another possibility may relate to the observed aberrant up-regulation of IL-2Rα expression on a majority of B1 cells in TG-F1 mice (Fig. 6). B1 cells in (NZB×NZW) F1 mice are intrinsically hyper-proliferative in response to IL-2, although the IL-2 signal does not induce their differentiation [22, 23]. One can speculate that in TG-F1 mice, up-regulated IL-2R expression induces skewed signaling for abnormal propagation of these anti-DNA clones, which eventually dysregulates their differentiation and affinity maturation; thus leading to reduction not only

in anti-DNA IgM of low-avidity nature but also in affinity-selected anti-DNA IgG.

In addition, a continuous stimulation of self-antigen-activated B1 cells with large amounts of IL-5 may cause a skewed IL-5 receptor signaling, *i.e.* positive for proliferation and negative for differentiation and maturation. Several distinct signaling cascades are involved in cell proliferation and maturation in the IL-5 receptor-signaling pathway. IL-5 activates a number of kinases, including Bruton-type tyrosine kinase (Btk), Janus kinase (Jak2), Lyn and Raf-1, as well as the phosphatase SHP2 [24–28]. Among these, Btk and Jak2 are essential for proliferation of B-lineage cell lines [24, 25]. Stimulation of B cells with IL-5 together with CD38 ligation induces expression of the Blimp-1 gene, capable of driving the terminal differentiation of B cells into IgM-secreting cells [29], and induces an μ–γ1 switch recombination to IgG1 secretion in a STAT5-dependent manner [30]. Whether there is any skewed signaling of the transgenic IL-5 in B1 cells of TG-F1 mice is now under investigation in our laboratory.

Although both IL-5-transgenic C3H mice and TG-F1 mice showed aberrant expansion of B1 cells, the extent was much greater in the latter. As (NZB×NZW) F1 mice have several susceptibility loci for abnormal expansion of B1 cells [31–35], IL-5 appears to augment this property of B1 cells in these mice. Lack of such genetic elements in C3H mice may account for the failure of the development of both SLE and B-CLL in IL-5-transgenic C3H mice [8]. Considering that the IL-2R expression is highly up-regulated on B1 cells in TG-F1 mice, aberrant, progressive proliferation of B1 cells in TG-F1 mice can be attributed to combined effects of both IL-5 and IL-2 cytokines and the heretofore unidentified genetic elements. Together with the long-lived capacity [1, 2], such B1 cells are likely to be highly susceptible for accumulation of genetic alterations, giving rise to malignant transformation.

Our present model suggests that certain regulatory abnormalities for proliferation and/or differentiation of B1 cells are a crossroad between B-CLL and autoimmune disease. Patients with B-CLL frequently develop intercurrent systemic autoimmune diseases, and genetic factors may predispose to this event [36]. In this context, we earlier found that while (NZB×NZW) F1 mice, carrying H-2^d from NZB and H-2^z from NZW, develop severe SLE [37–39], homozygous H-$2^{z/z}$-congenic (NZB×NZW) F1 mice do not develop SLE, but instead show abnormal proliferation of B1 cells, giving rise to B-CLL [31, 32], the same phenotypic change of the disease observed in TG-F1 mice. Thus, it may be that both genetic and environmental factors can induce a certain shared dysregulation of B1 cells. Taken collectively, further studies of our TG-F1 mouse model may provide clues to molecular mechanisms regulating proliferation and differentiation of self-reactive B1 cells, as related to autoimmune disease and B-CLL.

4 Materials and methods

4.1 Mice

To obtain IL-5 transgene-congenic NZB and NZW mice, NZB or NZW mice were crossed with murine IL-5 cDNA-transgenic C3H/HeN mice [8], and F1 mice were selectively backcrossed with NZB or NZW mice for eight generations. IL-5-transgenic C3H/HeN mice constitutively show increased frequencies of peripheral eosinophils [8]. Thus, mice with peripheral eosinophils of over 15% were regarded as positive for the IL-5 transgene. Percentages of eosinophils per total leukocytes were calculated under light microscopy with a blood smear stained with Giemsa solution. Selected mice were crossed to produce (NZB×NZW) F1 mice, and presence of the transgene was confirmed by Southern blot hybridization, using BamHI-digested tail DNA [8] (Fig. 1). The IL-5 probe used was the 461-bp SacI-AccI fragment of pSP6 K-mTRF23 [40].

4.2 Measurement of IL-5

Anti-IL-5 mAb NC17 (100 μg/ml) was coated onto ELISA plate, and nonspecific binding sites were blocked with 2% BSA in PBS. Samples were then applied to wells and incubated overnight at room temperature. After washing wells with PBS containing 0.05% Tween-20, polyclonal rabbit anti-mouse IL-5 antibodies were added, followed by a 4-h incubation at room temperature. Then, peroxidase-conjugated goat anti-rabbit Ig and 2,2'-azino-di(3-ethyl)-benzthiazoline sulfonic acid were used as a coloring system. Optical density was read at 405 nm. A standard curve was obtained using purified rIL-5.

4.3 Cell counts and cytological examination

Peripheral leukocyte counts were made using a MEK-6158 Automatic Blood Cell Counter (Nihon Koden, Tokyo, Japan). Leukocyte-rich populations were separated from 40 μl of heparinized blood using a density gradient lymphocyte separator M-SMF (Japan Immuno Research Laboratories Co. Ltd., Takasaki, Japan). Leukocyte film for cytological examination was prepared using the Cytospin 3 Cell Preparation System (Shandon Scientific Ltd., UK), then stained with Giemsa.

4.4 FCM analysis

Frequency of peripheral $CD5^+$ B1 cells per total B cells was examined using FACStar^{PLUS} (Becton Dickinson, San Jose, CA). Peripheral blood was treated with ammonium chloride to remove red blood cells. Aliquots of 5×10^5–10×10^5 cells in 20 µl of PBS containing 0.2% BSA/0.05% NaN_3 were incubated with FITC-labeled anti-mouse CD45R (B220) (clone RA3–6B2) and PE-labeled anti-mouse CD5 (clone 53–7.3) mAb at 4°C for 30 min. Percent frequency of $CD5^+$ B1 cells per total $B220^+$ B cells was calculated using the FITC/PE filter system.

Expression of IL-5Rα, IL-2Rα (CD25), CD80 and MHC class II (I-A) on B1 and B2 cells was examined by three-color FCM analysis. Spleen cell suspension was first incubated with FITC-labeled anti-mouse B220 (6B2) and PE-labeled anti-mouse CD5 mAb. Stained cells were further incubated with biotinylated mAb to IL-5Rα (H7) [41], CD25, CD80 (RM80) or I-A (m52) (K24–199), followed by allophycocyanin-labeled streptavidin. All incubations were done at 4°C for 30 min. Cells in the compartment with $CD5^+B220^{dull+}$ B1 and $CD5^-B220^+$ B2 phenotypes were gated and examined.

4.5 Cell sorting and culture

T cell-depleted spleen cells were obtained by treatment of cells with a mixture of mAb to CD4, CD8 and Thy1.2 plus rabbit complement at 37°C for 45 min. Contamination of T cells was under 2%. $CD5^+B220^{dull}$ B1 cells were FCM-sorted after staining cells with FITC-conjugated anti-B220 and PE-conjugated anti-CD5 mAb. Purity of sorted B1 cells always exceeded 95%.

For *in vitro* antibody assay, aliquots of 2×10^5 cells in 200 µl of RPMI 1640 consisting 5×10^{-5} M 2-mercapoethanol, penicillin (100 U/ml), streptomycin (100 mg/ml) and 10% FCS were cultured in 96-well flat-bottomed plates in the absence or the presence of mIL-5 (50 U/ml) or mIL-6 (500 U/ml) at 37°C for 7 days. For the proliferation assay, 2×10^5 cells per well in 96-well round-bottomed plates were cultured in the absence or the presence of mIL-5 (50 U/ml) or hIL-2 (5,000 U/ml) at 37°C for 3 days with addition of 0.5 µCi of [^{3}H]thymidine during the final 24 h and were then subjected to measurements of radioactivity.

4.6 Measurement of serum levels of Ig and class-specific anti-DNA antibodies

Isotype-specific Ig and anti-DNA autoantibodies were measured by ELISA with isotype-specific antibodies. The DNA-binding activities were expressed in U, referring to a standard curve obtained by serial dilution of a standard serum pool from 4- to 7-month-old NZB mice for IgM class and from over 9-month-old (NZB×NZW) F1 mice for IgG class, both containing 1,000 U activities/ml [39]. Double-stranded DNA was obtained from calf thymus DNA (Sigma Chemical Co., St. Louis, MO).

4.7 Histopathology and immunofluorescence staining

Organs were removed from necropsied animals, fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin or PAS solution. For immunofluorescence staining, frozen sections were incubated for 60 min at room temperature with FITC-labeled goat antibodies to IgG or to C3 (ICN Pharmaceuticals, Inc., Aurora, OH).

4.8 Southern blot analysis for B cell clonality

Approximately 10 µg of genomic DNA extracted from liver or T cell-depleted spleen cells was digested with EcoRI, fractionated on 0.7% agarose gels, transferred to nitrocellulose filters, and probed with radiolabeled J_H probe, a 2.0-kb BamHI/EcoRI fragment that induces J_H3 and J_H4 segments [42]. Hybridizations were done in 6× SSPE/5× Denhardt's solution/0.5% SDS/100 µg/ml of salmon sperm DNA at 65°C and washed in 2× SSPE/0.1% SDS and 0.2× SSPE/0.1% SDS at 65°C (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA; 1× Denhardt's solution = 0.02% BSA/0.02% Ficoll/0.02% polyvinylpyrrolidone).

4.9 Proteinuria

The onset of renal disease was monitored by biweekly measurement of proteinuria, as described [37]. A proteinuria of 111 mg/100 ml or more was regarded as being positive.

4.10 Statistical analysis

Statistical analysis was made using Student's *t*-test and a chi-square test. *p* values of <5% were considered to have a statistical significance.

Acknowledgment: We thank M. Ohara for language assistance. This work was supported in part by Grant-in-Aid for Scientific Research on Priority Areas and for COE Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and grant from The Organization for Pharmaceutical Safety and Research, Japan.

References

1 **Herzenberg, L. A., Kantor, A. B. and Herzenberg, L. A.,** Layered evolution in the immune system. A model for the ontogeny and development of multiple lymphocyte lineages. *Ann. N. Y. Acad. Sci.* 1992. **651:** 1–9.

2 **Hardy, R. R. and Hayakawa, K.,** CD5 B cells, a fetal B cell lineage. *Adv. Immunol.* 1994. **5:** 297–339.

3 **Kocks, C. and Rajewsky, K.,** Stable expression and somatic hypermutation of antibody V regions in B cell developmental pathways. *Ann. Rev. Immunol.* 1989. **7:** 537–559.

4 **Casali, P. and Notkins, A. L.,** $CD5^+$ B lymphocytes, polyreactive antibodies and the human B cell repertoire. *Immunol. Today* 1989. **10:** 364–368.

5 **Takatsu, K., Tominaga, A., Harada, N., Mita, S., Matsumoto, M., Takahashi, T., Kikuchi, Y. and Yamaguchi, N.,** T cell-replacing factor (TRF)/interleukin 5 (IL-5): molecular and functional properties. *Immunol. Rev.* 1988. **102:** 107–135.

6 **Hitoshi, Y., Yamaguchi, N., Mita, S., Sonoda, E., Takaki, S., Tominaga, A. and Takatsu, K.,** Distribution on IL-5 receptor-positive B cells. Expression of IL-5 receptor on Ly-1$(CD5)^+$ B cells. *J. Immunol.* 1990. **144:** 4218–4225.

7 **Tominaga, A., Mita, S., Nishikawa, S., Ogawa, M., Hitoshi, Y., Kikuchi, Y. and Takatsu, K.,** Establishment of IL-5 dependent early B cell lines from bone marrow cells by long term bone marrow culture. *Growth Factors* 1989. **1:** 135–146.

8 **Tominaga, A., Takaki, S., Koyama, N., Katoh, S., Matsumoto, R., Migita, M., Hitoshi, Y., Hosoya, Y., Yamaguchi, S., Kanai, Y., Miyazaki, J., Usaku, G., Yamamura, K. and Takatsu, K.,** Transgenic mice expressing a B cell growth and differentiation factor gene (interkeukin 5) develop eosinophilia and autoantibody production. *J. Exp. Med.* 1991. **173:** 429–437.

9 **Shirai, T., Hirose, S., Okada, T. and Nishimura, H.,** Immunology and immunopathology of the autoimmune disease of NZB and related mouse strains.In **Rihova, B. and Vetvicka, V.** (Eds.) *Immunological disorders in mice.* CRC Press Inc., Boca Raton 1991, pp 95–136.

10 **Okada, T., Abe, M., Takiura, F., Hirose, S. and Shirai, T.,** Distinct surface phenotypes of B cells responsible for spontaneous production of IgM and IgG anti-DNA antibodies in autoimmune-prone NZB×NZW F1 mice. *Autoimmunity* 1990. **7:** 109–120.

11 **Herron, L. R., Coffman, R. L., Bond, M. W. and Kotzin, B. L.,** Increased autoantibody production by NZB/NZW B cells in response to IL-5. *J. Immunol.* 1988. **141:** 842–848.

12 **Umland, S. P., Go, N. F., Cupp, J. E. and Howard, M.,** Responses of B cells from autoimmune mice to IL-5. *J. Immunol.* 1989. **142:** 1528–1535.

13 **Kanno, K., Okada, T., Abe, M., Hirose, S. and Shirai, T.,** Differential sensitivity to interleukins of $CD5^+$ and $CD5^-$ anti-DNA antibody-producing B cells in murine lupus. *Autoimmunity* 1993. **14:** 205–214.

14 **Jiang, Y., Hirose, S., Hamano, Y., Kodera, S., Tsurui, H., Abe, M., Terashima, K., Ishikawa, S. and Shirai, T.,** Mapping of a gene for the increased susceptibility of B1 cells to Mott cell formation in murine autoimmune disease. *J. Immunol.* 1997. **158:** 992–997.

15 **East, J., De Sousa, M. A. B. and Parrott, D. M. V.,** Immunopathology of New Zealand Black (NZB) mice. *Transplantation* 1965. **3:** 711–729.

16 **Shultz, L. D., Coman, D. R., Lyons, B. L., Sidman, C. L. and Taylor, S.,** Development of plasmacytoid cells with Russell bodies in autoimmune "viable motheaten" mice. *Am. J. Pathol.* 1987. **127:** 38–50.

17 **Taki, S., Hirose, S., Kinoshita, K., Nishimura, H., Shimamura, T., Hamuro, J. and Shirai, T.,** Somatically mutated IgG anti-DNA antibody clonally related to germ-line encoded IgM anti-DNA antibody. *Eur. J. Immunol.* 1992. **22:** 987–992.

18 **Tillman, D. M., Jou, N.-T., Hill, R. J. and Marion, T. N.,** Both IgM and IgG anti-DNA antibodies are the products of clonally selective B cell stimulation in (NZB×NZW) F1 mice. *J. Exp. Med.* 1992. **176:** 761–779.

19 **Labrecque, N., Madsen, L., Fugger, L., Benoist, C. and Mathis, D.,** Toxic MHC class II β chains. *Immunity* 1999. **11:** 515–516.

20 **Nishimura, H., Ishikawa, S., Nozawa, S., Awaji, M., Saito, J., Abe, M., Gotoh, Y., Tokushima, M., Kimoto, M., Akakura, S., Tsurui, H., Hirose, S. and Shirai, T.,** Effects of transgenic mixed-haplotype major histocompatibility complex class II molecules $A\alpha^dA\beta^z$ on autoimmune disease in New Zealand mice. *Int. Immunol.* 1996. **8:** 967–976.

21 **Hirose, S., Yan, K., Abe, M., Jiang, Y., Hamano, Y., Tsurui, H. and Shirai, T.,** Precursor B cells for autoantibody production in genomically Fas-intact autoimmune disease are not subject to Fas-mediated immune elimination. *Proc. Natl. Acad. Sci. USA* 1997. **94:** 9291–9295.

22 **Hasegawa, K., Abe, M., Okada, T., Hirose, S., Sato, H. and Shirai, T.,** Are Ly-1 B cells responsible for the IL2-hyperresponsiveness of B cells in autoimmune-prone NZB×NZW F1 mice? *Int. Immunol.* 1989. **1:** 99–103.

23 **Sekigawa, I., Noguchi, K., Hasegawa, K., Hirose, S., Sato, H. and Shirai, T.,** B cell hyperresponsiveness to interleukin 2 and the age-associated decline in murine lupus. *Clin. Immunol. Immunopathol.* 1989. **51:** 172–184.

24 **Satoh, S., Katagiri, T., Takaki, S., Kikuchi, Y., Hitoshi, Y., Yonehara, S., Tsukada, S., Kitamura, D., Watanabe, T., Witte, O. and Takatsu, K.,** IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus 2 kinases. *J. Exp. Med.* 1994. **180:** 2101–2111.

25 **Yousefi, S., Hoessli, D. C., Blaser, K., Mills, G. B. and Simon, H. U.,** Requirement of Lyn and Syk tyrosine kinase for the prevention of apoptosis by cytokines in human eosinophils. *J. Exp. Med.* 1996. **183:** 1407–1414.

26 **Yasue, T., Nishizumi, H., Aizawa, S., Yamamoto, T., Miyake, K., Mizoguchi, C., Uehara, S., Kikuchi, Y. and Takatsu, K.,** A critical role of Lyn and Fyn for B cell responses to CD38 ligation and interleukin 5. *Proc. Natl. Acad. Sci. USA* 1997. **94:** 10307–10312.

27 **Ogata, N., Kouro, T., Yamada, A., Koike, M., Hanai, N., Ishikawa, T. and Takatsu, K.,** Jak2 and Jak1 constitutively associate with an interleukin-5 (IL-5) receptor α and βc subunit, respectively, and are activated upon IL-5 stimulation. *Blood* 1998. **91:** 2264–2271.

28 **Pazdrak, K., Olszewska-Pazdrak, B., Stafford, S., Garofalo, R. P. and Alam, R.,** Lyn, Jak2, and Raf-1 kinases are critical for the antiapoptotic effect of interleukin 5, whereas only Raf-1 kinase is essential for eosinophil activation and degranulation. *J. Exp. Med.* 1998. **188:** 421–429.

29 **Kikuchi, Y., Yasue, T., Miyake, K., Kimoto, M. and Takatsu, K.,** CD38 ligation induces tyrosine phosphorylation of Bruton tyrosine kinase and enhanced expression of interleukin 5-receptor α chain: synergistic effects with interleukin 5. *Proc. Natl. Acad. Sci. USA* 1995. **92:** 11814–11818.

30 **Horikawa, K., Kaku, H., Nakajima, H., Davey, H. W., Henninghausen, L., Iwamoto, I., Yasue, T., Kariyone, A. and Takatsu, K.,** Essential role of STAT5 for IL-5-dependent IgH switch recombination in mouse B cells. *J. Immunol.* 2001. **167:** 5018–5026.

31 **Okada, T., Takiura, F., Tokushige, K., Nozawa, S., Kiyosawa, T., Nakauchi, H., Hirose, S. and Shirai, T.,** Major histocompatibility complex controls clonal proliferation of $CD5^+$ B cells in H-2-

congenic New Zealand mice: a model for B cell chronic lymphocytic leukemia and autoimmune disease. *Eur. J. Immunol.* 1991. **21:** 2743–2748.

32 **Shirai, T., Okada, T. and Hirose, S.,** Genetic regulation of CD5$^+$ B cells in autoimmune disease and in chronic lymphocytic leukemia. *Ann. N. Y. Acad. Sci.* 1992. **651:** 509–526.

33 **Hamano, Y., Hirose, S., Ida, A., Abe, M., Zhang, D., Kodera, S., Jiang, Y., Shirai, J., Miura, Y., Nishimura, H. and Shirai, T.,** *Susceptibility alleles for* aberrant B-1 cell proliferation involved in spontaneously occurring B cell chronic lymphocytic leukemia in a model of New Zealand White mice. *Blood* 1998. **92:** 3772–3779.

34 **Hirose, S., Jiang, Y., Hamano, Y. and Shirai, T.,** Genetic aspects of inherent B cell abnormalities associated with SLE and B cell malignancy: lessons from New Zealand mouse models. *Int. Rev. Immunol.* 2000. **19:** 389–421.

35 **Li, N., Nakamura, K., Jiang, Y., Tsurui, H., Matsuoka, S., Abe, M., Ohtsuji, M., Nishimura, H., Kato, K., Kawai, T., Atsumi, T., Koike, T., Shirai, T., Ueno, H. and Hirose, S.,** Gain-of-function polymorphism in mouse and human Ltk: implications for the pathogenesis of systemic lupus erythematosus. *Hum. Mol. Genet.* 2004. **13:** 171–179.

36 **Conley, C. L., Misiti, J. and Laster, A. J.,** Genetic factors predisposing to chronic lymphocytic leukemia and to autoimmune disease. *Medicine* 1980. **59:** 323–334.

37 **Hirose, S., Nagasawa, R., Sekikawa, I., Hamaoki, M., Ishida, Y., Sato, H. and Shirai, T.,** Enhancing effect of H-2-linked NZW gene(s) *on the autoimmune* traits of (NZB×NZW) F1 mice. *J. Exp. Med.* 1983. **158:** 228–233.

38 **Hirose, S., Ueda, G., Noguchi, K., Okada, T., Sekigawa, I., Sato, H. and Shirai, T.,** Requirement of H-2 heterozygosity for autoimmunity in (NZB×NZW) F1 hybrid mice. *Eur. J. Immunol.* 1986. **16:** 1631–1633.

39 **Hirose, S., Kinoshita, K., Nozawa, S., Nishimura, H. and Shirai, T.,** Effects of major histocompatibility complex on autoimmune disease of H-2-congenic New Zealand mice. *Int. Immunol.* 1990. **2:** 1091–1095.

40 **Tominaga, A., Matsumoto, M., Harada, N., Takahashi, T., Kikuchi, Y. and Takatsu, K.,** Molecular properties and regulation of mRNA expression for murine T cell-replacing factor (TRF)/IL-5. *J. Immunol.* 1988. **140:** 1175–1181.

41 **Yamaguchi, N., Hitoshi, Y., Mita, S., Hosoya, Y., Murata, Y., Kikuchi, Y., Tominaga, A. and Takatsu, K.,** Characterization of the murine interleukin 5 receptor by using a monoclonal antibody. *Int. Immunol.* 1990. **2:** 181–187.

42 **Stall, A. M., Farinas, M. C., Tarlington, D. M., Lalor, P. A., Herzenberg, L. A., Strober, S. and Herzenberg, L. A.,** Ly-1 B cell clones similar to human chronic lymphocytic leukemias routinely develop in older *normal mice and young* autoimmune (New Zealand Black-related) animals. *Proc. Natl. Acad. Sci. USA* 1988. **85:** 7312–7316.

Correspondence: Sachiko Hirose, Department of Pathology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
Fax: +81-3-3813-3164
e-mail: sacchi@med.juntendo.ac.jp

Spred-1 negatively regulates allergen-induced airway eosinophilia and hyperresponsiveness

Hiromasa Inoue,[1] Reiko Kato,[2] Satoru Fukuyama,[1,2] Atsushi Nonami,[2] Kouji Taniguchi,[2] Koichiro Matsumoto,[1] Takako Nakano,[1] Miyuki Tsuda,[1] Mikiko Matsumura,[1] Masato Kubo,[4] Fumihiko Ishikawa,[3] Byoung-gon Moon,[5] Kiyoshi Takatsu,[5] Yoichi Nakanishi,[1] and Akihiko Yoshimura[2]

[1]Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, [2]Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, and [3]The First Department of Internal Medicine, Faculty of Medicine, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan
[4]Laboratory for Signal Network, RIKEN Research Center for Allergy and Immunology, Institute of Chemical and Physical Research (RIKEN), Yokohama Institute, Tsurumi, Yokohama, Kanagawa 230-0045, Japan
[5]Department of Immunology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

T helper 2 cytokines, including interleukin (IL)-4, IL-5, and IL-13, play a critical role in allergic asthma. These cytokines transmit signals through the Janus kinase/signal transducer and activator of transcription (STAT) and the Ras–extracellular signal-regulated kinase (ERK) signaling pathways. Although the suppressor of cytokine signaling (SOCS) family proteins have been shown to regulate the STAT pathway, the mechanism regulating the ERK pathway has not been clarified. The Sprouty-related Ena/VASP homology 1–domain-containing protein (Spred)-1 has recently been identified as a negative regulator of growth factor–mediated, Ras-dependent ERK activation. Here, using Spred-1–deficient mice, we demonstrated that Spred-1 negatively regulates allergen-induced airway eosinophilia and hyperresponsiveness, without affecting helper T cell differentiation. Biochemical assays indicate that Spred-1 suppresses IL-5–dependent cell proliferation and ERK activation. These data indicate that Spred-1 negatively controls eosinophil numbers and functions by modulating IL-5 signaling in allergic asthma.

CORRESPONDENCE
Hiromasa Inoue:
inoue@kokyu.med.kyushu-u.ac.jp

Abbreviations used: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BMEo, BM–derived eosinophil; EGFP, enhanced GFP; ERK, extracellular signal-regulated kinase; PAS, periodic acid-Schiff; Spred, Sprouty-related EVH1-domain-containing protein.

Asthma is characterized by a variable degree of airflow obstruction, airway hyperresponsiveness (AHR; defined by enhanced airflow obstruction in response to nonspecific stimuli), mucus overproduction, and chronic airway inflammation. Numerous eosinophils and lymphocytes infiltrate peribronchial tissues in asthmatics. Th2 cells are the predominant lymphocyte population that infiltrates the airways of asthmatics, and the cytokine products of Th2 cells play essential roles in airway eosinophilia, AHR, and serum IgE in animal models (1). Eosinophils are produced in bone marrow, and recent observations in both mice and humans suggest that pulmonary allergen exposure results in both increased output of eosinophils from hemopoietic tissues and increased migration of these cells to the lung (2–4). It is the accumulation of activated eosinophils during the late-phase response to allergen exposure that ultimately results in progressive inflammatory tissue damage. In addition, pulmonary eosinophilia in response to allergen challenge is associated with elevated levels of eosinophil-derived proteins in both the lungs and peripheral blood (5, 6). However, the specific mechanisms that alter eosinophilopoiesis in asthma are poorly understood.

Eosinophil production is predominantly regulated by the Th2 cytokine, IL-5 (7, 8). IL-5 receptor consists of IL-5–specific α-chain and the common β-chain that is shared by the IL-3 and GM-CSF receptor (9). In addition to the JAK–STAT pathway, the Ras–extracellular signal-regulated kinase (ERK) pathway has also been implicated in signaling of IL-5 and other cytokines (10–12), and this pathway is shown to be important for IL-5–dependent cell survival (12). Therefore, the Ras-ERK signals seem to be important for eosinophilia in asthma; however, the regulation of this path-

H. Inoue, R. Kato, and S. Fukuyama contributed equally to this work.

JEM © The Rockefeller University Press $8.00
Vol. 201, No. 1, January 3, 2005 73–82 www.jem.org/cgi/doi/10.1084/jem.20040616

way and its contribution to the disease have not been clarified.

ERK activation is initiated by binding of Grb2 to the phosphorylated tyrosine residues of the receptor or phosphorylated adaptor molecules such as Shc, FRS-2, IRS-1/2, SHP-2, and Gab-1. The complex of Grb2 and SOS activates Ras by GTP loading. Ras-GTP recruits Raf-1 to the plasma membrane (13, 14). Raf-1 is phosphorylated and activated by not well-defined kinases with complex regulatory mechanisms (15). Activated Raf phosphorylates and activates the dual-specific kinase MEK, which phosphorylates and activates ERK. The regulation of this pathway has been suggested to be quite important for cell proliferation and differentiation. Recently, Sprouty family proteins were identified as negative regulators for several growth factor-induced ERK activation including FGF and EGF (9, 16). Four Sprouty homologues are found in mammals. We cloned an additional Sprouty-related family of novel membrane bound molecules, Sprouty-related Ena/VASP homology 1–domain-containing proteins (Spreds; reference 17). Three members of Spreds were identified in mammals (18), which have a Sprouty-related COOH-terminal cysteine-rich domain in addition to the NH_2-terminal Ena/VASP homology 1 domain. Like Sproutys, Spred-1, Spred-2, and Spred-3 also down-regulate Ras/ERK signaling. As Spred inhibits active Ras-induced ERK activation, Spred might modulate the unidentified activation steps of Raf by a novel mechanism. Spred/Sprouty family proteins have emerged as a negative regulator of the ERK pathway; however, details of their physiological function and molecular mechanism remain to be investigated.

In the present paper, we generated Spred-1 knockout mice and examined the function of Spred-1 on the development of asthma and associated eosinophilia. We show that Spred-$1^{-/-}$ mice exhibited exaggerated allergen-induced AHR, eosinophilia, and mucus production in a murine allergic asthma model. Spred-$1^{-/-}$ mice also showed increased responsiveness, especially ERK signals, to IL-5 and subsequent overexpression of IL-13 in eosinophils. Thus, it is conceivable that the down-regulation of Spred-1 in the airways has a significant role in prolonged airway eosinophilia and asthma phenotypes. We propose the possibility that Spred-1 may present a novel therapeutic target for the treatment of asthma.

RESULTS

Generation of Spred-$1^{-/-}$ mouse and assessment of T cell development

We found that Spred-1 is highly expressed in hematopoietic cells (unpublished data). To examine the function of Spred-1 in Th1/Th2 cell differentiation and diseases, we generated mice lacking the Spred-1 gene by homologous recombination techniques (Fig. 1). To obtain the loss-of-function mutant, exons containing the kit-binding domain and Sprouty-related COOH-terminal cysteine-rich domains were deleted. Loss of Spred-1 protein expression was confirmed by Western blotting of the tissue extracts. Spred-1 protein was detected in the brain of the WT mice but not in that of the mutant mice. Offspring were born within the Mendelian expectation ratio from intercrosses of heterozygotes as well as incrosses of homozygotes. This indicates that Spred-1 is not necessary for fertility and development. Adult Spred-$1^{-/-}$ mice appeared to be healthy, except that a slight lower body weight, a shortened face, and a kinky tail, but they showed no apparent abnormalities in most organs (unpublished data). No abnormalities of peripheral hematopoietic cell population and number, CD4/CD8 profiles of spleen and thymus, or B cell development were observed in Spred-$1^{-/-}$ mice (unpublished data).

Enhanced OVA-induced asthma phenotypes in Spred-$1^{-/-}$ mice

Because the ERK pathway is shown to be involved in the Th1/Th2 balance (19–21), we investigated the role of Spred-1 in a Th2-type disease, bronchial asthma, using an OVA-

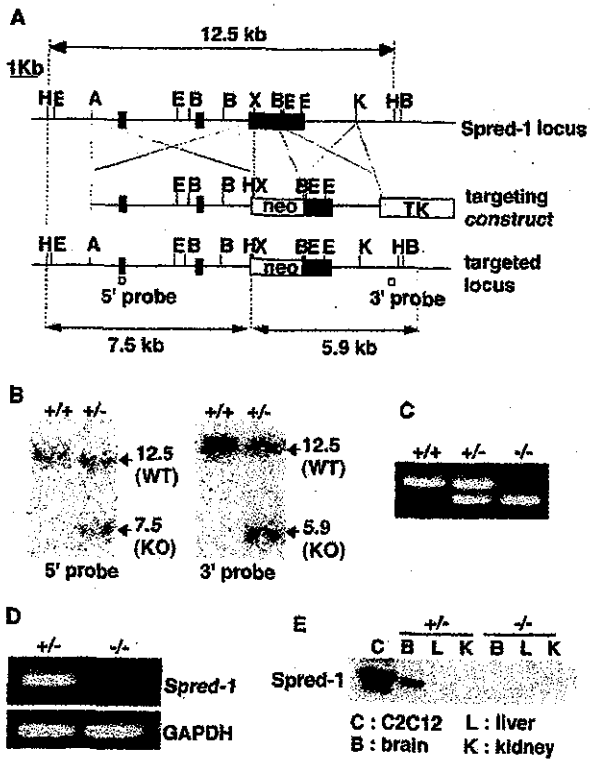

Figure 1. Generation of Spred-1–deficient mice. (A) Schematic representations of wild-type and mutant loci of the Spred-1 gene together with the targeting vector. Exons (from the second to fourth) for genes encoding Spred-1 are represented by black boxes. The neomycin-resistance gene driven by a PGK promoter (pgk-neo) and the gene coding the thymidine kinase driven by an HSV promoter (hsv-tk) are indicated by white boxes. EcoRV-digested genomic DNA fragments were detected by probe. Restriction sites: E, EcoRI; Ev, EcoRV; H, HindIII; A, ApaI; K, KpnI; X, XhoI. (B) Representative Southern blot analysis with HindIII-digested DNA. The positions of probes are shown in A. (C) Genomic PCR analysis of tails of indicated mice. (D) RT-PCR analysis of Spred-1 and control GAPDH expression in total RNA of brains of wild-type and Spred-$1^{-/-}$ mice. (E) Western blotting of brain, liver, and kidney with anti-Spred-1 antibody. C2C12 cells that express Spred-1 endogenously at high levels are shown as a positive control.

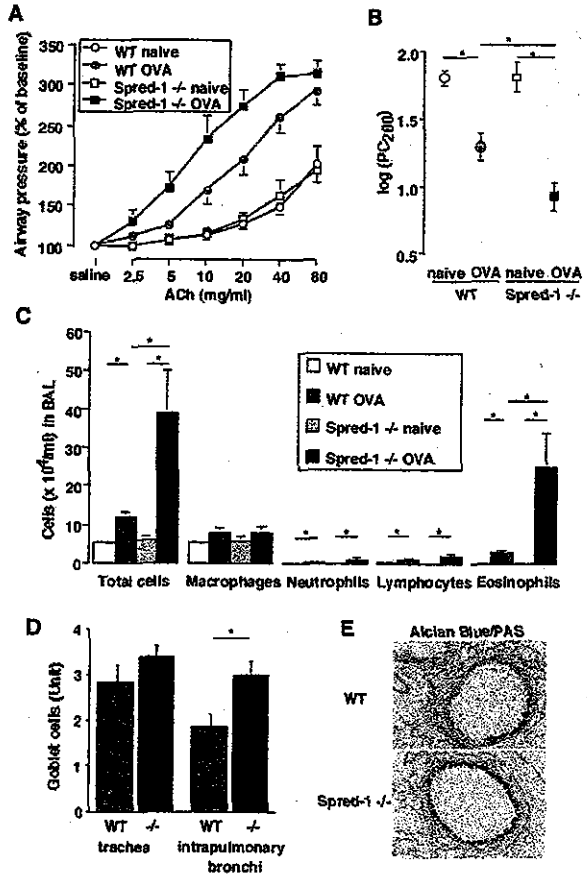

Figure 2. Effects of Spred-1 inactivation on asthmatic responses in an OVA-induced asthma model. Mice were sensitized and aerosol challenged with OVA. Airway responsiveness was determined by the acetylcholine-dependent change in airway pressure in saline-treated control and OVA-sensitized/-challenged LM and Spred-1–deficient mice (A) and the calculation of PC_{200} (B). (C) Effects of Spred-1 depletion on cell counts in BAL fluid. *, $P < 0.05$ by analysis of variance with Bonferroni correction. (D) Semiquantitative analysis of the abundance of PAS-positive mucus-containing cells. Lung tissue sections were obtained from formalin-fixed, paraffin-embedded lung tissue prepared and stained with Alcian blue/PAS. PAS-positive cells were defined as the average of the sore as described in Materials and Methods. *, $P < 0.05$ by Kruskal-Wallis test with Mann-Whitney U test. (E) Representative lung tissue sections. Original magnification, 200.

induced asthma model. After systemic sensitization to OVA and aerosolized OVA challenges, airway responsiveness to acetylcholine aerosol was measured using an invasive technique (22). WT mice demonstrated AHR to acetylcholine, and Spred-$1^{-/-}$ mice exhibited significantly enhanced AHR compared with WT mice (Fig. 2, A and B). OVA sensitization and challenge resulted in an increased number of eosinophils in bronchoalveolar lavage (BAL) fluid in WT mice. Spred-$1^{-/-}$ mice after an OVA challenge displayed a further increase in eosinophils in BAL fluids compared with WT mice, although there was no significant difference in the number of lymphocytes and neutrophils (Fig. 2 C). There was no difference in OVA-induced asthma between C57BL/6 mice and WT (Spred-$1^{+/+}$) littermates (unpublished data). Alcian blue/periodic acid-Schiff (PAS) staining was performed to examine the levels of mucus hyperproduction. After OVA challenge, mild staining in the airways of WT mice cells was noted, and staining levels were increased significantly in the intrapulmonary bronchi of Spred-$1^{-/-}$ mice (Fig. 2, D and E), indicating enhanced goblet cell metaplasia in Spred-$1^{-/-}$ lung. In the absence of sensitization and challenges, no substantial differences were apparent in AR.H to acetylcholine, inflammatory cells in BAL fluids, and Alcian blue/PAS staining cells in the airways between Spred-$1^{-/-}$ and WT mice.

Next, we directly measured cytokines and eotaxin levels in mouse BAL fluids. The challenge with OVA increased the concentrations of IL-13 and eotaxin in Spred-$1^{-/-}$ mice significantly more than in WT mice (Fig. 3 A), reflecting marked eosinophilia in the airways of Spred-$1^{-/-}$ mice. However, there was no difference in the concentration of IL-4, IL-5, or IFN-γ in BAL fluids after OVA challenges between Spred-$1^{-/-}$ and WT mice.

Th1/Th2 responses in Spred-$1^{-/-}$ T cells

Enhanced AHR in Spred-$1^{-/-}$ mice may be explained by a simple increased Th2 response, although IL-4 levels were not altered. Therefore, we measured serum IgE levels that are determined by Th1/Th2 balance. The concentrations of total IgE and OVA-specific IgE in Spred-$1^{-/-}$ mice were similar to those in WT controls (Fig. 4 A). There were no significant differences in serum IgA levels among the groups (Fig. 4 A). $CD4^+$ T cells were isolated from draining lymph node cells from paratracheal and mediastinal lymph nodes after OVA

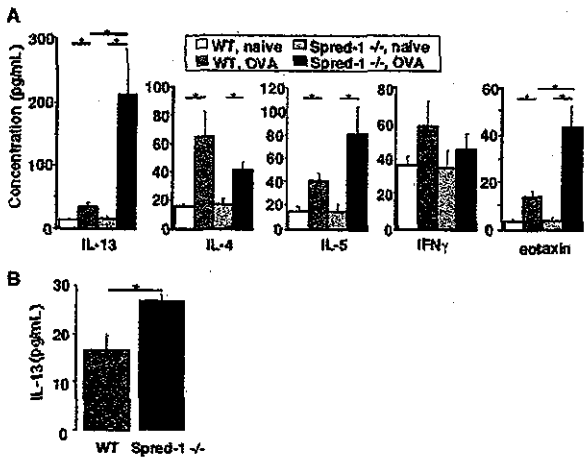

Figure 3. Elevated IL-13 levels in BAL fluid of Spred-1–deficient mice. (A) Effects of Spred-1 inactivation on cytokine levels in BAL fluid in an OVA-induced asthma model. Data are means ± SEM of at least six mice per group. *, $P < 0.05$ by analysis of variance with Bonferroni correction. (B) Eosinophils isolated from BAL of OVA-sensitized/-challenged WT and Spred-1–deficient mice were incubated at 10^6 cells/ml for 24 h. The secretion of IL-13 from Spred-1–deficient mouse eosinophils was significantly higher than that from WT mouse eosinophils ($n = 6$). Data are expressed as the mean ± SEM of six mice. *, $P < 0.05$ by Student's t test.

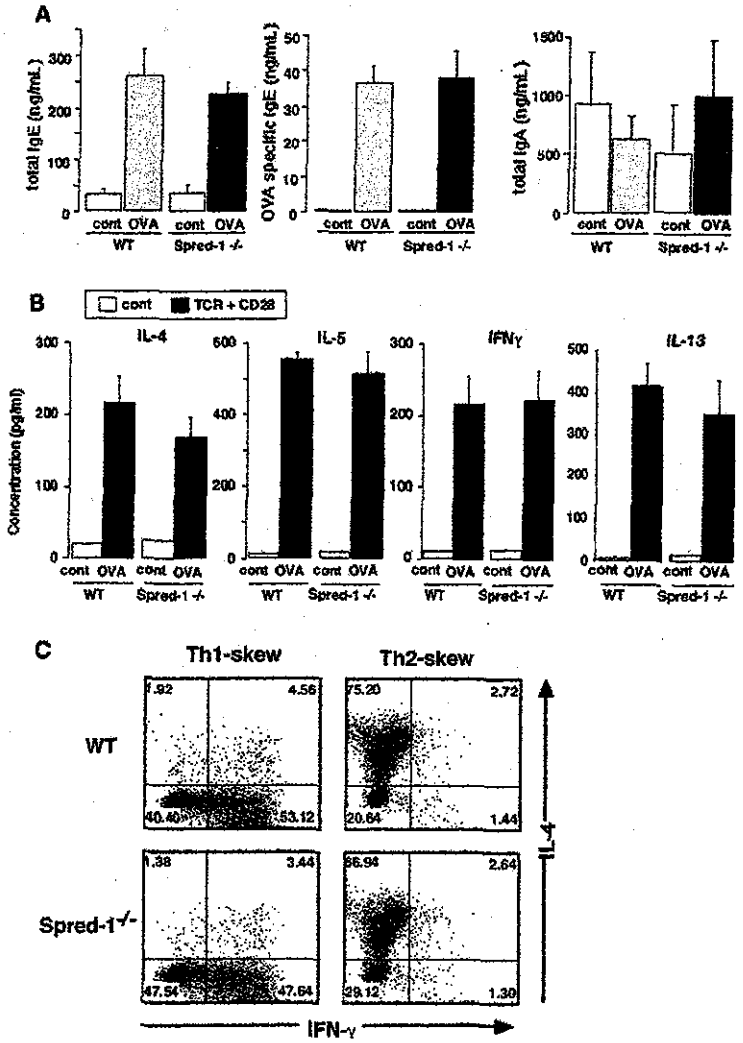

Figure 4. Intact Th1/Th2 response in Spred-1–deficient mice. (A) Serum concentration of total IgE, OVA-specific IgE, and total IgA in an OVA-induced asthma model. Data are means ± SEM of at least six mice per group. (B) Cytokine production from CD4+ T cells after stimulation with anti-TCR plus anti-CD28 mAbs. CD4+ T cells were isolated from paratracheal and mediastinal lymph node cells from WT and Spred-1–deficient mice after OVA challenges and cultured with anti-TCR plus anti-CD28 mAbs in vitro for 48 h. Cytokine production in the supernatants was assessed by ELISA. All data are means ± SEM. (C) CD4+ T cells from WT and Spred-1–deficient mice were stimulated for 5 d with anti-TCR plus anti-CD28 mAbs in the presence of IL-12 and anti–IL-4 mAb (Th1-skewed condition) or IL-4 and anti–IL-12 mAb (Th2-skewed condition). The generation of Th1 and Th2 cells was assessed by cytoplasmic staining with IL-4 and IFN-γ. The absolute numbers of cells harvested were similar in these cultures. The percentages of cells in each quadrant are shown. Three independent experiments were performed with similar results.

challenges and stimulated in vitro with anti-TCR plus anti-CD28 mAbs. IL-4, IL-5, IL-13, and IFN-γ production from CD4+ T cells was similar between Spred-1$^{-/-}$ and WT mice (Fig. 4 B). These data suggest that Spred-1 does not affect Th1/Th2 differentiation and cytokine production from T cells.

To confirm these conclusions, we assessed the development of Th1 and Th2 cells from naive CD4+ T cells in WT and Spred-1$^{-/-}$ mice. T cells were stimulated with anti-TCR plus anti-CD28 mAbs, and the population of IL-4– or IFN-γ–producing cells was analyzed by intracellular FACS staining. The ratio of IL-4– and IFN-γ–producing T cells was comparable between Spred-1$^{-/-}$ and WT mice. IL-4 and IFN-γ levels in the culture supernatant were also comparable (unpublished data). We also tested the generation of Th1 and Th2 cells under a Th1- or Th2-skewed condition. Naive CD4+ T cells were stimulated with anti-TCR mAb in the presence of IL-4 plus anti–IL-12 mAb or of IL-12 plus anti–IL-4 mAb. There were no significant differences in the generation of IL-4–dependent Th2 cells or IL-12–dependent Th1 cells between Spred-1$^{-/-}$ and WT T cells (Fig. 4 C). These data suggested that the development of asthma phenotypes is enhanced in Spred-1$^{-/-}$ mice through the up-regu-

lation of a limited repertoire of Th2 cytokines, such as IL-13, and that this is not due to the enhancement of antigen-specific Th2 immune responses.

In addition to T cells, eosinophils produce Th1 and Th2 cytokines, including IL-5 and IL-13 (23, 24). Next, we investigated IL-13 expression in accumulated eosinophils in the airways of OVA-challenged mice (Fig. 3 B). Eosinophils were isolated from BAL fluids and incubated in vitro for the evaluation of IL-13 production. Airway eosinophils isolated from Spred-1$^{-/-}$ mice secreted significantly higher levels of IL-13 than those from WT mice (Fig. 3 B). These findings indicate that the IL-13 production from eosinophils is augmented in Spred-1$^{-/-}$ mice.

Increased eosinophilia in response to IL-5 but not to IL-13 in Spred-1$^{-/-}$ mice

It has been shown that IL-5 is critical for the induction of eosinophilia (25–27), as is IL-13 for the development of eosinophilic inflammation and AHR (28, 29). Thus, to elucidate the mechanism of enhanced OVA-induced asthma phenotypes and preferential IL-13 production in Spred-1$^{-/-}$ mice, we analyzed the response of eosinophils to these cytokines. Intratracheal administration of recombinant mouse IL-13 increased eosinophil counts in BAL fluids in WT mice; however, the level of eosinophilia after IL-13 instillation in Spred-1$^{-/-}$ mice was comparable to that in WT mice (unpublished data). Intraperitoneal injection of recombinant mouse IL-5 into WT mice induced an increase in the eosinophil number in peripheral blood as reported previously (30). In Spred-1$^{-/-}$ mice, IL-5 injection induced a prominent and prolonged increase in peripheral eosinophil counts (Fig. 5 A).

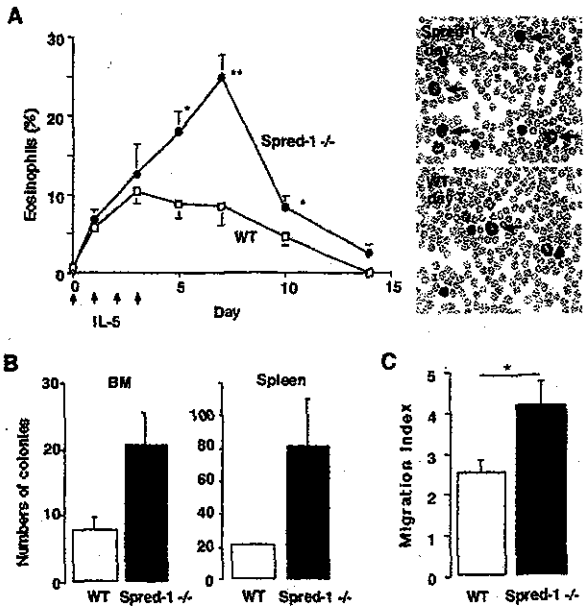

Figure 5. Eosinophil response to IL-5 or to eotaxin and IL-13 production from isolated eosinophils. (A) Recombinant mouse IL-5 (10,000 U/d) was injected intraperitoneally into WT or Spred-1–deficient mice for 4 d. Differential counts were performed by examination of blood smear stained with a modified Wright-Giemsa stain at various time points (left). Typical appearances of blood smear from WT and Spred-1–deficient mice at day 7 are shown (right). Original magnification, 400. *, $P < 0.05$ and **, $P < 0.01$ by Student's t test. (B) Colony formation of eosinophil progenitors in bone marrow and splenic cells. Bone marrow or splenic cells were plated in methylcellulose containing 10 ng/ml IL-5. On day 14, the number of colonies was counted microscopically. (C) Eotaxin-induced eosinophil chemotaxis. Eosinophils from bone marrow of WT or Spred-1–deficinet mice were allowed to migrate to 100 ng/ml eotaxin through polycarbonate filter for 60 min. The migrated cells were counted. Data are reported as migration index and they are calculated as follows: (number of cells migrating to chemoattractant)/(number of cells migrating to vehicle). Data are means ± SEM of six experiments. * $P < 0.05$ by Mann-Whitney U test.

Because the Ras–ERK pathway has been shown to regulate eosinophil chemotaxis (31), we performed the chemotaxis experiment with eosinophils derived from bone marrow of Spred-1$^{-/-}$ or WT mice. Chemotaxis of eosinophils from Spred-1$^{-/-}$ mice was accelerated in response to eotaxin compared with WT mice (Fig. 5 C). These findings indicate that Spred-1$^{-/-}$ mice exhibit the hyperresponsiveness of eosinophil to IL-5 and to eotaxin and enhanced/sustained eosinophilia.

Spred-1 inhibits IL-5–mediated ERK activation and cell proliferation

IL-5 activates the propagation of signals principally via the JAK–STAT pathways, especially STAT5, and the Ras-MAPK pathways (32). To elucidate the molecular mechanism by which the inactivation of Spred-1 enhances IL-5 responses, we analyzed the JAK-STAT and Ras–MAPK pathways using an IL-5–dependent cell line, Y16 (33). Although forced expression of WT or ΔC-Spred-1 did not affect IL-5–induced JAK2 and STAT5 activation, WT Spred-1 reduced Raf-1 and ERK activation, and the dominant negative ΔC mutant of Spred-1 augmented Raf-1 and ERK activation (Fig. 6 A).

Other tyrosine kinases such as Lyn and JAK2 have been shown to enhance eosinophil differentiation (34). Therefore, we analyzed the interaction of Spred-1 with Raf-1, Lyn, and JAK2 kinases in Y16 cells transfected with WT Flag-Spred-1. The expression levels of Lyn in Y6 cells was very low, so we could not conclude the interaction between Lyn and Spred-1. Raf-1 was coimmunoprecipitated with Spred-1 as reported previously (17). However, JAK2 was not coimmunoprecipitated with Spred-1 (Fig. 6 B). These data further support our